

Identification of two duplicate etiolation genes (py1, py2) in pakchoi (*Brassica rapa* L. ssp. *chinensis*)

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Abstract

We identified a stably inherited yellow leaf mutant derived from 'Huaguan' pakchoi variety via isolated microspore culture and designated as *pylm*. This mutant displayed yellow leaves after germination. Its etiolated phenotype was nonlethal and stable during the whole growth period. Its growth was weak and its hypocotyls were markedly elongated. Two recessive nuclear genes named *py1* and *py2* had a duplicate effect on etiolation. BSR-Seq revealed that *py1* and *py2* were mapped on chromosomes A09 and A07, respectively. The genes were single Mendelian factors in F_{3:4} populations based on a 3:1 phenotypic segregation ratio. The *py1* was localized to a 258.3-kb interval on a 34-gene genome. The differentially expressed gene BraA09004189 was detected in the *py1* mapping region and regulated heme catabolism. One single-nucleotide polymorphism of BraA09004189 occurred in *pylm*. A candidate gene-specific SNP marker in 1,520 F_{3:4} yellow-colored individuals co-segregated with *py1*. For *py2*, 1,860 recessive homozygous F_{3:4} individuals were investigated and localized *py2* to a 4.4-kb interval. Of the five genes in this region, BraA07001774 was predicted as a candidate for *py2*. It encoded an embryo defective 1187 and a phosphotransferase related to chlorophyll deficiency and hypocotyl elongation. One single-nucleotide polymorphism of BraA07001774 occurred in *pylm*. It caused a single amino acid mutation from Asp to Asn. According to qRT-PCR, BraA07001774 was downregulated in *pylm*. Thus, BraA09004189 and BraA07001774 are candidate genes for *py1* and *py2*. These findings will elucidate the molecular mechanisms of the gene interactions controlling pakchoi etiolation.

Introduction

The photosynthetic pigment chlorophyll (Chl) is ubiquitous in cyanobacteria and the chloroplasts of higher plants. Chl converts the energy of sunlight into bioavailable chemical energy which drives carbohydrate biosynthesis (Fromme et al. 2003). Chl is an essential component of leaf color which influences dry matter accumulation and crop yield. In general, leaves appear green because Chl predominates and has a vital role in them. When the Chl content changes in plants, various leaf color mutant phenotypes result including chlorina, virescent, albino, yellow-green, and stay-green (Kurata et al. 2005). Leaf color mutants develop from the inhibition of genes regulating Chl biosynthesis and chloroplast development. Downregulation of these genes directly or indirectly influences Chl synthesis and degradation and produces the leaf color mutations (Schultes et al. 2000; Motohashi et al. 2003; Song et al. 2018). Thus, leaf color mutants may be ideally suited for the elucidation of the mechanisms of photosynthesis, Chl biosynthesis, chloroplast development, and the expression and regulation of the genes associated with these processes (Stern et al. 2004; Kusaba et al. 2007; Bang et al. 2008; Chen et al. 2018). Leaf color mutants have been characterized in *Arabidopsis* (Jarvis et al. 2000), rice (Zhang et al. 2006; Moon et al. 2008), wheat (Hui et al. 2012), *Brassica napus* (Zhu et al. 2014), *Brassica oleracea* (Liu et al. 2016), barley (Mueller et al. 2012), kale (Zhou et al. 2013), tobacco (Wu et al. 2014), soybean (Fang et al. 2014), cotton (Song et al. 2012), and cucumber (Gao et al. 2016; Song et al. 2018). Much research has been invested in the analysis of the genetics, physiology, and molecular mechanisms of Chl biosynthesis and chloroplast development via leaf color mutants.

Several studies in genetic analysis have categorized leaf color mutation inheritance as nuclear and cytoplasmic. Most leaf color mutations are recessively inherited and conferred by a single nuclear gene (Wang et al. 2010; Ansari et al. 2013; Gao et al. 2016; Wang et al. 2018). Leaf color mutations involving two recessive genes are rare. Moreover, their inheritance is complex and it is difficult to apply genetic analysis and gene mapping on them. Wu et al. (2014) identified the light color mutant *ws1* in *Nicotiana tabacum* and determined that this phenotype was controlled by the recessive nuclear genes *ws1a* and *ws1b* localized by different BC₁F₂ groups to linkage groups 5 and 24, respectively. *BnChd1-1* and *BnChd1-2* are responsible for the light green leaf mutant phenotype in *Brassica napus*. Fine mapping of *BnChd1-1* was achieved using the BC₃F₁ population. Candidate gene prediction suggested that *BnChd1-1* encodes a subunit of the NAD(P)H complex in the thylakoid lumen (Zhao et al. 2014). Chl-deficient mutant phenotypes in durum wheat (Li et al. 2013) and *Brassica juncea* (Tian et al. 2012) are also controlled by two recessive genes. Cytoplasmic mutants are uncommon compared to nuclear mutants. However, they have been reported for tobacco (Barak et al. 2000), barley (Colombo et al. 2008), and *Brassica campestris* (Tang et al. 2018).

In plants, Chl biosynthesis comprises 15 enzymatic steps regulated by at least 27 genes (Nagata et al. 2005). Inactivation mutations of the Chl biosynthetic genes usually results in Chl-deficient mutants (Frick et al. 2003; Zhang et al. 2006; Wang et al. 2010; Sheng et al. 2017). Mutations in the genes governing Chl degradation metabolism generally produce stay-green mutants which retain their green leaf phenotype even during senescence (Yoo et al. 2007; Morita et al. 2009; Wang et al. 2018). Chl and heme biosynthesis are two types of tetrapyrrole formation and share a common metabolic pathway from 5-aminolevulinic acid (ALA) to protoporphyrin IX (Proto IX) (Weller et al. 1996). Heme is essential for both respiration and photosynthesis. In contrast, excessive heme accumulation inhibits glutamyl-tRNA reductase activity and ALA synthesis, reduces the rate of tetrapyrrole biosynthesis, and affects Chl biosynthesis (Terry and Kendrick 1999). Leaf color mutants arising from abnormal heme metabolism were identified for *Arabidopsis* (Xie et al. 2012), rice (Xu et al. 2012; Chen et al. 2013; Li et al. 2014), pea (Linley et al. 2006), and maize (Shi et al. 2013).

In a previous study, we developed a pakchoi (*Brassica rapa* L. ssp. *chinensis*) yellow leaf mutant (*pylm*) derived from the 'Huaguan' pakchoi variety by isolated microspore culture. This strain is a double haploid (DH) with a stable yellow leaf phenotype (Zhang et al. 2017). In the present study, we conducted genetic analysis on *pylm* using BSR-Seq with linkage analysis to map the corresponding genes. Then, the candidate genes associated with the mutant phenotype were predicted. The information derived from this work may help facilitate the cloning of etiolation genes and elucidate the molecular mechanisms of gene interactions.

Materials And Methods

Plant materials and mapping population development

The DH line *pylm* was obtained by isolated microspore culture of the ‘Huaguan’ pakchoi variety introduced from Japan musashino seed company. This strain was characterized by yellow leaves and elongated hypocotyls (Zhang et al. 2017). The *pylm* and the green Chinese cabbage DH line ‘FT’ were used as parents for the segregating population. The *pylm* was reciprocally crossed with ‘FT’ to produce the F₁, F₂, and BC₁ generations. Twenty F₂ individuals with green leaves were self-pollinated to produce F_{2:3} progenies. Eight green F_{2:3} individuals per group were randomly selected from the corresponding populations and self-pollinated to produce the F_{3:4} populations. Those with character segregation were used in linkage analysis and gene mapping. All plants were grown in the greenhouse at Shenyang Agricultural University, China.

Detection of variations by BSR-Seq

One hundred individuals with extreme leaf color phenotype were separately collected from the F₂ progeny and pooled for RNA extraction (RNAprep Pure Plant Kit; Tiangen, China). The RNA concentration and integrity were analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The extreme mixed pools green-leaf (G-pool) and yellow-leaf (Y-pool) were constructed by mixing equal amounts of each RNA sample. RNA-Seq was run on an Illumina HiSeq 2500 by GENEWIZ Suzhou Biological Technology Co., Ltd., China.

The quality of the raw RNA-Seq reads was assessed with FastQC (v. 0.10.1). Adapter sequences and low-quality reads containing N and < 70 were deleted from the raw reads with Cutadapt (v. 1.9.1). Low-quality bases at the 5’ or 3’ end were filtered out. Those with mean quality < 20 were trimmed by the 4-bp sliding window method. Clean data were aligned to the *Brassica* database (<http://brassicadb.org/brad/>) with Hisat v. 2.0.14 (Kim et al. 2015). Candidate SNPs between the pools were obtained with the Mpileup module in SAM Tools (v. 0.1.18) SNP loci with depth coverage > 3× were screened for differential SNP analysis in the mutant and wild type pools. Euclidean distances (ED) for the differential SNP loci were calculated. The ED for each differential SNP locus was raised to the power five, namely, ED⁵, to eliminate background noise (Su et al. 2016). All ED⁵ were sorted and the differential SNP loci with ED⁵ in the top 1% were screened and mapped to specific chromosome regions based on the SNP locus distributions. Chromosome regions associated with the target traits were predicted according to the distributions of the ED⁵ for the differential SNP loci on the chromosomes.

Differential gene expression analysis

To detect differentially expressed genes (DEGs) between the pools, a gene expression level analysis was performed with Htseq (v. 0.6.1). The reads per kilobases per million mapped reads (RPKM) were calculated (Mortazavi et al. 2008). DEGs were screened using a preset threshold ($|\log_2 \text{fold change}| \geq 1$ and false discovery rate (FDR) ≤ 0.05).

DNA isolation, polymerase chain reaction (PCR), and linkage analysis

A modified CTAB method (Murray and Thompson 1980) was used to extract genomic DNA from young leaves of the parental and $F_{3:4}$ populations with a phenotypic segregation ratio of 3:1. The primers for polymorphism analysis were designed in Primer Premier (v. 5.0). PCR amplifications were performed following the instruction as described by Wang et al. (2018). PCR products were separated on 5% (w/v) denaturing polyacrylamide gels and examined by silver staining. The genetic linkage map was constructed by Join Map v. 4.0 (Van Ooijen 2006) using the segregation data. Map distances were calculated and reported in centimorgans (cM) according to Kosambi's mapping function (Kosambi 1944).

Candidate gene prediction and qRT-PCR validation

Using the chromosome location of the target gene, all adjacent genes were annotated with the *Brassica* database (<http://brassicadb.org/brad/>). Candidate genes were predicted according to gene annotation. PCR primers were designed to span the entire putative gene length between the mutant *pylm* and wild type 'CK-51'. Candidate genes were cloned following the method referenced from Huang et al. (2016). Sequences were determined by GENEWIZ Biological Technology Co. Ltd. (Suzhou, China) and aligned with DNAMAN software.

Leaves of *pylm* and 'CK-51' were collected for total RNA extraction as previously described. After confirming its **concentration** and **integrity**, the RNA was reverse-transcribed with a FastQuant RT Kit (Tiangen, China). Quantitative real-time (qRT)-PCR was performed with a Bio-Rad IQ5 Real-Time PCR System (Bio-Rad Laboratories, USA) and SYBR Green PCR Master Mix (Tiangen, China). All reactions were run on three biological replicates. Two independent technical replicates per sample were processed to confirm data accuracy. Primers for the candidate genes *BraA09004189* (F: 5'-GCTTCCACTTCGCTTTTTCCT-3'; R: 5'-TCTTTTTCTGCTTCTCTGATGCTG-3') and *BraA07001774* (F: 5'-GGATACGACTGTGATTACACCTTCTAC-3'; R: 5'-CCGACTCCACTTCACCAACG-3') were used in the qRT-PCR analysis. The *Actin* gene (F: 5'-CGAAACAACCTTACAACCTCA-3'; R: 5'-CTCTTTGCTCATACGGTCA-3') served as an endogenous control. The qRT-PCR reaction conditions and program were those cited in Huang et al. (2015). The relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Bio-Rad IQ5 (Bio-Rad Laboratories, USA) analyzed the data.

Results

Phenotypic characterization of mutant *pylm*

The wild type 'CK-51' and *pylm* were both obtained from isolated microspore culture of the 'Huaguan' pakchoi variety. However, the latter displayed yellow leaves at germination and this phenotype was stable throughout its lifetime (Fig. 1). The mutant had a slender phenotype and weak growth. However, its

yellow leaf color was nonlethal. Moreover, relative to 'CK-51', *pylm* displayed an elongated hypocotyl at the seedling stage (Fig. 1c) and early flowering at the bolting stage (Fig. 1b).

Genetic analysis of mutant *pylm*

F₁ and F₂ populations were constructed from crosses between *pylm* (Fig. S1a) and the Chinese cabbage DH line 'FT' (Fig. S1b). The F₁ individuals from the reciprocal crosses had the same green leaf phenotype as 'FT'. Therefore, inheritance of the etiolation phenotype in *pylm* is nuclear rather than cytoplasmic. Segregation statistics data for the green and yellow leaf phenotypes of the F₂ population accorded with the expected Mendelian ratio of 15:1 ($\chi^2 < \chi^2_{0.05} = 3.84$). Thus, the Chl deficiency trait is controlled by two duplicate recessive nuclear genes. The BC₁ progeny was obtained from F₁ separately backcrossed with *pylm* and 'FT'. Segregation statistics data for the green and yellow leaf phenotypes of the BC₁F₁ population from the cross of F₁ with *pylm* fitted the expected Mendelian ratio of 3:1 ($\chi^2 < \chi^2_{0.05} = 3.84$). This finding confirms that the mutant trait is conferred by two duplicate recessive nuclear genes. They were designated as *py1* and *py2*. Neither gene alone can induce the yellow leaf phenotype. Phenotypic data for all generations are listed in Table 1.

Segregation of *py1* and *py2*

According to the genetic analysis of *pylm*, the reduced-Chl phenotype is controlled by the recessive nuclear genes *py1* and *py2* with duplicate effects on etiolation. In consequence, the F₂ and BC₁F₁ populations could not be used to map these genes separately. To separate *py1* from *py2*, F₂ individuals with green leaves may be randomly selected and self-pollinated to produce F_{2:3} progeny. Green-colored F_{2:3} plants with a statistical segregation ratio of 3:1 (green-colored:yellow-colored) may be self-pollinated to generate F_{3:4} progeny. In theory, ~2/3 of the F_{3:4} families should display the expected Mendelian segregation ratio of 3:1. Some of these could map *py1* while the others could map *py2* (Fig. 2).

Twenty green-colored individuals from F₂ were randomly selected and self-pollinated to produce F_{2:3}. For the twenty F_{2:3} families, phenotypic segregations were investigated. There were three distinct groups. Eleven populations showed no yellow-colored plants, five segregated with 15:1, and the other four with 3:1 (Table S1). These results corroborated the theoretical segregation ratio of "all green-colored:(green-colored:yellow-colored = 3:1):(green-colored:yellow-colored = 15:1) = 7:4:4" for F_{2:3}.

Of the four F_{2:3} families segregated with 3:1, eight plants with green leaves per family were selected and self-pollinated to produce F_{3:4}. Phenotypic segregations revealed that twenty F_{3:4} families (Nos. 1–20) segregated with 3:1 while the other twelve showed no yellow-colored individuals (Nos. 21–32). Thus, their F_{2:3} genotypes should be *Py1 py1 py2 py2/py1 py1 Py2 py2* and *Py1 Py1 py2 py2/py1 py1 Py2 Py2*, respectively (Table S2). Phenotypic segregations of the F_{3:4} families fitted the theoretical segregation

ratio of "(green-colored:yellow-colored = 3:1):all green-colored = 2:1". Therefore, the F_{3:4} families (Nos. 1–20) could be used to map the *py1* and/or *py2* loci.

BSR-Seq analysis

A total of 47,526,126 and 49,119,466 raw reads (150-bp) were generated from the G-pool and Y-pool, respectively. After quality evaluation and data filtering, 97% of the read pairs (46,456,174 for the G-pool and 47,581,728 for the Y-pool) remained. Clean reads were mapped against the *Brassica* reference genome with Hisat v. 2.0.14. Of these, > 66% were uniquely mapped in both pools.

Relative to the reference genome, 154,863 and 157,022 SNPs were detected in the G-pool and Y-pool, respectively. Differential SNP loci were screened for ED⁵ calculation and 412 target differential SNP loci were obtained between the pools according to the top 1% ED⁵ threshold. Two distinct peaks were observed on chromosomes A07 and A09 (Fig. 3). This finding was consistent with the hypothesis that the mutant trait is controlled by two duplicate recessive nuclear genes. Thus, it was predicted that the etiolation genes were located on chromosomes A07 and A09 within five chromosome regions (Table 2).

Identification of differentially expressed genes

RPKM was used to measure gene expression level. By setting RPKM ≥ 0.1 , 55,250 genes were detected. These were divided into six RPKM distribution intervals (Table S3). There were 181 DEGs between the G-pool and Y-pool according to the constraint ($|\log_2$ fold change| ≥ 1 and FDR ≤ 0.05). Ninety genes were upregulated and the others were downregulated when the G-pool was compared with the Y-pool (Fig. S2). The DEGs are shown in Table S4.

Fine mapping of *py1*

Ninety-six SSR markers were developed around the three predicted chromosome regions on chromosome A09. They were used to detect polymorphisms between *py1m* and 'FT'. After screening, thirty-seven SSR markers displayed polymorphisms between parents. They were used to test twelve green-colored and yellow-colored individuals each from the No. 1 F_{3:4} family. SSRzk5 and SSRzk12 were located near the 23,811,435–27,563,122 region on chromosome A09 and showed linkage to *py1* on the opposite side.

A total of 1,520 yellow-colored individuals of the No. 1 F_{3:4} family were selected as the *py1* mapping population. A linkage analysis disclosed that *py1* was located between SSRzk5 and SSRzk12 at estimated genetic distances of 3.2 cM and 1.8 cM, respectively (Fig. 4a). To identify the molecular markers tightly linked to *py1* and narrow the *py1* mapping interval, new SSR and Indel markers were developed between SSRzk5 and SSRzk12. The polymorphic markers SSRzk17, SSRzk28, SSRzk29, SSRzk36, Indelzk72, and Indelzk125 were linked to *py1* (Table S5). SSRzk17, Indelzk72, and Indelzk125

were located on one side of *py1* as SSRzk5 while SSRzk28, SSRzk29, and SSRzk36 were located on the other side of *py1* as SSRzk12. The *py1* was mapped between Indelzk125 and SSRzk36 at 0.13 cM and 0.2 cM, respectively (Fig. 4b). Therefore, *py1* was mapped in a 258.3-kb region between the most tightly linked markers (Fig. 4c).

Candidate *py1* analysis

The target DNA sequences of the 258.3-kb region between Indelzk125 and SSRzk36 were obtained from the *Brassica* database. A genomic sequence analysis revealed that the candidate region contained 34 genes (Fig. 4c, Table S6). Differential gene expression analysis disclosed only *BraA09004189* in the *py1* mapping region. *BraA09004189* is a heme oxygenase (HO1) which participates in heme catabolism. Mutants with yellow leaf phenotype induced by defective HOs were reported in earlier studies (Chen et al. 2013; Li et al. 2014). *BraA09004189* was predicted to be the most probable candidate *py1* gene.

To confirm this hypothesis, two pairs of primers were designed to sequence *BraA09004189* in *pylm* and 'CK-51' (Table S7). The BraA sequence did not differ between parents whereas the BraB sequence in *pylm* presented with one single nucleotide polymorphism (SNP) (Fig. 5). Based on the position of *BraA09004189*, an SNP marker was designed to screen 1,520 yellow-colored individuals from the No. 1 F_{3:4} family. The bands of whole mapping individuals co-segregated with *py1*.

A qRT-PCR was performed to determine *BraA09004189* expression in *pylm* and 'CK-51'. In accordance with the differential gene expression analysis, the results indicated that *BraA09004189* expression level was much higher in 'CK-51' than that in *pylm* (Fig. 6). This finding confirms that *BraA09004189* is the candidate gene for *py1*.

Fine mapping of *py2*

Considering the constructed populations size, we screened the Nos. 2–5 F_{3:4} families using the same research strategy applied for SSRzk5 and SSRzk12 linked to *py1*. The etiolation gene *py1* was identified in the Nos. 2, 4, and 5 F_{3:4} families. In theory, then, the No. 3 F_{3:4} family may be used to establish the *py2* locus.

Forty-eight SSR markers were developed around the two predicted regions on chromosome A07 to detect polymorphisms between *pylm* and 'FT'. After screening, eleven SSR markers displayed polymorphisms between the parents. They were used to test twelve green-colored and twelve yellow-colored individuals of the No. 3 F_{3:4} family. SSR84 and SSR103 were located around the region 11,166,810–15,034,483 on chromosome A07 and presented with linkages to *py2* on the opposite side (Fig. 7a; Table S8).

There were 1,860 yellow-colored individuals from the No. 3 F_{3:4} family selected as the *py2* mapping population. The *py2* was located between SSR11 and SSR15 at estimated genetic distances of 0.24 cM

and 0.03 cM, respectively (Fig. 7b). To narrow the *py2* mapping interval and identify the molecular markers tightly linked to *py2*, new single-nucleotide variant (SNV) markers were developed between SSR11 and SSR15. Only the polymorphic marker SNV11 was linked to *py2*. Based on the recombinant individuals, the *py2* interval was narrowed to 14,851,951–14,896,902 and contained five genes (Fig. 7c).

Candidate *py2* analysis

Annotation data for the five candidate genes in the *py2* target region were obtained from the *Brassica* database (Table S9). Primers were designed to cover the cDNA for each gene and predict the candidate genes (Table S10). There were no differences between *pylm* and 'CK-51' in terms of *BraA07001775*, *BraA07001776*, or *BraA07001777*. After PCR amplification, the *BraA07001773* sequence was disordered and the sequence comparisons were inconsistent over serial repetitions. There was SNP variation between parents for the first exon in *BraA07001774* (Fig. 8). It caused a single amino acid mutation from Asp (GAT) in the wild type to Asn (AAT) in *pylm* (Fig. 9). Therefore, *BraA07001774* was taken as the most probable candidate gene for *py2*.

BraA07001774 is an *embryo defective 1187* (*emb 1187*) and a phosphotransferase. The albino mutants (*pds1*, *pds2*) phenotypes in *Arabidopsis thaliana* may be caused by *emb 71* (Franzmann et al. 1995). For *Arabidopsis* seeds with silique defects, hypocotyl elongation was characterized during the development of F₂ generation mutant seedlings (Patton et al. 1991). We proposed that the mutant phenotype is determined by mutations in *BraA07001774*. To validate our prediction, *BraA07001774* expression in *pylm* and 'CK-51' was analyzed by qRT-PCR. *BraA07001774* was dramatically downregulated in *pylm* (Fig. 10). Thus, it probably is the candidate gene for *py2*.

Discussion

Mutations in leaf color are widespread in nature. The main type of leaf color mutation is Chl deficiency. Dwarfism, retarded growth, attenuated photosynthetic capacity, low yield, and death are associated with this defect (Jung et al. 2003; Wu et al. 2007; Zhao et al. 2014). Here, we identified the pakchoi yellow leaf mutant *pylm* from isolated microspore culture. Unlike previously reported Chl deficient mutants, *pylm* presented with substantially elongated hypocotyls at the seedling stage and early flowering at the bolting stage. The etiolation phenotype in *pylm* was nonlethal and stable throughout the growth period. The Chl deficiency in *pylm* was controlled by two duplicate recessive genes. These characteristics suggested that *pylm* was of high value for research in revealing the Chl biosynthesis mechanism regulated by gene interactions.

Map-based cloning is an effective gene isolation strategy. It has been extensively used for plant gene function analysis (Jander and Last 2002; Sang et al. 2010; Xie et al. 2018). However, it is contingent upon fine mapping of the target gene. For most leaf color mutants, the traits are recessively inherited and controlled by a single nuclear gene. The F₂ populations are instinctively applied to map the target gene

(Wu et al. 2007; Li et al. 2014; Song et al. 2018). With regard to the character conferred by two recessive nuclear genes, F_2 populations may also be used in preliminary mapping. An efficient way to isolate allele pairs from each other and separately map them is to construct advanced backcrosses and other populations. The recessive white stem (*ws*) loci in *Nicotiana tabacum* and the Chl deficiency (*Bnchd1*) loci in *Brassica napus* were successfully mapped using constructed BC_1F_2 and BC_3F_1 populations, respectively (Wu et al. 2014; Zhao et al. 2014). In the present study, genetic analysis revealed that the duplicate recessive nuclear genes designated as *py1* and *py2* were responsible for the etiolation trait. We successfully segregated *py1* from *py2* and constructed an inheritance model for the Chl deficiency trait in pakchoi. Twenty $F_{3:4}$ families with a phenotypic segregation ratio of 3:1 were constructed. Various $F_{3:4}$ families were successfully used to map the *py1* and *py2* loci separately. Compared to using advanced backcross populations to map pairs of recessive nuclear genes, creating and using $F_{2:3}$ or $F_{3:4}$ families avoid the selection errors and interference in genetic analysis caused by the incomplete emasculation of *Brassica rapa*.

BSR-Seq efficiently combines the respective superiorities of bulk segregation analysis (BSA) and RNA sequencing (RNA-Seq) for rapid gene mapping (Trick et al. 2012; Lee et al. 2014; Lu et al. 2014; Ramirez-Gonzalez et al. 2015). BSR-Seq is targeted at the mRNA level. It selects phenotypically opposite individuals from segregated populations and constructs two RNA mixing pools to find SNPs at the transcript level. The transcriptome data localize the target genes and detect potentially associated DEGs (Schneeberger and Weigel 2011). BSR-Seq has been extensively applied to map the causal genes linked to a single target trait (Nestler et al. 2014; Tang et al. 2014; Wang et al. 2018). Two independently inherited traits may also be localized by BSR-Seq. Tan et al. (2019) applied BSR-Seq to locate the genes controlling male sterility on chromosome A05 and white petal on chromosome A02. Here, the mutant *pylm* and Chinese cabbage DH 'FT' lines were chosen as parents to construct the F_2 separation population. The phenotypes of the wild type and mutant individuals significantly differed. Thus, extreme mixed pools could be accurately and conveniently created for BSR-Seq. Release of *Brassica rapa* genomic data enhanced the reliability of these populations in BSR-Seq applications. Five candidate regions related to the yellow leaf phenotype in *pylm* were identified on chromosomes A07 and A09. Molecular markers were developed according to the locations of the candidate regions. The etiolation genes *py1* and *py2* were separately mapped with different $F_{3:4}$ families. The findings confirmed the feasibility of BSR-Seq for mapping two recessive nuclear genes. They also showed that BSR-Seq simplifies molecular marker development and screening in traditional mapping methods and greatly improves their efficiency.

New molecular markers were developed near the target regions based on preliminary *py1* mapping by BSR-Seq. The *py1* was mapped between the markers Indelzk125 and SSRzk36 on A09 chromosome over a 258.3-kb localization interval containing 34 predicted genes. No new polymorphic SSR or Indel markers were available to limit the localization interval. The gene expression patterns determined by BSR-Seq disclosed only one differentially expressed gene (*BraA09004189*) within the *py1* mapping region. A gene annotation referenced from the *Brassica* database indicated that *BraA09004189* encodes heme

oxygenase-1 (HO1). This enzyme plays a vital role in phytochrome chromophore metabolism, the photoresponse mechanism, adventitious root formation, and oxidative damage mitigation (Davis et al. 1999; Ortiz de Montellano and Wilks 2001; Quail 2002; Emborg et al. 2006). HO1 stabilizes and maintains the heme content by transforming heme into BV-IX α (Terry et al. 1993). As embranchments of tetrapyrrole biosynthesis, Chl and heme biosynthesis share a common **metabolic pathway** from ALA to Proto IX. Excessive heme accumulation caused by abnormal heme metabolism leads to feedback inhibition of Chl biosynthesis (Weller et al. 1996). Therefore, a decrease in HO1 activity may influence Chl biosynthesis. The *hy1* mutant of *Arabidopsis* and the *yellow-green leaf 2* mutant of rice presented with the reduced-Chl phenotype because of free heme inhibition resulting from HO1 mutations (Davis et al. 1999; Chen et al. 2013). HO1 defects strongly affected thylakoid development in rice (Li et al. 2014). Davis et al. (1999) found that the abnormally elongated hypocotyl phenotype of mutant *Arabidopsis* seedlings may be associated with HO1 defects. Thus, *py1* may encode HO and a mutation thereof may influence Chl biosynthesis and leaf color. In this study, qRT-PCR demonstrated that *BraA09004189* was downregulated in *pym*. This finding was consistent with those obtained by BSR-Seq. The SNP *BraA09004189* was detected between *pym* and 'CK-51'. A candidate gene-specific SNP marker in 1,520 F_{3:4} yellow-colored individuals co-segregated with *py1*. Thus, *BraA09004189* corresponds to the yellow leaf locus *py1* in *pym*.

It was already known that certain Chl deficiency traits are controlled by two recessive nuclear genes. However, there was a lack of appropriate mapping populations or reliable molecule markers. Therefore, they were either approximately mapped without definite locations (Li et al. 2013; Wu et al. 2014) or only one of the pair could be localized (Zhao et al. 2014). In previous studies, little progress was made in the simultaneous fine mapping or accurate prediction of the candidate genes. Here, we used the same mapping strategy as that for *py1* to accomplish fine mapping for *py2*. The linkage analysis disclosed that *py2* was mapped between SSR11 and SSR15 on chromosome A07. The mapping interval was narrowed to 4.4 kb by the SNV11 marker linked to *py2*. Sequence analysis of the five genes in the *py2* localization interval showed that only *BraA07001774* expression significantly differed between *pym* and 'CK-51'. For *pym*, *BraA07001774* had a SNP missense mutation on the first exon such that the wild type had an Asp residue whereas *pym* had an Asn. The qRT-PCR revealed that *BraA07001774* was downregulated in the mutant relative to the wild type. Gene annotation in the *Brassica* database indicated that *BraA09004189* encodes *embryo defective 1187* (*emb 1187*) and a phosphotransferase. In *Arabidopsis*, the *emb* (*embryo defective*) genes are essential for seed development (Despres et al. 2001). *EMB* genes encode various proteins. Thirty percent of them are active in the plastids (Shen et al. 2013). Most *emb* mutations result in albinism or etiolated seeds and embryos which are secondary effects of mutations in chloroplast biogenesis and function (Li and Thomas 1998). The albino mutants (*pds1*, *pds2*) and hypocotyl elongation phenotypes in *Arabidopsis* may be related to mutations in *EMB* genes (Patton et al. 1991; Franzmann et al. 1995). Thus, *BraA07001774* is a candidate gene for *py2*. Here, we successfully achieved fine mapping and predictions for the two candidate etiolation genes *py1* and *py2*. These discoveries may help elucidate the molecular mechanisms underlying the trait controlled by two duplicate recessive nuclear genes. In future studies, functional validation will be conducted to clarify the functions

of these candidate genes. In this manner, the molecular mechanism of gene interactions may be better understood.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

All data are fully available without restriction.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KZ, YM, XS and NW conducted the experiments and collected the data. KZ and WL analyzed the data and drafted the manuscript. HF designed the experiments and revised the manuscript. All authors read and approved the final manuscript.

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References

- Ansari MJ, Al-Ghamdi A, Kumar R, Usmani S, AL-Attal Y, Nuru A, Mohamed AA, Singh K, Dhaliwal HS (2012) Characterization and gene mapping of a chlorophyll-deficient mutant *clm1* of *Triticum monococcum* L. *Biol Plant* 57(3):442–448
- Bang WY, Jeong IS, Kim DW, Im CH, Ji C, Hwang SM, Kim SW, Son YS, Jeong J, Shiina T, Bahk JD (2008) Role of *Arabidopsis* CHL27 protein for photosynthesis, chloroplast development and gene expression profiling. *Plant Cell Physiol* 49(9):1350–1363
- Barak S, Heimer Y, Nejidat A, Volkita M (2000) The peroxisomal glycolate oxidase gene is differentially expressed in yellow and white sectors of the *DP1* variegated tobacco mutant. *Physiol Plant* 110(1):120–126
- Chen H, Cheng Z, Ma X, Wu H, Liu Y, Zhou K, Chen Y, Ma W, Bi J, Zhang X, Guo X, Wang J, Lei C, Wu F, Lin Q, Liu Y, Liu L, Jiang L (2013) A knockdown mutation of *YELLOW-GREEN LEAF2* blocks chlorophyll biosynthesis in rice. *Plant Cell Rep* 32(12):1855–1867
- Chen P, Hu H, Zhang Y, Wang Z, Dong G, Cui Y, Qian Q, Ren D, Guo LB (2018) Genetic analysis and fine-mapping of a new rice mutant, *white and lesion mimic leaf1*. *Plant Growth Regul* 85(3):425–435
- Colombo N, Emanuel C, Lainez V, Maldonado S, Prina AR, Börner T (2008) The barley plastome mutant *CL2* affects expression of nuclear and chloroplast housekeeping genes in a cell-age dependent manner. *Mol Gen Genomics* 279(4):403–414
- Davis SJ, Kurepa J, Vierstra RD (1999) The *Arabidopsis thaliana* *HY1* locus, required for phytochrome-chromophore biosynthesis, encodes a protein related to heme oxygenases. *Proc Natl Acad Sci* 96(11):6541–6546
- Despres B, Delseny M, Devic M (2001) Partial complementation of *embryo defective* mutations: a general strategy to elucidate gene function. *Plant J* 27(2):149–159
- Emborg TJ, Walker JM, Noh B, Vierstra RD (2006) Multiple heme oxygenase family members contribute to the biosynthesis of the phytochrome chromophore in *Arabidopsis*. *Plant Physiol* 140(3):856–868

- Fang C, Li C, Li W, Wang Z, Zhou Z, Shen Y, Wu M, Wu Y, Li G, Kong LA, Liu C, Jackson SA, Tian Z (2014) Concerted evolution of *D1* and *D2* to regulate chlorophyll degradation in soybean. *Plant J* 77(5):700–712
- Franzmann LH, Yoon ES, Meinke DW (1995) Saturating the genetic map of *Arabidopsis thaliana* with embryonic mutations. *Plant J* 7(2):341–350
- Frick G, Su Q, Apel K, Armstrong GA (2003) An *Arabidopsis porB porC* double mutant lacking light-dependent NADPH: protochlorophyllide oxidoreductases B and C is highly chlorophyll-deficient and developmentally arrested. *Plant J* 35(2):141–153
- Fromme P, Melkozernov A, Jordan P, Krauss N (2003) Structure and function of photosystem I: interaction with its soluble electron carriers and external antenna systems. *FEBS Lett* 555(1):0–44
- Gao M, Hu L, Li Y, Weng Y (2016) The chlorophyll-deficient golden leaf mutation in cucumber is due to a single nucleotide substitution in *CsChll* for magnesium chelatase I subunit. *Theor Appl Genet* 129(10):1961–1973
- Huang S, Liu Z, Yao R, Li D, Feng H (2015) Comparative transcriptome analysis of the petal degeneration mutant *pdm* in Chinese cabbage (*Brassica campestris ssp. pekinensis*) using RNA-Seq. *Mol Genet Genomics* 290(5):1833–1847
- Huang S, Liu Z, Yao R, Li D, Zhang T, Li X, Hou L, Wang Y, Tang X, Feng H (2016) Candidate gene prediction for a petal degeneration mutant, *pdm*, of the Chinese cabbage (*Brassica campestris, ssp. pekinensis*) by using fine mapping and transcriptome analysis. *Mol Breed* 36(3):26–35
- Hui Z, Tian FX, Wang GK, Wang GP, Wang W (2012) The antioxidative defense system is involved in the delayed senescence in a wheat mutant *tasg1*. *Plant Cell Rep* 31(6):1073–1084
- Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL (2002) Arabidopsis map-based cloning in the post-genome era. *Plant Physiol* 129(2):440–450
- Jarvis P, Dörmann Peter, Peto CA, Lutes J, Benning C, Chory J (2000) Galactolipid deficiency and abnormal chloroplast development in the *Arabidopsis MGD synthase 1* mutant. *Proc Natl Acad Sci* 97(14):8175–8179
- Jung KH, Hur J, Ryu CH, Choi Y, Chung YY, Miyao A, Hirochika H, An G (2003) Characterization of a rice chlorophyll-deficient mutant using the T-DNA gene-trap system. *Plant Cell Physiol* 44(5):463–472
- Kim D, Langmead B, Salzberg SL (2015) HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 12(4):357–362
- Kosambi DD (1944) The estimation of map distance from recombination values. *Ann Eugen* 12(1):172–175

- Kurata N, Miyoshi K, Nonomura KI, Yamazaki Y, Ito Y (2005) Rice mutants and genes related to organ development, morphogenesis and physiological traits. *Plant Cell Physiol* 46(1):48–62
- Kusaba M, Ito H, Morita R, Iida S, Sato Y, Fujimoto M, Kawasaki S, Tanaka R, Hirochika H, Nishimura M, Tanaka A (2007) Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. *Plant Cell* 19(4):1362–1375
- Lee YP, Cho Y, Kim S (2014) A high-resolution linkage map of the *Rfd1*, a restorer-of-fertility locus for cytoplasmic male sterility in radish (*Raphanus sativus* L.) produced by a combination of bulked segregant analysis and RNA-Seq. *Theor Appl Genet* 127(10):2243–2252
- Li N, Jia J, Xia C, Liu X and Kong X (2013) Characterization and mapping of novel chlorophyll deficient mutant genes in durum wheat. *Breed Sci* 63(2):169–175
- Li Q, Zhu FY, Gao X, Sun Y, Li S, Tao Y, Lo C, Liu H (2014) *Young leaf chlorosis 2*, encodes the stroma-localized heme oxygenase 2 which is required for normal tetrapyrrole biosynthesis in rice. *Planta* 240(4):701–712
- Li Z, Thomas LT (1998) *PEI1*, an embryo-specific zinc finger protein gene required for heart-stage embryo formation in *Arabidopsis*. *Plant Cell* 10(3):383–398
- Linley PJ, Landsberger M, Kohchi T, Cooper JB, Terry MJ (2006) The molecular basis of heme oxygenase deficiency in the *pcd1* mutant of pea. *Febs J* 273(12):2594–2606
- Liu XP, Yang C, Han FQ, Fang ZY, Yang LM, Zhuang M, Lv HH, Liu YM, Li ZS, Zhang YY (2016) Genetics and fine mapping of a yellow-green leaf gene (*ygl-1*) in cabbage (*Brassica oleracea* var. *capitata* L.). *Mol Breed* 36(6):82–89
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25(4):402–408
- Lu H, Lin T, Klein J, Wang S, Qi J, Zhou Q, Sun J, Zhang Z, Weng Y, Huang S (2014) QTL-seq identifies an early flowering QTL located near *Flowering Locus T* in cucumber. *Theor Appl Genet* 127(7):1491–1499
- Moon S, Giglione C, Lee DY, An S, Jeong DH, Meinel T, An G (2008) Rice peptide deformylase PDF1B is crucial for development of chloroplasts. *Plant Cell Physiol* 49(10):1536–1546
- Morita R, Sato Y, Masuda Y, Nishimura M, Kusaba M (2009) Defect in non-yellow coloring 3, an alpha/beta hydrolase-fold family protein, causes a stay green phenotype during leaf senescence in rice. *Plant J* 59(6):940–952
- Motohashi K, Koyama F, Nakanishi Y, Ueoka-Nakanishi H, Hisabori T (2003) Chloroplast Cyclophilin is a target protein of thioredoxin THIOL MODULATION OF THE PEPTIDYL-PROLYL *CIS-TRANS* ISOMERASE ACTIVITY. *J Biol Chem* 278(34):31848–31852

- Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 5(7):621–628
- Mueller AH, Dockter C, Gough SP, Lundqvist U, von Wettstein D, Hansson M (2012) Characterization of mutations in barley *fch2* encoding chlorophyllide a oxygenase. *Plant Cell Physiol* 53:1232–1246
- Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* 8(19):4321–4326
- Nagata N, Tanaka R, Satoh S, Tanaka A (2005) Identification of a vinyl reductase gene for chlorophyll synthesis in *Arabidopsis thaliana* and implications for the evolution of Prochlorococcus Species. *Plant Cell* 17(1):233–240
- Nestler J, Liu S, Wen TJ, Paschold A, Marcon C, Tang HM, Li D, Li L, Meeley RB, Sakai H, Bruce W, Schnable PS, Hochholdinger F (2014) Roothairless5, which functions in maize (*Zea mays* L.) root hair initiation and elongation encodes a monocot-specific NADPH oxidase. *Plant J* 79(5):729–740
- Ortiz de Montellano PR, Wilks A (2000) Heme oxygenase structure and mechanism. *Adv Inorg Chem* 51(1):359–407
- Patton DA, Franzmann LH, Meinke DW (1991) Mapping genes essential for embryo development in *Arabidopsis thaliana*. *Mol Gen Genet* 227(3):337–347
- Quail P (2002) Phytochrome photosensory signalling networks. *Nat Rev Mol Cell Biol* 3(2):85–93
- Ramirez-Gonzalez RH, Segovia V, Bird N, Fenwick P, Holdgate S, Berry S, Jack P, Caccamo M, Uauy C (2015) RNA-seq bulked segregant analysis enables the identification of high-resolution genetic markers for breeding in hexaploid wheat. *Plant Biotechnol J* 13(5):613–624
- Sang XC, Fang LK, Vanichpakorn Y, Ling YH, Du P, Zhao FM, Yang ZL, He GH (2010) Physiological character and molecular mapping of leaf-color mutant *wyv1* in rice (*Oryza sativa* L.). *Genes Genomics* 32(2):123–128
- Schneeberger K, Weigel D (2011) Fast-forward genetics enabled by new sequencing technologies. *Trends in Plant Sci* 16(5):282–288
- Schultes NP, Sawers RJH, Brutnell TP, Krueger RW (2000) Maize *high chlorophyll fluorescent 60* mutation is caused by an *Ac* disruption of the gene encoding the chloroplast ribosomal small subunit protein 17. *Plant J* 21(4):317–327
- Shen Y, Li C, McCarty DR, Meeley R, Tan BC (2013) *Embryo defective12* encodes the plastid initiation factor 3 and is essential for embryogenesis in maize. *Plant J* 74(5):792–804

- Sheng Z, Lv Y, Li W, Luo R, Wei X, Xie L, Jiao G, Shao G, Wang J, Tang S, Hu P (2017) *Yellow-Leaf 1* encodes a magnesium-protoporphyrin IX monomethyl ester cyclase, involved in chlorophyll biosynthesis in rice (*Oryza sativa* L.). PLoS ONE 12(5):e0177989
- Shi D, Zheng X, Li L, Lin W, Xie W, Yang J, Chen S, Jin W (2013) Chlorophyll deficiency in the maize *elongated mesocotyl2* mutant is caused by a defective heme oxygenase and delaying grana stacking. PLoS One 8(11):e80107
- Song M, Wei Q, Wang J, Fu W, Qin X, Lu X, Cheng F, Yang K, Zhang L, Yu X, Li J, Chen J, Lou Q (2018) Fine mapping of *CsVYL*, conferring virescent leaf through the regulation of chloroplast development in cucumber. Front Plant Sci 9:432–443
- Song M, Yang Z, Fan S, Zhu H, Pang C, Tian M, Yu S (2012) Cytological and genetic analysis of a virescent mutant in upland cotton (*Gossypium hirsutum* L.). Euphytica 187(2):235–245
- Stern DB, Hanson MR, Barkan A (2004) Genetics and genomics of chloroplast biogenesis: Maize as a model system. Trends Plant Sci 9(6):293–301
- Su A, Song W, Xing J, Zhao Y, Zhang R, Li C, Duan M, Luo M, Shi Z, Zhao J (2016) Identification of genes potentially associated with the fertility instability of s-type cytoplasmic male sterility in maize via bulked segregant RNA-Seq. Plos One 11(9):e0163489
- Tan C, Liu Z, Huang S, Feng H (2019) Mapping of the male sterile mutant gene *ftms* in *Brassica rapa* L. ssp. *pekinensis* via BSR-Seq combined with whole-genome resequencing. Theor Appl Genet 132(2):355–370
- Tang HM, Liu S, Hill-Skinner S, Wu W, Reed D, Yeh CT, Nettleton D, Schnable PS (2014) The maize *brown midrib2 (bm2)* gene encodes a methylenetetrahydrofolate reductase that contributes to lignin accumulation. Plant J 77(3):380–392
- Tang X, Wang Y, Zhang Y, Huang S, Liu Z, Fei D, Feng H (2018) A missense mutation of plastid RPS4 is associated with chlorophyll deficiency in Chinese cabbage (*Brassica campestris* ssp. *pekinensis*).. BMC Plant Biol 18(1):130–140
- Terry MJ, Kendrick RE (1999) Feedback inhibition of chlorophyll synthesis in the phytochrome chromophore-deficient *aurea* and *yellow-green-2* mutants of tomato. Plant Physiol 119(1):143–152
- Terry MJ, Maines MD, Lagarias JC (1993) Inactivation of phytochrome- and phycobiliprotein-chromophore precursors by rat liver biliverdin reductase. J Biol Chem 268(35):26099–26106
- Tian Y, Huang Q, Liu H, Wang J, An R, Liu X, Hu W (2012) Inheritance of chlorophyll-deficient mutant *L638-y* in *Brassica juncea* L. and molecular markers for chlorophyll-deficient gene *gr1*. J Northwest Agric Forest Univ (Nat Sci Ed) 40(12): 90–96 (in Chinese)

- Trick M, Adamski NM, Mugford SG, Jiang CC, Febrer M, Uauy C (2012) Combining SNP discovery from next-generation sequencing data with bulked segregant analysis (BSA) to fine-map genes in polyploid wheat. *BMC Plant Biol* 12(1):14–30
- Van Ooijen J (2006) Joinmap 4.0 software for the calculation of genetic linkage maps in experimental populations. Wageningen, Kyazma BV
- Wang N, Liu Z, Zhang Y, Li C, Feng H (2018) Identification and fine mapping of a stay-green gene (*Brnye1*) in pakchoi (*Brassica campestris* L. ssp. *chinensis*).. *Theor Appl Genet* 131(3):673–684
- Wang P, Gao J, Wan C, Zhang F, Xu Z, Huang X, Sun X, Deng X (2010) Divinyl chlorophyll (ide) *a* can be converted to monovinyl chlorophyll (ide) *a* by a divinyl reductase in rice. *Plant Physiol* 153(3):994–1003
- Weller JL, Terry MJ, Rameau C, Reid JB, Kendrick RE (1996) The phytochrome-deficient *pcd1* mutant of pea is unable to convert heme to biliverdin IX α . *Plant Cell* 8(1):55–67
- Wu Q, Wu X, Zhang X, Jiang C, Xiao B, Zhang Y, Wang Y, Liu G (2014) Mapping of two white stem genes in tetraploid common tobacco (*Nicotiana tabacum* L.). *Mol Breed* 34(3):1065–1074
- Wu Z, Zhang X, He B, Diao L, Sheng S, Wang J, Guo X, Su N, Wang L, Jiang L, Wang C, Zhai H, Wan J (2007) A chlorophyll-deficient rice mutant with impaired chlorophyllide esterification in chlorophyll biosynthesis. *Plant Physiol* 145(1):29–40
- Xie K, Wu S, Li Z, Zhou Y, Zhang D, Dong Z, An X, Zhu T, Zhang S, Liu S, Li J, Wan X (2018) Map-based cloning and characterization of *Zea mays male sterility33* (*ZmMs33*) gene, encoding a glycerol–3-phosphate acyltransferase. *Theor Appl Genet* 131(6):1363–1378
- Xie YJ, Xu DK, Cui WT, Shen WB (2012) Mutation of *Arabidopsis HY1* causes UV-C hypersensitivity by impairing carotenoid and flavonoid biosynthesis and the down-regulation of antioxidant defence. *J Exp Bot* 63(10):3869–3883
- Xu S, Wang L, Zhang B, Han B, Xie Y, Yang J, Zhong W, Chen H, Wang R, Wang N, Cui W, Shen W (2012) RNAi knockdown of rice *SE5* gene is sensitive to the herbicide methyl viologen by the down-regulation of antioxidant defense. *Plant Mol Biol* 80(2):219–235
- Yoo SC, Cho SH, Zhang H, Paik HC, Lee CH, Li J, Yoo JH, Lee BW, Koh HJ, Seo HS, Paek NC (2007) Quantitative trait loci associated with functional stay green *SNU-SG1* in rice. *Mol Cells* 24(1):83–94
- Zhang K, Liu Z, Shan X, Li C, Tang X, Chi M, Feng H (2017) Physiological properties and chlorophyll biosynthesis in a Pak-choi (*Brassica rapa*, L. ssp. *chinensis*) yellow leaf mutant, *pylm*. *Acta Physiol Plant* 39(1):22–32
- Zhang H, Li J, Yoo JH, Yoo SC, Cho SH, Koh HJ, Seo HS, Paek NC (2006) Rice *Chlorina–1* and *Chlorina–9* encode ChlD and ChlI subunits of Mg-chelatase, a key enzyme for chlorophyll synthesis and chloroplast

development. *Plant Mol Biol* 62(3):325–337

Zhao H, Yu L, Huai Z, Wang X, Ding G, Chen S, Li P, Xu F (2014) Mapping and candidate gene identification defining *BnChd1-1*, a locus involved in chlorophyll biosynthesis in *Brassica napus*. *Acta Physiol Plant* 36(4):859–870

Zhou S, Hu Z, Zhu M, Zhang B, Deng L, Pan Y, Chen G (2013) Biochemical and molecular analysis of a temperature-sensitive albino mutant in kale named “White Dove”. *Plant Growth Regul* 71(3):281–294

Zhu L, Zeng X, Chen Y, Yang Z, Qi L, Pu Y, Yi B, Wen J, Ma C, Shen J, Tu J, Fu T (2014) Genetic characterisation and fine mapping of a chlorophyll-deficient mutant (*BnaC.ygl*) in *Brassica napus*. *Mol Breed* 34(2):603–614

Tables

Table 1 Genetic analysis of leaf color mutant phenotype

Generation	Total	Green-colored	Yellow-colored	Segregation ratio	χ^2
P ₁ ('FT')	92	92	0		
P ₂ (<i>pylm</i>)	120	0	120		
F ₁ (P ₁ × P ₂)	258	258	0		
F ₁ (P ₂ × P ₁)	226	226	0		
BC ₁ (F ₁ × 'FT')	669	669	0		
BC ₁ (F ₁ × <i>pylm</i>)	720	551	169	3.26:1	0.82
F ₂	2,376	2,243	133	16.86: 1	1.62

Table 2 Localization of chromosome regions related to etiolation genes

Chromosome	Start position	End position	Number of differential SNV loci	Interval length
A07	9,207,067	10,833,976	19	1,626,909
A07	11,475,098	15,522,445	54	4,047,347
A09	19,610,472	20,763,415	11	1,152,943
A09	23,811,435	27,563,122	33	3,751,687
A09	32,067,464	35,505,463	32	3,437,999

Figures

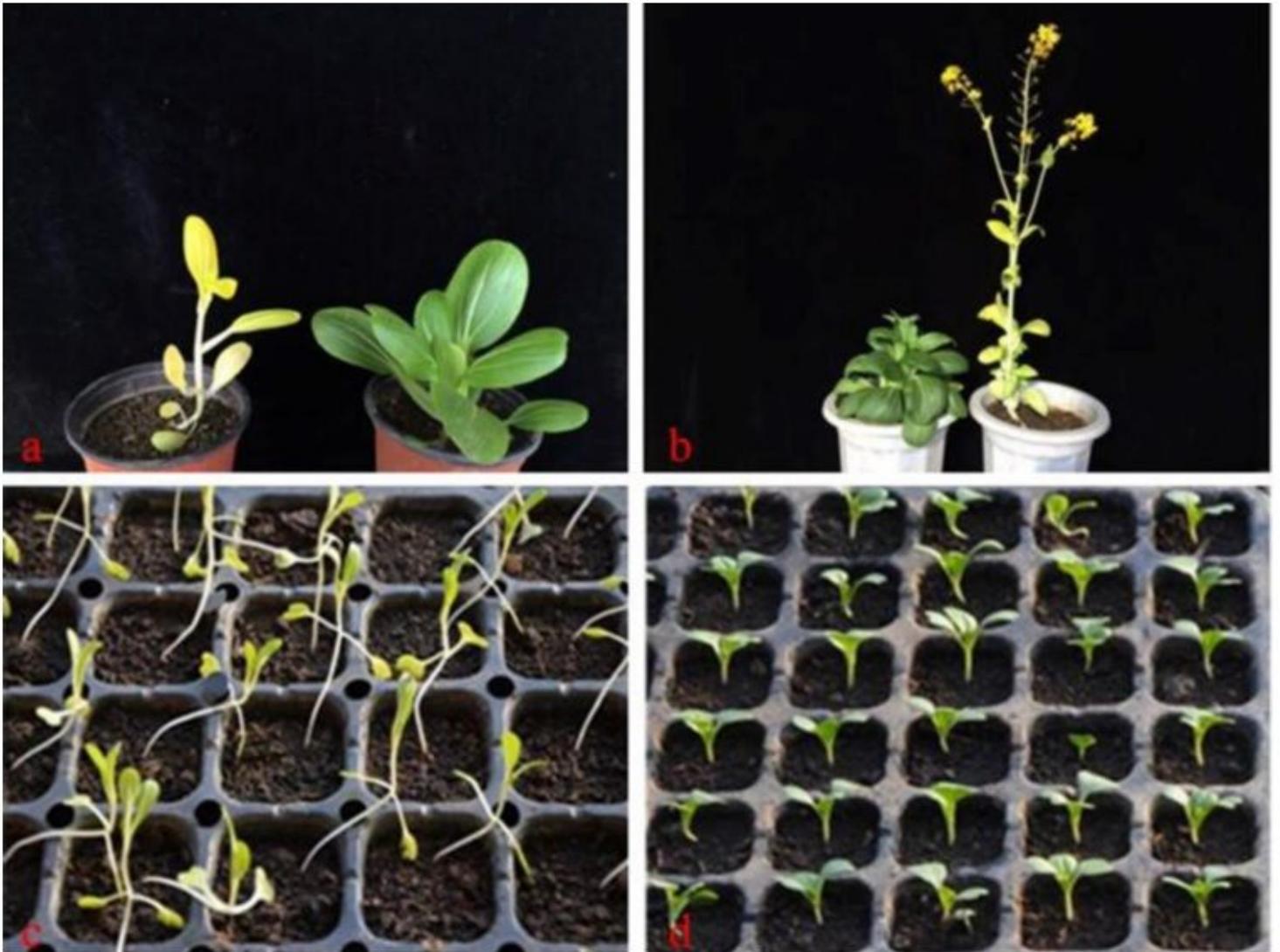


Figure 1

Phenotypes of mutant *pylm* and wild type 'CK-51'. a *pylm* (left) and 'CK-51' (right) at the seedling stage; b 'CK-51' (left) and *pylm* (right) at the bolting stage; c Seedling morphology of 2-wk *pylm* plants; d Seedling morphology of 2-wk 'CK-51' plants

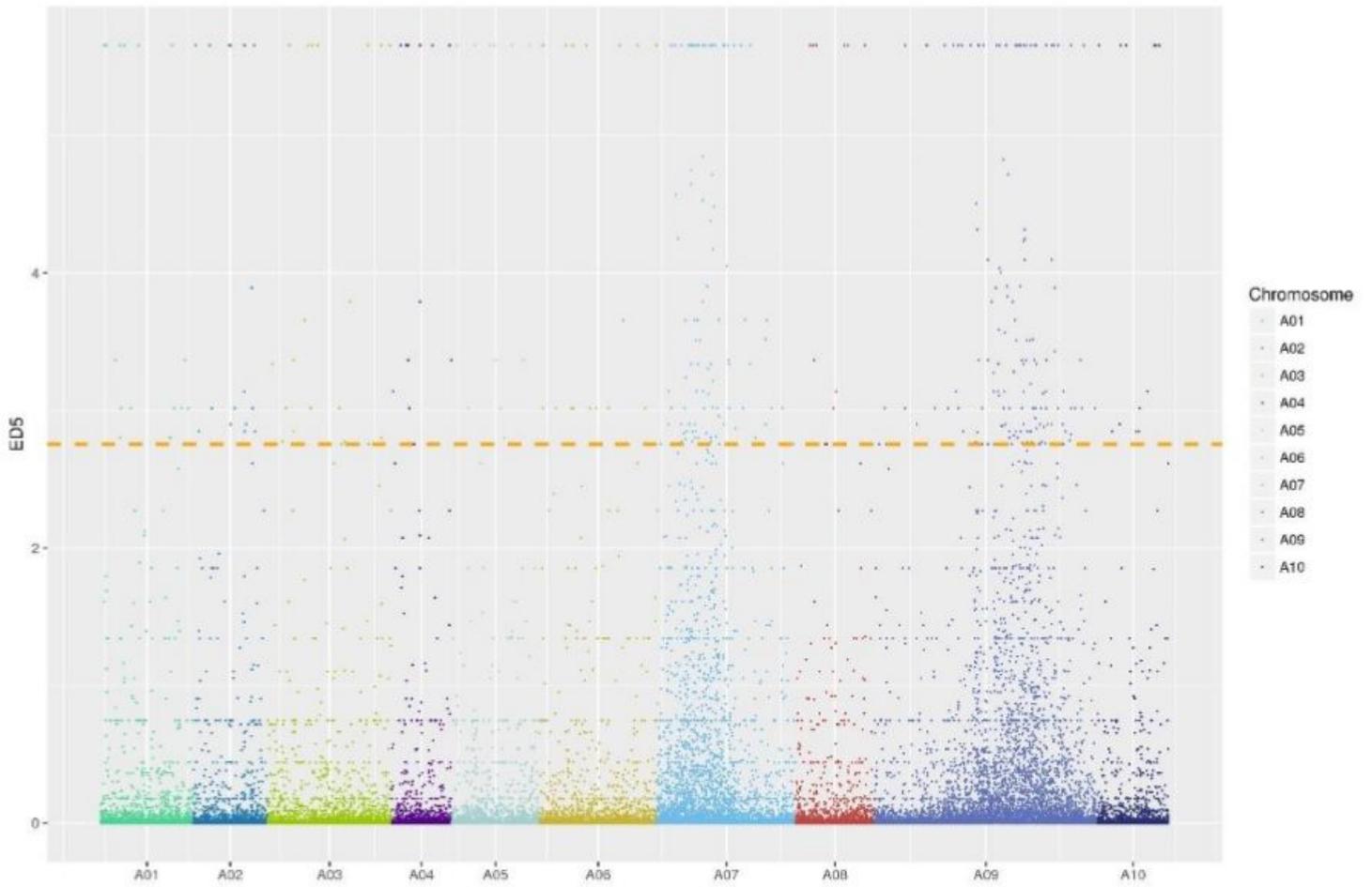


Figure 3

ED5 distributions on chromosomes. Each color on the X-axis represents different chromosomes of *Brassica rapa*. Y-axis represents ED5 for each differential SNP locus. Horizontal line is the threshold of the top 1% ED5

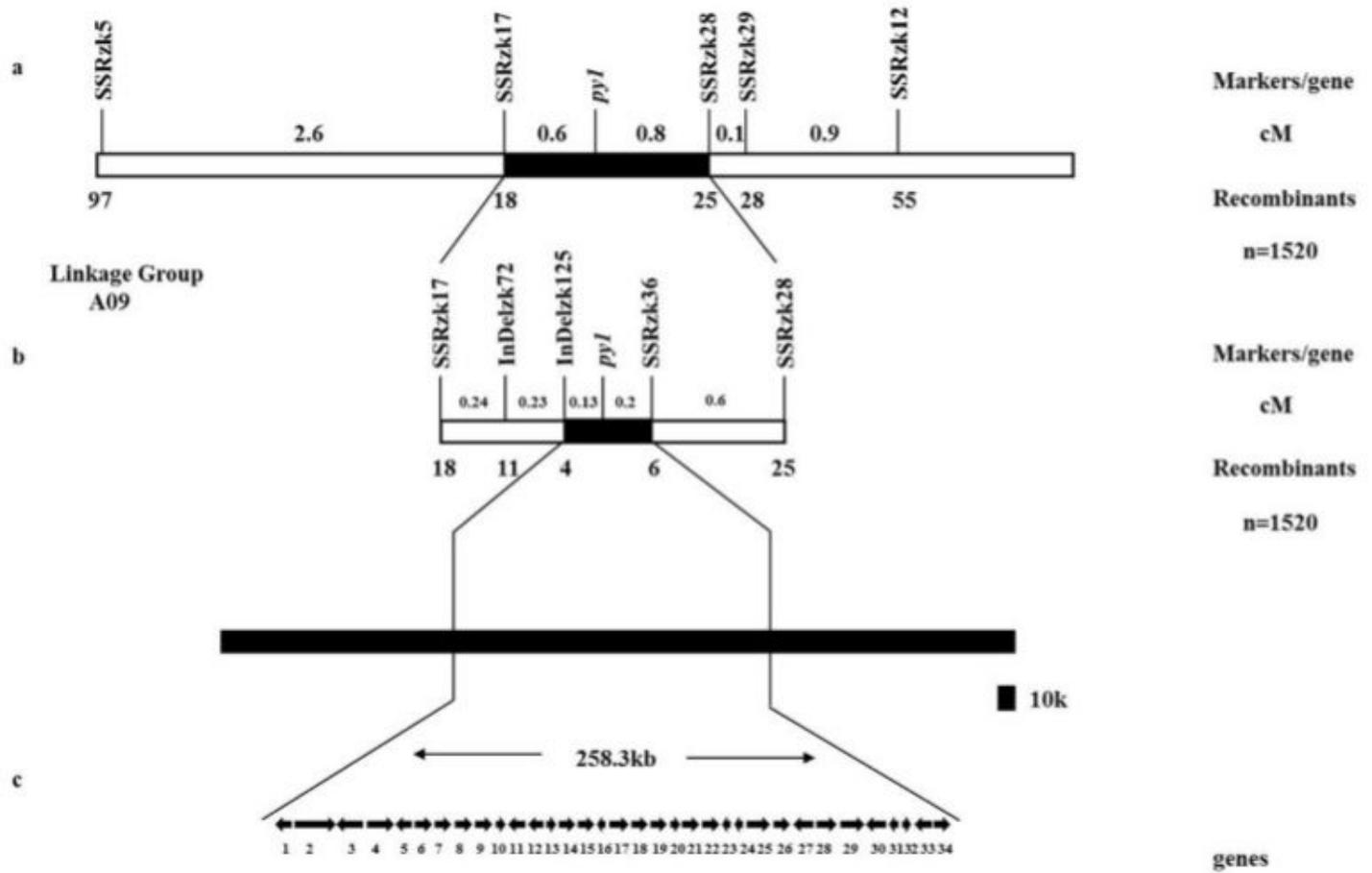


Figure 4

Genetic and physical *py1* maps and candidate gene analysis. a: Chromosome A09 linkage map was constructed with 1,520 *py1m* individuals from the No. 1 F3:4 family. The *py1* was preliminarily mapped between SSRzk17 and SSRzk28; b: Fine mapping of *py1*. The *py1* was restricted to the region between InDelzk125 and SSRzk36. Number of recombinants between the markers and *py1* is shown below the genetic map. The mapping distance above the linkage map is in centimorgan (cM) units; c: Candidate *py1* region and the annotated genes in the Brassica database. The *py1* locus was narrowed to a 258.3-kb region comprising 34 predicted genes. The numbers 1–34 refer to the candidate genes. The arrows indicate the direction of gene expression. Detailed information on the 34 genes is presented in Table S6

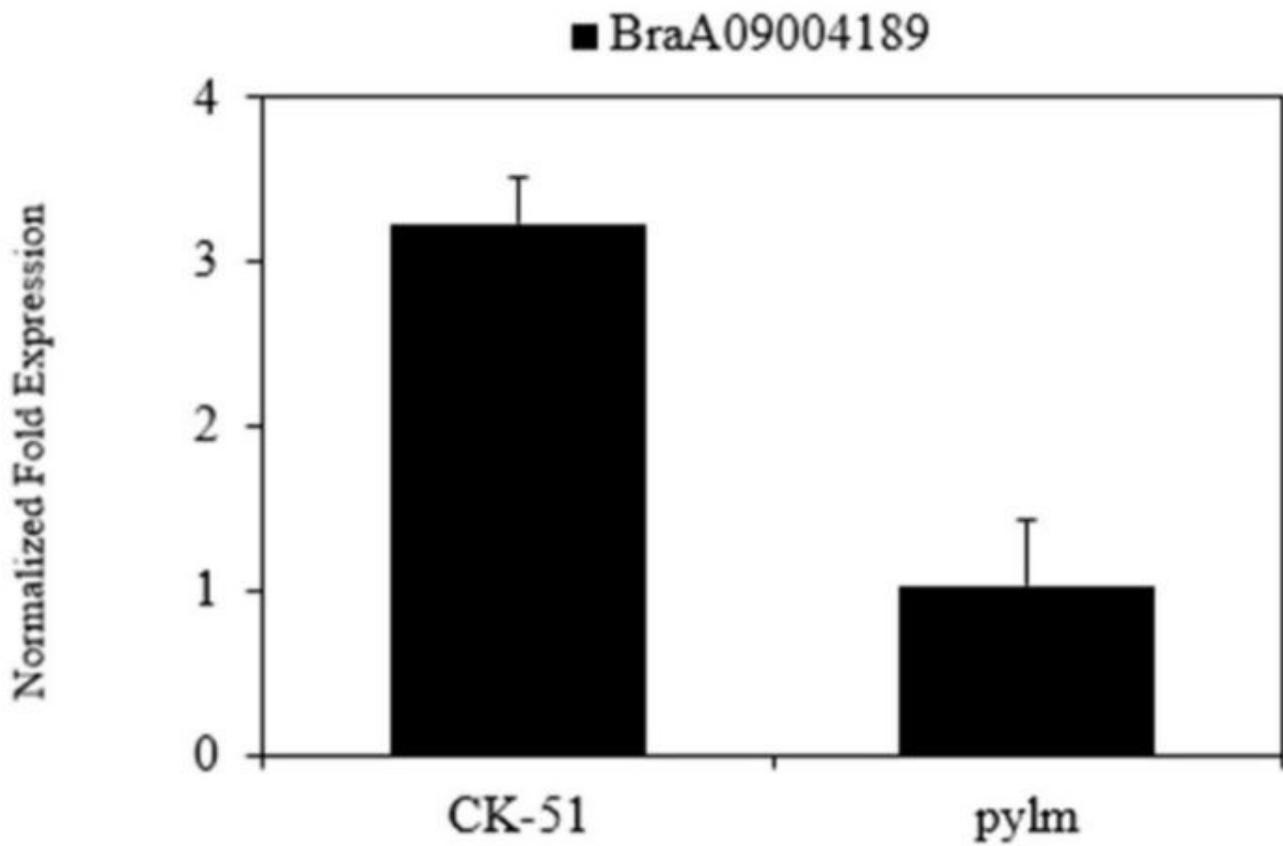


Figure 6

BraA09004189 expression analyses by qRT-PCR for pylm and 'CK-51'. Error bars indicate standard errors of the means of three replicates

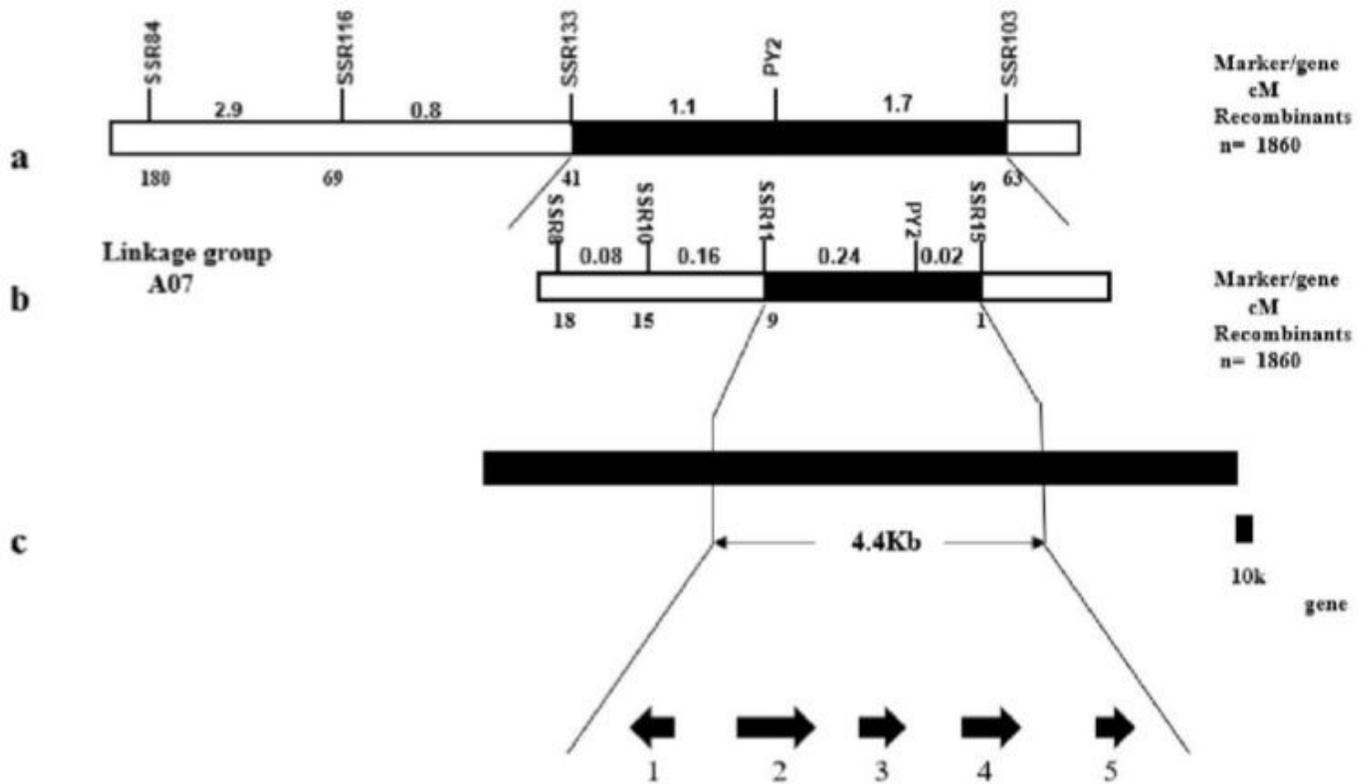


Figure 7

Genetic and physical maps of *py2* and candidate gene analysis. a: Linkage map of chromosome A07 was constructed with 1,860 individuals bearing the *pylm* phenotype in the No. 3 F3:4 family. The *py2* was preliminarily mapped between SSR133 and SSR103; b: Fine mapping of *py2*. The *py2* was restricted to the region between SSR11 and SSR15. The number of recombinants between the markers and *py2* is shown under the genetic map. The distance above the linkage map is in centimorgan (cM) units; c: Candidate *py2* region and annotated genes in the Brassica database. The *py2* locus was narrowed to a 4.4-kb region containing the five predicted genes BraA07001773–BraA07001777. Arrows indicate the direction of gene expression. Detailed information on these genes is presented in Table S9

CK-51	MGAAKNNNNWAILEGEGGGSSGGNDASSSSQIPYSSSVVDTSLPLPLMIPRIIELCKDLF	60
pylm	MGAAKNNNNWAILEGEGGGSSGGNDASSSSQIPYSSSVVDTSLPLPLMIPRIIELCKDLF	60
Consensumgaaknnnnwailegegggssggndassssqipysssvvdtslplplmipriielckdlf		
CK-51	SNWRELIDSLFSVERVSGGITNLLKVSVKKEDKESSITVRLYGPNTDYVINRQRELQAI	120
pylm	SNWRELIDSLFSVERVSGGITNLLKVSVKKEDKESSITVRLYGPNTDYVINRQRELQAI	120
Consensusnwrelidslfsvervsggitnllkvsvkkedkessitvrlygpntdyvinrqrelqai		
CK-51	KYLSAAGFGAKLLGGFGNGMVQSFINARTLAPSDMRQPKIAAEIAKELGKFHKVNIPGPK	180
pylm	KYLSAAGFGAKLLGGFGNGMVQSFINARTLAPSDMRQPKIAAEIAKELGKFHKVNIPGPK	180
Consensukylsaagfgakllggfgngmvqsfinarlapsdmrqpkiaaeiakelgkfhkvnipgpk		
CK-51	EPQLWVDILKFFEKASTLTFEEDRQKLYETISFEELYKEI IELREFTGLLNAPVVFVFAHN	240
pylm	EPQLWVDILKFFEKASTLTFEEDRQKLYETISFEELYKEI IELREFTGLLNAPVVFVFAHN	240
Consensuepqlwvdilkffekastltfeepdrqklyetisfeelykei ielreftgllnapvfvfahn		
CK-51	DLLSGNMLNEEEERLYLIDFEYGSYNYRQFDIGNHFNEYAGYDCDYTFYPTKEEQYHFI	300
pylm	DLLSGNMLNEEEERLYLIDFEYGSYNYRQFDIGNHFNEYAGYDCDYTFYPTKEEQYHFI	300
Consensudllsgnmlneeeerlylidfeygsynyrqfdignhfneyagydcdytfyptkeeqyhfi		
CK-51	KHYLQPKPDEVSVGEVESVFIETDAYKLASHLYWAVWAI IQARMSPIDFDYLGYYFFLRY	360
pylm	KHYLQPKPDEVSVGEVESVFIETDAYKLASHLYWAVWAI IQARMSPIDFDYLGYYFFLRY	360
Consensukhylqpkpdevsvgevesvfietdayklashlywawwai iqarmspidfdylgyfflry		
CK-51	NEYKKQKPLTFSLVTSYLSAS	381
pylm	NEYKKQKPLTFSLVTSYLSAS	381
Consensuneykkqkpltfslvtsylsas		

Figure 9

Amino acid sequence alignments of BraA07001774 in 'CK-51' and pylm

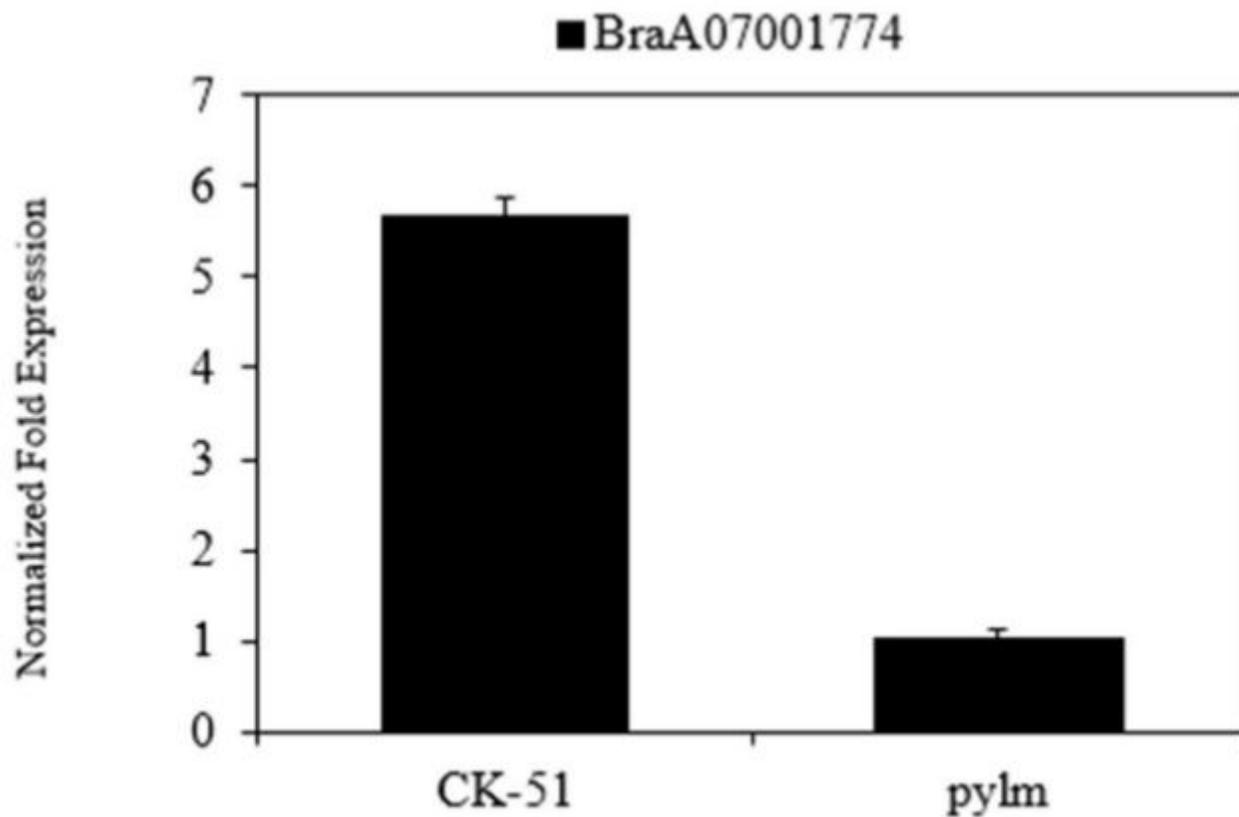


Figure 10

Expression analysis of BraA07001774 in pylm and 'CK-51' by qRT-PCR. Error bars indicate standard errors of the means of three replicates

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS.docx](#)
- [Fig.S1.pdf](#)
- [Fig.S2.pdf](#)