



Full Length Article

Development of High-Density Genetic Map by Specific-Locus Amplified Fragment (SLAF) Sequencing and Identification of QTLs Governing Flowering and Bolting Time in Chinese Kale

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Abstract

This study was aimed on finding quantitative trait loci (QTL) controlling flowering (FT) and bolting (BT) time traits in Chinese kale (*Brassica oleracea* var. *Alboglabra*) along with the candidate gene analysis. Firstly, a genetic map of high density was created by Specific-locus amplified fragment (SLAF) sequencing technique using an F₂ population of 159 individuals of *B. oleracea* var. *Alboglabra* cultivars 'Dasun' and 'LB07M' with varying flowering times. The genetic map contained 4,296 loci, 8,500 SNPs spanning 1,075.66 cM over 9 chromosomes. The linkage analysis fine mapped the loci controlling BT and FT within 0.315 and 0.314 cM, respectively. Quantitative trait loci (QTLs) analysis showed a locus on Chr 2 governing bolting time (BT) and flowering time (FT) traits, explaining 41.14 and 48.08% of phenotypic variation (PVE), respectively along with one minor QTL of BT explaining 15% of PVE on chromosome 7. The QTLs were aligned with the physical map positions of a *B. oleracea* reference genome. The candidate genes were preliminary annotated based on homology, located within the QTLs regions for the BT and FT traits. Regarding BT trait, two candidate genes were identified as Bo2g024555 and Bo2g009900, which control shoot apical and floral meristems, respectively. But, the gene Bo2g048220, which controls gibberellic acid production, was selected as being putatively responsible for the FT trait.

In conclusion, the QTLs found in this study provide information for marker-assisted selection and could contribute to develop early maturing Chinese kale varieties. © 2020 Friends Science Publishers

Keyword: Bolting time; Chinese kale; Flowering time; Genetic map; SLAF-seq

Introduction

The genus *Brassica* includes different vegetables and oil crops that are grown in different countries. Chinese kale (*Brassica oleracea* var. *Alboglabra*) was originated in Southern China. This vegetable was cultivated on nearly 50000 ha in Guangdong Province of China during 2014 (Li *et al.* 2015). Commonly, three to four crops of Chinese kale are produced in a single year. Along with the domestic consumption, Chinese kale is also exported to Asia, Europe and USA.

Bolting (BT) and flowering (FT) time are important agronomic traits of crop quality, production (Zhang *et al.* 2018). Understanding genetic mechanisms governing bolting and flowering time is an essential factor in plant breeding (Oded *et al.* 2014). Plant flowering is a series of

developmental events. Budding and bolting mainly controls flowering time (FT) of plants. Several genes control FT trait that is an important determinant for the evolution and domestication. Genes controlling FT trait have been explored in *Arabidopsis thaliana* (Koornneef *et al.* 1998). Nearly 80 genes have been shown to control the flowering process either by direct or indirect mechanisms (Levy and Dean 1998). During the entire growth period of Chinese kale, BT and FT traits directly influence the time of harvest, yield and quality.

Genetic map and QTL analysis are important approaches for marker assisted breeding of plants. High-density genetic map is an essential resource for mapping of the phenotypic traits of interest (Liu *et al.* 2016a, b). Next-generation sequencing techniques have leveraged the advantages of molecular markers. SLAF sequencing, when

assisted by next-generation sequencing technologies, is a useful strategy for discovering single nucleotide polymorphisms (SNPs) (Li *et al.* 2019). SLAF-seq has significantly improved the resolution of QTL mapping in numerous crops by large-scale SNPs discovery (Sun *et al.* 2013b). Previously, different QTLs controlling flowering time in *B. oleracea* have been identified using genetic linkage maps developed by crossing the members of different sub-species and old molecular markers such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSR) (Li *et al.* 2015; Honghao *et al.* 2016; Rahman *et al.* 2018). These maps cannot serve effectively for molecular assisted selection of traits for breeding Chinese kale as these were of low genetic density. Hence, there is a need to develop a high-density genetic linkage map that can be used to find loci governing BT and FT traits in Chinese kale plants. The availability of a high-density genetic map of Chinese kale along with the presence of reference genome of Arabidopsis can further assist in the identification of novel genetic resources controlling the flowering time trait of Chinese kale.

Keeping in view the importance of high-density genetic resources, it deems necessary to build a genetic map of relatively high density to perform QTL mapping of flowering traits of Chinese kale. Hence, this study was carried out to identify the loci controlling BT and FT traits in Chinese kale plants, construction of a high density genetic map and candidate gene analysis.

Materials and Methods

Mapping population

An F₂ population was raised by crossing 'Dasun' and 'LB07M' lines of Chinese kale. The line 'Dasun' has long bolting (BT) and flowering (FT) times and was the male in the cross. 'LB07M' has BT and FT and acted as female during cross. An F₂ population consisting on 159 lines was consisted derived from single seed.

Phenotypic evaluation of flowering and bolting time

The two parents (30 plants of each), F₁ (30 plants) and the F₂ plants were sown on 26 September of 2018 year in the field at Research station of Guangdong Academy of Agricultural Sciences, Guangzhou (25.1N, 115.0E), China. The soil at the research station is a sandy loam with adequate fertility. Field layout consisted of two-row plots, 20 (L) × 25 (W) cm, with planting following a randomized block design. The flowering and bolting times were evaluated using the values of each line and parent. Two rows were planted in each plot for avoidance of errors caused by the border effects. BT was noted as the day when the main flower stalk had grown to 1.0 cm. FT was noted when the first flower was fully opened.

DNA extraction

Young leaves from F₂ individuals and two parental lines were collected. For SLAF library construction, DNA was extracted using a Sangon DZup plant DNA extraction kit following the provided instructions. DNA quality was assessed by standard electrophoresis method.

SLAF library construction and data analysis

SLAF sequencing was performed in accordance to the methodology proposed by Sun *et al.* (2013a). Briefly, preliminary studies were performed to select suitable restriction enzymes for the production of restriction fragments and high-quality SLAFs. Different restriction enzymes were screened in a pilot study and finally the genomic DNA of Chinese kale was digested with "HaeIII" and "Hpy166II" enzymes. Afterwards, a single nucleotide overhang was joined with the obtained fragments and duplex tag-labeled sequencing adaptors were ligated with "A-tailed" fragments. SLAFs (DNA fragments) of 400 to 500 bp were separated and sequenced on an Illumina HiSeq 2500 system.

The identification and genotyping of SLAF markers were performed according to the procedure of Sun *et al.* (2013a). Raw reads with < Q30 scores were removed. The left-over reads were allotted to the plant samples according to the duplex barcode sequences and clustered on the basis of sequence homologies perceived by BLAST analysis.

Genetic linkage map construction

The genetic map was constructed in accordance to the Wu *et al.* (2018). The incomplete significant segregation distortion markers were removed, leaving 4,337 Marker loci showing MLOD values larger than 5.0 were used for linkage group (LG) creation (Zhang *et al.* 2015). By calculating the MLOD value between two tags, the labels with MLOD value less than 3 of other SLAF tags were filtered out; a total of 4,296 loci was generated in the 9 linkage groups. Efficient maximum likelihood estimation method was used for each chromosome to construct a genetic map using HighMap software.

QTL analysis

QTL analysis was performed by rQTL using an interval mapping method (Broman *et al.* 2003). The walking speed of 1 cM was set during composite interval mapping (CIM) (Zhiwen *et al.* 2012). The statistical likelihood ratio (LOD score) was calculated to denote the significance of each QTL interval. Afterwards, the threshold of the LOD score for significance ($P=0.05$) was sorted using 10,000 permutations as suggested by Liu *et al.* (2016a, b).

Annotation of candidate genes

The candidate genes found in major QTL regions were annotated by performing sequence alignments with a reference genome of *Brassica oleracea* in the Ensembl plant

genome database and the Bolbase database (Yu *et al.* 2013) using the Blastn function with default parameters. Information regarding the gene functional annotations and protein features were acquired from different public databases as described by (Liu *et al.* 2016a, b).

Results

Genetic analysis of the BT and FT traits

The F₂ population of *B. oleracea* was created by crossing ‘Dasun’ and ‘LB07M’ lines. This population was used to investigate the inheritance of FT and BT. The values for FT and BT ranged 42 to 80 and 37 to 61 days respectively (Fig. 1). The phenotypic distribution patterns of both traits in the F₂ population (Fig. 1) showed that both the traits are inherited quantitatively.

Analysis of SLAF sequencing data and genotyping

DNA sequencing provided nearly 4.8 Gb raw data containing 423.21 M clean reads with fragments length ranging from 264 to 314 bp (Table 1). The Q30 score was 94.78%, and the guanine-cytosine proportion was 36.10%. The details of SLAF sequencing are provided in Table 1. A total of 11.5 M and 15.4 M high quality reads were obtained (Table 2). The aa × bb segregation patterns with 13,329 SLAFs were used for further analysis (Fig. 2).

High-density linkage map construction

A total of 4,296 markers were mapped onto nine chromosomes, designated Chr 1-Chr 9 (Table 3). The average mapped markers integrity was 99.77%, as indicative of the high quality of the genetic map. Ultimately, a genetic linkage map of 1,075.66 cM was obtained (Fig. 3). On an average basis, chromosomes contained 477.3 spanning at an average distance of 119.52 cM. The map lengths of the 9 chromosomes ranged from 83.86 cM (Chr 2) to 164.24 cM (Chr 6). Chromosome 9 and 2 contained the maximum (592) and minimum (295) numbers of markers, respectively (Table 3).

QTL mapping of FT and BT traits

Phenotypic data of FT and BT traits is provided in Fig. 1. Three QTL were detected for both traits (Table 3). The primary QTL for BT (LOD=17.77) was identified as the map position of the chromosome 2 (Table 3 and Fig. 5). This accounted for 41.14% of the total phenotypic variance for the trait. Whereas, one minor QTL controlling the same trait was located at the chromosome 7 (LOD=6.56) and accounted for 15.00% of the total phenotypic variance for the BT trait (Table 4).

The primary QTL for the FT was also located at the chromosome 2, and accounted for 45.08% of the total phenotypic variance for the FT trait (Table 4 and Fig. 4, 5).

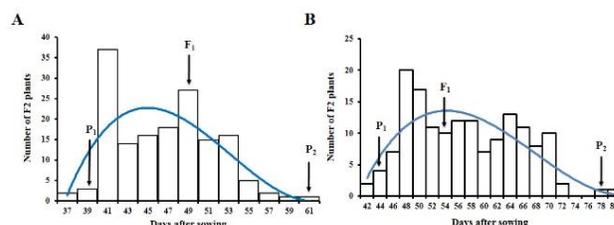


Fig. 1: The phenotypic distribution of the bolting (A) and flowering time (B) traits in the F₂ population of *B. oleracea*. P₁ shows the average bolting or flowering time of parent one (LB07M). P₂ shows the average bolting or flowering time of parent two Dasun. F₁ shows the average bolting or flowering time of F₁ generation

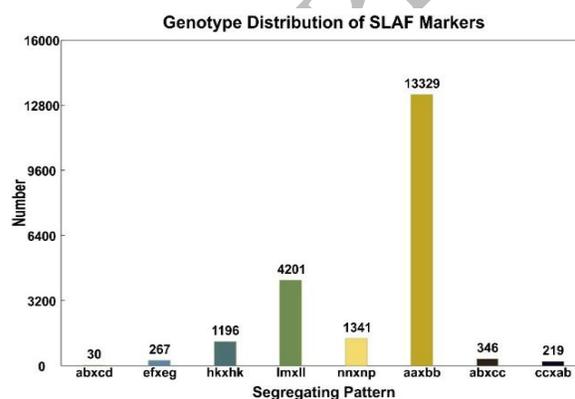


Fig. 2: Numbers of each marker segregation type on the linkage maps of Chinese kale. X-axis represents segregation patterns whereas y-axis shows number of SLAFs

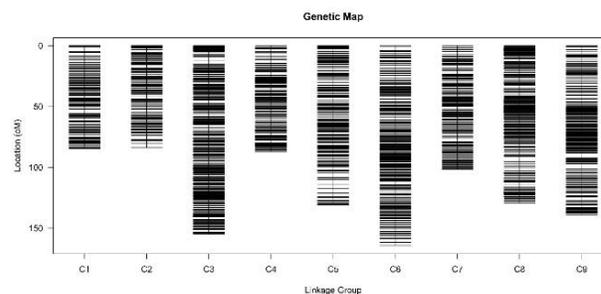


Fig. 3: Distribution of SLAF markers on nine chromosomes of Chinese kale. A black bar indicates a SLAF marker. The x-axis represents chromosome number and the y-axis indicates genetic distance in centi-morgan (cM)

It is worth mentioning that both major QTLs controlling the FT and BT traits were located on chromosome 2 and have a total spanned distance of 83.86 cM. This indicated that a pleiotropic or neighbor gene(s) are governing the FT and/or BT traits.

Association of SNP markers and functional genes

Based on the evidences from previously published literature

Table 1: Results summary of the SLAF sequencing

Parameter	Value
Enzyme digestion protocol	HaeIII + Hpy166II
Digestion normally	91.37%
Digestion partly	8.63%
Fragment length	264-314bp
Clean reads	423.21M
Average Q30	94.78%
Average GC content	40.53%
Develop the total number of SLAF	191,209
Paired-end mapped reads	74.01%
Singled-end mapped reads	5.46 %
Polymorphic SLAF	27,875
Number of linkage groups	9
Number of markers above	4,296
Genetic map total map distance	1,075.66cM
Genetic map average distance	0.25cM

Table 2: SLAF-seq data summary for Chinese kale F₂ population

Samples	Clean reads	Q30 %	GC %	SLAF number	Total depth	Average depth
Dasun	11,506,750	95.05	40.12	146,077	5,487,967	37.57
LB07M	15,469,125	94.63	40.50	155,551	7,134,346	45.86
Offspring	2,492,061	94.77	40.53	122,703	1,174,674	9.57

Table 3: Basic characteristics of Chinese kale linkage groups

Chromosome	Marker no.	Map length	Maximum distance	Marker interval	Integrity (%)	Double crossover (%)	Missing (%)
Chr 1	286	84.71	3.27	0.30	99.82	0.01	0.18
Chr 2	295	83.86	3.59	0.29	99.80	0.00	0.20
Chr 3	760	154.81	3.34	0.20	99.75	0.01	0.25
Chr 4	390	87.12	2.58	0.22	100.0	0.01	0.00
Chr 5	460	130.89	3.91	0.29	99.79	0.02	0.21
Chr 6	368	164.24	2.64	0.45	99.51	0.07	0.49
Chr 7	527	101.59	3.21	0.19	99.73	0.01	0.27
Chr 8	618	129.33	3.65	0.21	99.79	0.02	0.21
Chr 9	592	139.11	4.22	0.24	99.74	0.02	0.26
Total	4296	1075.66	4.22	0.25	99.77		

Table 4: An overview of QTLs related with FT and BT traits

QTL ID	Chromosome	Start (cM)	End (cM)	max LOD	PVE
1.1	2	6.983	7.298	17.77	41.14
1.2	7	48.81	49.12	6.56	15.00
2.1	2	6.04	6.35	18.80	48.08

PVE= Phenotypic contribution rate

Table 5: Selected candidate genes associated with BT and FT traits in *B. oleracea* found on major QTLs

Gene name	Gene ID	Function	Reference
SAM1	Bo2g089890	Regulation of shoot apical meristem.	(Takada <i>et al.</i> 2001; Vroemen <i>et al.</i> 2003)
WNK8	Bo2g009900	Regulating floral meristem, phyllotactic patterning	(Chandler 2014; Zhang <i>et al.</i> 2016)
GA20OX3	Bo2g048220	Initiation of flowering.	(Mutasa-Göttgens and Hedden 2009; Plackett <i>et al.</i> 2011; Rebers <i>et al.</i> 1999; Tenreira <i>et al.</i> 2017)

(Table 5) three candidate genes (Bo2g089890, Bo2g009900 and Bo2g048220) were selected mainly governing BT and FT traits. The gene Bo2g089890 (SAM1) is homologous of Arabidopsis gene AT3G15170 which encodes the cup-shaped cotyledon protein-2 that regulates shoot apical meristem (Arús and Orton 1983; Takada *et al.* 2001; Vroemen *et al.* 2003). The second selected gene Bo2g009900 (WNK8) is homologous to Arabidopsis gene AT5G41990 which regulates the floral meristem and phyllotactic patterning (Chandler 2014; Zhang *et al.* 2016).

Discussion

In this study, SLAF sequencing was used to construct a high-density genetic map of *B. oleracea* using an F₂ segregating population of 159 individuals obtained by crossing plants of Chinese kale lines 'Dasun' and 'LB07M'. Parents of the mapping population had different bolting time and flowering time profiles. Particularly, 'Dasun' has longer bolting and flowering times than 'LB07M'. Our map spanned 1,075.66 cM in 9 linkage groups, designated Chr1–

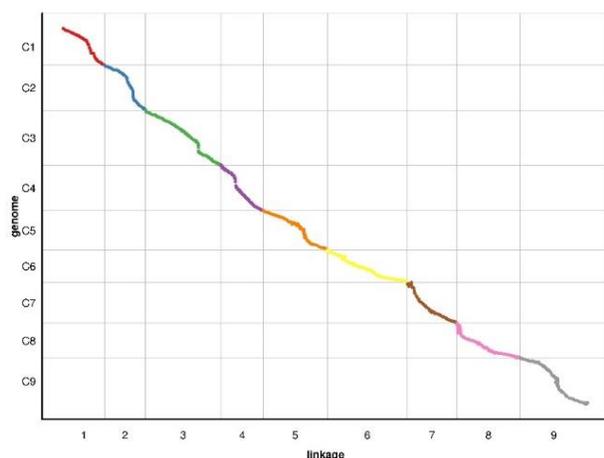


Fig. 4: The collinearity of chromosomes with the *B. oleracea* reference genome. The x-axis indicates the genetic distance of *B. oleracea* chromosomes accordingly, and the y-axis represents the linearity order of the physical position in the soybean genome

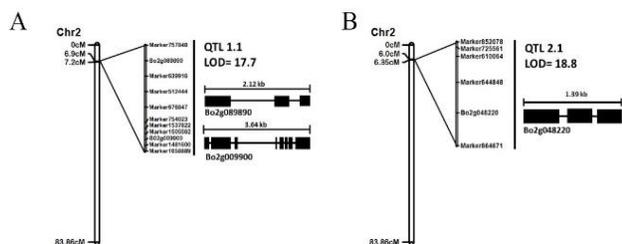


Fig. 5: Distribution of QTLs and selected candidate genes of BT and FT traits on Chromosome-2 of Chinese Kale. (A) Candidate genomic region putatively controlling BT trait on chromosome-2. (B) Candidate genomic region putatively controlling FT trait on chromosome-2

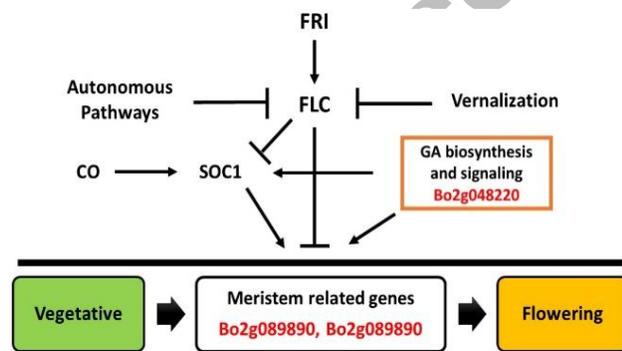


Fig. 6: Possible involvement of selected candidate genes in floral pathway of Chinese kale plant. Selected genes ID are shown in red colour

Chr9. There were a few maps available for Chinese kale despite of many linkage maps have been reported for *B. oleracea*. Compared with previously reported genetic map of *B. oleracea* (Babula *et al.* 2003; Huang *et al.* 2017), this newly constructed map had fewer gaps and high linearity

between genetic and physical distance.

Flowering time correlates with production and quality of Chinese kale. In a previous study, loci controlling flowering time were mapped in F_2 populations using SSR and SRAP markers (Li *et al.* 2015). The two QTLs were found on LG5 at a distance of 1.0 cM, governing bolting and flowering times in Chinese kale (Li *et al.* 2015). In this study both major QTL of BT and FT (1.1 and 2.1) were located at chromosome 2 at the distance less than 1.0 cM, showing their presence at an identical locus (Table 4 and Fig. 5). The correlation coefficient between BT and FT time in F_2 was 0.872, showing a very significant positive correlation. Some previous studies *e.g.*, Chaim *et al.* (2001); Fazio *et al.* (2003) and Rao *et al.* (2003) have also reported that QTLs for closely related traits are likely to be located on identical or same regions of chromosomes.

Three candidate genes were selected based on the reference genome, previously published literature (Table 5) and their homologous genes related to same traits in other plants. The putative role of these candidate genes in floral pathway is shown in Fig. 6. Two candidate genes, Bo2g089890 and Bo2g009900, that were selected on the major QTL 1.2, which controls the traits for bolting time or first flowering bud. These genes encode the cup-shaped cotyledon protein-2 (Bo2g089890) and serine/threonine-protein kinase (Bo2g009900), which have been shown to regulate shoot apical and flower meristems, respectively, according to the GO annotations and previously published literature (Table 5). The gene Bo2g009900, is homologous to Arabidopsis gene AT5G41990, and encodes a WNK8 like serine/threonine-protein kinase. The same gene in Arabidopsis plants has been shown to regulate the flowering time by modulating the photoperiod pathway (Wang *et al.* 2010). The WNK8 protein also interacts with the EDM2 protein that in turn, modulates floral meristem and developmental processes (Tsuchiya and Eulgem 2010). The flower organ specification processes are centered within the flower meristem (FM) to generate floral organs (Chandler 2014), with the FM differentiation determined by the stem cells within the shoot apical meristem (Zhang *et al.* 2016). This coordinated role of SAM and FM highlights the importance of both of these meristems in bolting time or appearance of the first flower node. When considering the importance of the shoot apical and flower meristems in the development of first flower node (Zhang *et al.* 2018), the genes Bo2g024555 and Bo2g009900 are important candidates for bolting time or appearance of the first flower node trait in *B. oleracea*. Chromosome 02 appears to be a strong candidate having major QTLs with the genes controlling bolting time.

The candidate gene for flowering time, Bo2g048220, is homologous to *Arabidopsis* AT5G07200, and encodes the gibberellic acid (GA) 3-oxidase (GA3ox) protein. The GA oxidases proteins primarily dictate early floral initiation (Rebers *et al.* 1999; Mutasa-Göttgens and Hedden 2009; Tenreira *et al.* 2017) and fruit development (Rebers *et al.*

1999) by regulating different floral networks (Mutasa-Göttgens and Hedden 2009). The GA3ox catalyzes the last step in bioactive GA biosynthesis. GA is a regulator of flowering initiation (Plackett et al. 2011). The exogenous application of GA₃ and GA₄ has shown early bud development in apple plants (Bertelsen et al. 2002).

As both major QTLs are residing on chromosome 02, this genomic region can be helpful for developing early maturing Chinese kale varieties using marker assisted breeding technology.

Conclusion

This is the first report highlighting the involvement GA3ox, WNK8 and SAM1 genes in the quantitative inheritance of early BT and FT traits in Chinese kale plants. Secondly, the high-density genetic map of Chinese kale constructed in this study will offer a suitable basis for further study of Chinese kale, such as gene mapping, map-based cloning of specific genes, quantitative trait locus mapping and marker-assisted selection.

Acknowledgements

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