Molecular markers based on sequence variation in BoFLC1.C9 for characterizing early- and late-flowering cabbage genotypes

Md. Abuyusuf1,2, Ujjal Kumar Nath1,3, Hoy-Taek Kim1,4, Md. Rafiqul Islam1, Jong-In Park1* and Ill-Sup Nou1* 

Abstract

Background: Cabbage (Brassica oleracea var. capitata) is popular worldwide for consumption as a leafy vegetable. Premature flowering is triggered by low temperature, and deteriorates quality of cabbage as vegetable. In general, growers prefer late-flowering varieties to assure good quality compact head. Here, we report BoFLC1.C9 as a gene with clear sequence variation between cabbage lines with different flowering times, and proposed as molecular marker to characterize early- and late-flowering cabbage lines.

Results: We identified sequence variation of 67 bp insertions in intron 2, which were contributed in flowering time variation between two inbred lines through rapid down-regulation of the BoFLC1.C9 gene in early-flowering line compared to late-flowering one upon vernalization. One set of primer ‘F7R7’ proposed as marker, of which was explained with 83 and 80% of flowering time variation in 141 F2 individuals and 20 commercial lines, respectively.

Conclusions: This F7R7 marker could be used as genetic tools to characterize flowering time variation and to select as well to develop early- and late-flowering cabbage cultivars.

Keywords: F7R7 marker, FLC, Early- and late-flowering, Cabbage

Background

Cultivated as a leafy vegetable, cabbage belongs to the Brassicaceae family and is popular worldwide. Flowering makes rapid elongation of the stem linked to the development of an indeterminate inflorescence [1] and the crucial transition from the vegetative phase to the reproductive phase of the plant’s life cycle [2], which reduces its market quality and consumer preference. Early-flowering at premature condition leads in reduction of yield and commercial values. Therefore, vegetable growers prefer late-flowering varieties to produce high quality and economically valuable vegetables [3, 4]. Considering the value of early- and late-flowering cabbage varieties, it is important to predict flowering time before planting. The genetic features of flowering pathways, which are mediated by environmental signals, have been previously well characterized in Arabidopsis thaliana [5, 6].

The FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), and LEAFY (LFY) genes are characterized through functional analysis and denoted as the main floral integrators [7–9]. FT acts as florigen and this protein is conserved in the most flowering plants [10]. SOC1 and the transcription factor LFY encoding MADS-box protein, of which act as floral activator for controlling floral patterning and floral meristem to identity the male and female reproductive organs during the process of flower development [11–13]. In Brassicaceae, expression of the key gene FLOWERING LOCUS C (FLC) is regulated by the perception of vernalization (passing the vegetative phase at low temperature for certain duration) [14, 15]. MADS-box transcription factor is encoded by the FLC genes and repressed flowering of the plants through inhibiting downstream floral integrator genes [6, 16–20].

Brassica plants showed natural variation in flowering time, which provides an excellent resource for explaining the molecular mechanism behind it. In B. oleracea, four
BoFLCs have been identified [21], but differences in the alleles of these FLC genes have not been confirmed; thus it is unknown how they were contributed in variation of flowering time? A functional allele of BoFLC2 was identified in an annual Brassica [15], the mutant allele boflc2 explained the role of FLC in B. oleracea and Arabidopsis. A recent study on sequence polymorphism of four FLC paralogs in B. oleracea indicated that they are not candidate in flowering time variation [22].

Many crops of B. oleracea species show remarkable morphological diversity and popular for their diverse edible parts, like inflorescences, axillary buds, leaves and stems [23]. Presently, two reference genomes of B. oleracea are available [24, 25], however based on only one reference sequence is not enough to collect the entire gene repertoire in the species, like structural variants, presence/absence variants (PAVs) and copy number variants [26–28]. A pangenome has been published for explaining such variation in B. oleracea [23]. Tettelin et al. [29] introduced the concept of pangenome in 2005 for full genomic makeup of a given species, which represents possible structural variation absent in the reference sequence.

To date, there has been little analysis of the molecular markers involved in the variation of flowering time by FLC in cabbage (a sub-family member of B. oleracea). Herein, we propose molecular markers based on pangenome data of flowering integrator genes of B. oleracea for characterization of early- and late-flowering genotypes before planting in the field.

**Results**

**Selection of the genes and their phylogenetic positions**

The AtFLC and central floral integrator genes of Arabidopsis thaliana [AtFT, AtSOC1, AtLFY, AtCO (CONSTANS LIKE), AtVRN (VERNALIZATION), AtSVP (SHORT VEGETATIVE PHASE) and AtSPL (SQUAMOSA PROMOTER BINDING LIKE)] sequences were collected from TAIR (https://www.arabidopsis.org/) and a syntenic gene search for B. oleracea using the BRAD database (http://brassicadb.org/brad/) retrieved syntenic genes of BolFLC, BolFT, BolSOC1, BolLFY, BolCO, BolVRN, BolSVP, and BolSPL. The genes were also cross-checked in the Bolbase (http://www.ocri-genomics.org/bolbase/genes.htm). Finally, Hidden Markov Models (HMM) profiling against EnsemblPlants (http://plants.ensembl.org/) and Bolpangenome (http://www.brasicca genome.net/) revealed 3, 2, 1, 6, 3, 2 and, 5 BoFLC, BoFT, BoSOC1, BoLFY, BoCO, BoVRN, BoSVP, and BoSPL genes, respectively in B. oleracea (Table 1).

**Analysis of sequences and similarity of BoFLC, and integrator genes**

The structures of the selected twenty five genes were accomplished to know their protein length, locations on chromosomes, and distribution of domains (Table 1). Our analysis showed that the genes contained: MADS-box, K-box, MADS MEF2-like, MADS AFFECTING FLOWERING 5-like isoform X1, Phosphatidylethanolamine-binding protein, Floricaula/Leafy protein, SAM, DNA-binding C-terminal, B-box type zinc finger protein with CCT, CONSTANS-LIKE 14, CONSTANS-LIKE 3, B3 domain-containing transcription factor VRN1, VEFS-Box of polycomb protein, and Squamosa promoter-binding-like protein domains. We also searched Ensembl Plants (https://plants.ensembl.org/index.html) to identify the ortholog sequences of these genes, and calculated their percent identity, query coverage, and gene order conservation (GOC) with A. thaliana and B. napus (Additional file 1: Table S1).

**Detection of DNA polymorphism in the selected gene**

PCR (polymerase chain reaction) was used to amplify gene-specific forward and reverse primer pairs covering the promoter (1000 bp at the 3’ UTR, untranslated region) to the stop codon of each of the genes (BoFLC, BoFT, BoSOC1, BoLFY, BoCO, BoVRN, BoSVP, and BoSPL). Different combinations of the designed primer pairs were used to detect any size polymorphism in PCR amplicons between early- and late-flowering cabbage lines. However, no size polymorphism was detected for most of the genes (Additional file 2: Figure S1 and Additional file 3; for BoFLC genes as representation), except in BoFLC1.C9. Further, we analyzed details of the BoFLC1.C9 gene to confer the nature of polymorphism. By using primer sets F7R7 with expected product sizes of 438 bp, revealed distinct size polymorphisms with insertion/deletion (Indel) mutations in the second intron (Fig. 1, Table 2).

**Cloning, sequencing, and sequence alignment**

PCR amplicons from early- and late-flowering lines were cloned, sequenced and aligned to detect the number and position of the sequence polymorphism. Alignment of cloned sequences against the reference genes confirmed the presence of a 67-bp insertion in the second intron of early-flowering BN623 line at 2711–2712 bp position only for BoFLC1.C9 (Additional file 2: Figure S2).

This mutation of the target gene may have altered gene expression and flowering time. We analyzed the second intron of BoFLC1.C9 (Bo9g173400) because of the presence of a 67 bp insertion in the second intron in the early-flowering line BN623. We found inserted conserved segments of ‘A’ and ‘F’ of intron 1 of AtFLC sequence, of which contains the six segments A – F, in the second intron of BoFLC1.C9 (Fig. 2), which reported as the suppressor of FLC gene [30] and produce early-flowering.
**Table 1** In silico analysis of 25 flowering pathway genes collected from the Bolpangenome database for their protein length, chromosomal location (based on the Bolpangenome and EnsemblPlants database), and distribution of domains for each gene

| Gene Name ID | Protein (aa) | Chromosomal Location | Domains |
|--------------|--------------|----------------------|---------|
| BoFLC1.C9    | Bo9gl73400   | C9:3033935–35,038,038 (+) | MADS-box superfamily, K-box, MADS MEF2-like |
| BoFLC3.C3    | Bo3g005470   | C3:2126630–1,299,928 (+) | MADS-box, K-box, MADS MEF2-like |
| BoFLC4.C3    | Bo3g100540   | C3:33574670–35,750,749 (+) | MADS AFFECTING FLOWERING S-like isoform X1, MADS-box, K-box |
| BoFT.C5      | Bo5g025100   | C5:8644739–8,466,343 (-) | Phosphatidyethanolamine-binding protein |
| BoFT.C8      | Bo8g104520   | C8:3663547–36,665,731 (+) | Phosphatidyethanolamine-binding protein |
| BoSOC1.C3    | Bo3g038880   | C3:17200121–17,202,561 (+) | MADS MEF2-like |
| BoSOC1.1.C4  | Bo4g024850   | C4:40213854–40,235,941 (+) | MADS MEF2-like |
| BoSOC1.2.C4  | Bo4g105720   | C4:53013986–53,017,593 (+) | MADS MEF2-like |
| BoFLY.C2     | Bo2g161690   | C2:5096976–50,959,525 (+) | Floricaula/Leafy protein, MADS-box, SAM domain, DNA-binding C-terminal domain |
| BoCO.C1      | Bo1g105550   | C1:32389932–32,391,351 (+) | Zinc finger protein CONSTANS-LIKE 3 |
| BoCO.C3      | Bo3g143010   | C3:51379117–51,380,915 (+) | Zinc finger protein CONSTANS-LIKE 14 |
| BoCO.C4      | Bo4g002240   | C4:153423–154,846 (+) | Zinc finger protein CONSTANS-LIKE 3 |
| BoCO.C5      | Bo5g073010   | C5:24568350–24,569,416 (+) | Zinc finger protein CONSTANS-LIKE 14 |
| BoCO.C7      | Bo6g031220   | C6:6726663–67,277,299 (+) | VeFS-Box of polycomb protein |
| BoCO.C8      | Bo8g295190   | C8:40723422–40,726,294 (+) | VeFS-Box of polycomb protein |
| BoVRN1.C1    | Bo1g115980   | C1:34825349–34,828,013 (+) | VeFS-Box of polycomb protein |
| BoVRN2.C8    | Bo9g163730   | C9:48,282,858–48,283,872 (+) | MADS-box protein 8 |
| BoVRN3.C3    | Bo3g095330   | C3:43476716–34,748,076 (+) | VeFS-Box of polycomb protein |
| BoSVP.C4     | Bo4g149800   | C4:40723422–40,726,294 (+) | VeFS-Box of polycomb protein |
| BoSVP.C8     | Bo8g010000   | C8:35302475–35,305,535 (+) | VeFS-Box of polycomb protein |
| BoSPL.C2     | Bo2g062840   | C2:18471026–18,472,176 (+) | VeFS-Box of polycomb protein |
| BoSPLC4      | Bo4g042390   | C4:947,145–9,472,001 (+) | VeFS-Box of polycomb protein |
| BoSPL1.C6    | Bo6g029290   | C6:6726663–67,277,299 (+) | VeFS-Box of polycomb protein |
| BoSPL2.C6    | Bo6g031220   | C6:7482570–7,483,508 (+) | VeFS-Box of polycomb protein |
| BoSPL2.C8    | Bo8g118210   | C8:41670596–41,672,303 (+) | VeFS-Box of polycomb protein |

**Intron sequence variation interferes with gene expression**

To understand the effect of vernalization on early- and late-flowering lines, the relative gene expression of **BoFLC1.C9** (Bo9gl73400) was estimated by qPCR using cDNA synthesized from leaf samples of pre-vernalized (0 week), weekly vernalized plants (1–11 week) and the samples of control plants (without vernalization). In both early- and late-flowering lines, expression of **BoFLC1.C9** (Bo9gl73400) decreased with increasing duration of vernalization. However, the expression of the gene in the early-flowering line was significantly lower (at least 5 times) at any given time point compared to the late-flowering line. In fact, even after 11 weeks of vernalization, the expression of this gene in the late-flowering line was never as low as in the pre-vernalized early-flowering line. In the early- and late-flowering lines, expression started to decline significantly after 3 and 6 weeks of vernalization, respectively (Fig. 3). In case of control (without vernalization), gene expression was non-significantly declined with the advance of time points (0–11 weeks) for both of the early- and late-flowering lines. In addition, unresponsiveness to the vernalization of the early-flowering line confirmed by the non-significant difference in gene expression between vernalized and non-vernalized plants at each time point. We concluded that a 67 bp insertion in the second intron may cause a loss of normal function and lower expression of **BoFLC1.C9**.
Characterization of flowering time with proposed markers in the F$_2$ population

We recorded the flowering times, which were 140 to 150 days after sowing (DAS) for the early-flowering line BN623 and $\geq$190 DAS for the late-flowering line BN3848, while flowering times were varied from 142 to 210 DAS in the F$_2$ generation (Fig. 4). Among the F$_2$ individuals, fraction of heterozygous plants (presence of both parental bands) showed flowering time variations between 143 to 200 DAS. Our proposed marker ‘F7R7’ can explained 83% of the phenotypic variations of 141 F$_2$ individuals regarding DAS (Additional file 2: Figure S3 and S4). While, 20 commercial lines showed 80% match with this marker considering the PCR amplification of early- and late-flowering lines (Fig. 5). The marker ‘F7R7’ (Table 2) was designed on the BoFLC1.C9 gene, which was able to explain 83% phenotypic variations of the early- and late-flowering corresponded to PCR amplifications in the F$_2$ generation (Additional file 2: Figure S3 and S4).

![Gene structure of BoFLC1.C9, in which different primers are designed to detect polymorphism. a Schematic representation of the positions of primers used to detect polymorphism covering the promoter to stop codon region. Red box, promoter; black box, exon; black line, intron; forward arrow, forward primer; reverse arrow, reverse primer. b Polymorphic PCR amplicons with the F7R7 marker for the late-flowering line (P$_1$) = BN3848 and the early-flowering line (P$_2$) = BN623, as well as their F$_1$ hybrid. M: 100 bp DNA marker (bioD, Gwangmyaong, Korea)](image_url)

**Table 2** List of primers used to follow the mutation of BoFLC1.C9 gene in parental lines, F$_1$ and segregating F$_2$ by polymerase chain reaction (PCR); and relative expression analysis of the target and Actin genes in quantitative PCR (qPCR)

| Gene name | Accession number | Primer code | Sequences (5’→3’) | Product size (bp) | Marker type | References |
|-----------|------------------|-------------|--------------------|-------------------|-------------|------------|
| BoFLC1.C9 | Bo9g173400       | F7:         | GGAAAGCAACATGGTGATGA CATGGTGTAACAGAGTCC | 438               | Indel       | Present study |
|           |                  | R7:         |                    |                   |             |            |
|           |                  | F:          | CTCTACAGCTTCTCTCCGG TGGAACCAAGATCCTCAAAGC | 119               | qPCR        | Present study |
|           |                  | R:          |                    |                   |             |            |
| Actin     | AF044573         | 1F:         | TTCTCTCTCCACACGGCAT CGGGGATGTTGATGCT | 235               | qPCR        | [62]       |
|           |                  | 1R:         |                    |                   |             |            |
|           |                  | 2F:         | GTGGTGCCGCGGTAATCCG | 251               | qPCR        | [63]       |
|           |                  | 2R:         |                    |                   |             |            |
| XM_013753106 | 3F: | ATCACACTTTTACATGAGC TGGTAGATGAGTCCAGAGG | 241               | qPCR        | [64]       |
|           |                  | 3R:         |                    |                   |             |            |
Discussion

Flowering is a multipart physiological characteristic controlled by a series of integrator genes, vernalization genes, and by the plant spending its vegetative phase exposed to cold temperatures for a certain period. It is necessary to develop molecular markers to select early-and late-flowering cabbage lines before sowing from a breeding population. Overwinter types must be exposed to cold (vernalization) to transition from vegetative growth to flowering, but this is not obligatory for

![Diagram of intron 1 from Arabidopsis thaliana FLC (AtFLC) and sequence alignment of BoFLC1.C9 in the early-flowering line BN623.](image)

**Fig. 2** Pictogram of intron 1 from Arabidopsis thaliana FLC (AtFLC) and sequence alignment of BoFLC1.C9 in the early-flowering line BN623. Red-colored (AtFLC region A) and green-colored (AtFLC region F) sequences are conserved in the inserted portion. Exons are shown in black. Intron 1 from AtFLC is partitioned to show the regulatory segments involved in various FLC activities [30]. Segments A and F are essential for down-regulation of the gene and other segments are maintained the regulatory system. Sixty-seven base pair-long segments inserted into intron 2 of BoFLC1.C9 are shown in red and green; +1 indicates the start codon (ATG).

![Relative expression of BoFLC1.C9 using cDNA from vernalized (V) and non-vernalized (C: control) early-flowering line BN623 and late-flowering line BN3848.](image)

**Fig. 3** Relative expression of BoFLC1.C9 using cDNA from vernalized (V) and non-vernalized (C: control) early-flowering line BN623 and late-flowering line BN3848. Bars indicate the mean values of five replications ± SD. Different lower case letters indicate statistically significant differences at p < 0.05 according to Tukey’s method.
spring varieties, which are generally grown in areas with shorter seasons. Many genetic and environmental factors are involved in this transition, and signals of these factors are integrated into the causal effect of flowering [31]. Transition of flowering from vegetative phase is the resultant of the interactions between transducer proteins and integrator signals, which either promote or inhibit the transition process [10, 32]. In Arabidopsis, the key flowering genes have been identified and characterized [6, 33]. Flowering genes are involved in a ‘floral integrator’ network, comprised with six regulatory gene pathways [31]. FLC is a key member of the floral integrator network, and its expression is influenced by low temperature.

There are multiple FLC and ‘floral integrator’ homolog genes in cabbage (B. oleracea var. capitata) were duplicated before divergence of Brassica from their ancestor [21]. The group of genes with MADS-box domains consists of five MADS AFFECTING FLOWERING (MAF) proteins [34]. FLOWERING LOCUS M (FLM, known as MAF1) represses flowering [35, 36], through vernalization dependent repression of FLM and MAF1 genes and accelerates flowering [37–39].

Among the selected 25 genes (3 BoFLC, 2 BoFT, 3 BoSOC1, 1 BoLFY, 6 BoCO, 3 BoVRN, 2 BoSVP, and 5 BoSPL), we found DNA size polymorphisms only in BoFLC1.C9 (Bo9gl73400) gene between early- and late-flowering lines by using several primers at different positions. The polymorphic BoFLC1.C9 (Bo9gl73400) gene contains MADS-box and K-box domain proteins, thus it might be involved in flowering time variation in cabbage. By contrast, in the Brassicaceae, suppression of flowering is mediated by vernalization, which mainly involves major-effect changes at a few loci [40–43]. Of
these, FLC appears to be the predominant source of variation in the vernalization response [44, 45], and ultimately causes the variation in flowering time. Association and/or quantitative trait locus (QTL) mapping corroborated that variation in flowering time of B. oleracea, B. rapa, and B. napus is linked to polymorphism in FLC homologs and responded upon vernalization [46–49].

In BoFLC1.C9 (Bo9g173400), the ‘F7R7’ marker (Table 2) showed polymorphism across intron 2. We linked this mutation with flowering time variation in the F2 population of the cross (BN3848 (late-flowering) × BN623 (early-flowering)) and twenty commercial cultivars. FRI gene was identified as a major determinant of flowering time variation in A. thaliana population through its effect on FLC [50, 51]. In addition, indel polymorphism of CONSTANS LIKE 1 (COL1) has also been associated with flowering time variation in B. nigra [52].

In this study, lower relative expression of the BoFLC1.C9 gene was found in the early-flowering line, where presence the Indel variation, compared to the late-flowering line (Fig. 3). The presence of spliced long noncoding RNA in COOLAIR locus of late-flowering line leading to higher FLC expression and delay flowering. This is supported by the identification of a single nucleotide polymorphism (SNP) in A. thaliana haplotype which caused splicing of long noncoding RNA (lnRNA) at COOLAIR locus leading to higher FLC expression and increased requirement for vernalization or delayed flowering [38]. COOLAIR splicing disrupted COOLAIR production due to splice at the COOLAIR proximal acceptor site [53] and suggested that splicing of lnRNA could be modulated FLC expression quantitatively through co-transcriptional coupling mechanisms [53]. Conservation of COOLAIR [54] and presence of the AtFLC antisense RNA in Arabis alpina [55] and B. rapa [56] have been pointed as strong commonality in the regulation of FLC across the Brassicaceae.

Noncoding sequence alteration has recently been identified in AtFLC haplotype groups with different degree of AtFLC expression and epigenetic silencing [57]. In B. oleracea, one of two major FLC haplotypes is transcriptionally repressed by exposure to cold more slowly than others [46]. Intron 2 of the BoFLC1.C9 gene might be responsible for FLC repression activities by accommodating segments of intron 1 of Arabidopsis FLC (Fig. 2). The intron 1 of Arabidopsis FLC segments are required to maintain FLC repression [30]. The segregating F2 population showed wide range of variation in flowering time followed neither in continuous variation nor in Mendelian inheritance (Fig. 4).

Results obtained from DNA size polymorphism analysis of BoFLC1.C9 and the F2 phenotypes suggest that BoFLC1.C9 is a likely candidate for causing variation in flowering time in this population. It has previously been reported that regulation of FLC is controlled by the regions in its intron [30, 58], with polymorphisms leading to differential expression and splicing patterns. The proposed marker ‘F7R7’ based on 67 bp insertion in intron 2 accounted for 83% of flowering time variation, among the F2 population. While, in commercial lines ‘F7R7’ makers matched up to 80%. Ridge et al. [15] found 65% flowering time variation in the F2 population of a cross of late- and early-flowering cauliflower lines. A 67 bp insertion in the second intron of BoFLC1.C9 gene in the early bolting line made a distinct mutation and disrupted the function of the gene and showed lower expression caused early-flowering.

Conclusions
Using molecular markers and relative expression-based approaches, we reported the sequence variations in BoFLC1.C9 gene for characterizing early- and late-flowering cabbage lines. Our result suggests that naturally occurring ‘Indel’ confirmed by ‘F7R7’ marker in intron 2 in the BoFLC1.C9 gene is able to characterize early- and late-flowering cabbage lines up to 80% variation. This marker might be useful for selecting desired early- and/or late-flowering cabbage cultivars before cultivation. Further experiments on presence of copy number of BoFLC1.C9 gene could elucidate the mismatched fractions of the ‘F7R7’ marker more clearly.

Methods
Plant materials and evaluation of flowering time
Two inbred lines (late-flowering BN3848 and early-flowering BN623) with distinct flowering times varying by 40–45 days, were used to develop F1 and F2 generation plants by crossing and selfing, respectively. The average flowering time was 140 to 150 DAS for the early-flowering line BN623 and ≥ 190 DAS for the late-flowering line BN43848. The F1 generation was designated as intermediate flowering with 160–175 DAS. Whereas, twenty commercial cabbage cultivars collected from Sunchon National University Cabbage breeding germplasm, which were characterized as early-flowering (140–150 DAS) and late-flowering (≥ 195 DAS). A pot-based glasshouse trial was conducted at Sunchon National University, South Korea using five plants of parental lines, five from the F1 generation, and 141 from the F2 generation to record variations in flowering time. Seeds of the plant materials were germinated in a multi-pot tray using cocopit soil, and were allowed to grow in a growth chamber at 24 °C, 60% relative humidity, and under 16 h/8 h (light/dark) conditions for up to 60 days. Then, in September 2015, eight weeks old plants at 8
leaf stage were transferred to larger pots (30 × 25 cm) filled with a mixture of 50% cocopit and 50% soil. Plants were allowed to overwinter inside the glasshouse, where temperature and day length were recorded in the range of −5°C to 8°C with 10 h/14 h (light/dark) during the winter and 12–17°C with 12 h/12 h (light/dark) during the spring. The number of days to flowering was recorded as the day on which the first flower of individual plants was observed to open after seedlings were transplanted to larger pots in the glasshouse. Plants that did not flower within 190 days were considered to be late flowering.

Gene selection and sequence analyses
The flowering pathway gene sequences of *A. thaliana* were collected from TAIR (https://www.arabidopsis.org/) and the syntenic genes of *B. oleracea* were collected from the BRAD database (http://brassicadb.org/brad/) using syntenic gene search and cross-checked against the Bolbase (http://www.ocri-genomics.org/bolbase/gens.htm) as well as EnsemblPlants (http://plants.ensembl.org/) databases. A complementary method, Hidden Markov Models (HMM) profiling was performed using the Bopangenome (http://www.braonica-genome.net/) database to increase the accuracy of the identified genes. The National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) web tool was used for searching the domains.

Isolation of DNA and detection of DNA polymorphism
The protocol described by Ishizawa et al. [59] with slight modifications was followed for extracting DNA from four weeks leaf samples of parental lines, five F1 plants, 141 F2 plants, and 20 commercial cultivars. Primer3Plus online tool was used for designing locus-specific primers. The newly designed and previously reported primers [15, 22, 46, 60] (listed in Table 2, and Additional file 1: Table S2A,B) were used to identify DNA polymorphisms between the contrasting lines. A total volume of 20 μl was used in PCR, which contained 1 μl DNA (80 ng), 1 μl (10 pmol) of forward and 1 μl (10 pmol) of reverse primers, 8 μl Prime Taq-prenix (2×) (GENETBIO Inc., Gwangmyaong, Korea), and 9 μl ultra-pure H2O. PCR was carried out in a thermo-cycler set as 5-min initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 1 min, final elongation at 72°C for 10 min, and cool down at 4°C. PCR products were separated in 2% agarose gel stained with HiQ blue mango (20,000×) (bioD, Gwangmyaong, Korea) and visualized with ultraviolet light.

Cloning and sequencing of the polymorphic gene
Promega Purification Kit (Promega, Madison, WI, USA) was used for purification of the amplified DNA fragments following manufacturer’s instructions. TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) was used for cloning. Three independent clones were separated from the PCR amplicon of the polymorphic gene, amplified in both early- and late-flowering lines. The universal primers M13F and M13RpUC were used for sequencing the cloned DNA by using ABI3730XL sequencer (Macrogen Co., Seoul, South Korea). Cloned sequences were aligned with a reference sequence using ClustalOmega [61] to identify the types and positions of sequence variations.

Isolation of RNA, synthesis of cDNA and expression profiling of the polymorphic gene
The RNaseq Mini Kit (Qiagen, Hilden, Germany) was used for isolating total RNA from leaf samples collected from the different levels of vernalized plants, and total RNA was purified with a Qiagen RNase-free DNase1 Kit (Qiagen, Hilden, Germany). For the vernalization treatment, a set of plants at 8th leaf stage was transferred to incubators (TOTA clean system; model: TOGA UGSR01, Daejong, Korea) maintained at 4°C with 14 h/10 h (light/dark) until 11 weeks of vernalization. Leaf samples were excised from five replicated plants of prevernalized (0 week), 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, and 11-week of vernalization and control (no vernalization) as checked. The leaf samples were immersed quickly in liquid nitrogen and stored at −80°C to avoid degradation of RNA. The RNA was quantified by using NanoDrop™ 1000 Spectrophotometer (Wilmington, DE, USA) and 6 ng of RNA per sample was used for synthesizing first-strand cDNA by using the Superscript™III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) with oligo-dT primer. Gene-specific primers of the candidate *FLC* (BoFLC1.C9) and *Actin* genes were used for qPCR (Table 2). A total volume 10 μl PCR mastermix for each sample contained 1 μl of cDNA (70 ng), 1 μl (10 pmol) of each forward and reverse primer, 2 μl double distilled water, and 5 μl Taq™ from the SYBR®Green PCR Kit (ThermoFisher, California, USA)of was used for conducting qPCR. A Lightcycler®96SW 1.1 (Roche, Dusseldorf, Germany) programmed as pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 15 s was used for carrying out the qPCR. Gene expression levels for each sample were normalized by using the average ‘Ct’ value of the 3 *Actin* genes as a reference. The 2^ΔΔCt method was used to calculate relative expression [34].
Additional files

**Additional file 1:** Table S1. Ortholog species, ortholog ID, percent identity, percent query coverage and GOC of 25 genes of BoFLC, BoFT, BoSLOC1, BoLFY, BoCCO, BoVRN, BoVIN, BoSVP and BoSPL of *B. oleracea*.

**Additional file 2:** List of newly designed primers on the identified 25 genes used for searching polymorphism by PCR. Table S2 B. List of previously published primers on the reported FLCs in *B. oleracea* used in this study. (DOC 272 kb)

**Additional file 3:** PCR amplicons of BoFLC1.C9, BoFLC3.C3 and BoFLC4.C3 in late-flowering line BN3848 (P1) and early-flowering line BN623 (P2). PCR products with respective primers from start to stop codons of the genes (Additional file 1: Table S1A) were run on a 1.5% agarose gel and their corresponding amplicon sizes are mentioned. M is a 100-bp size marker. Figure S2. Sequence alignments of the BoFLC1.C9 gene cloned from early- and late-flowering lines. Variation of a 67-bp insertion in the early-flowering line BN623 is highlighted in red color.

**Additional file 4:** PCR-amplicons of 141 F2 segregating population with F7R7 primers of Indel marker of BoFLC1.C9 gene. P1 = Late-flowering parent (BN3848), P2 = Early-flowering parent (BN623), F1 = (BN3848 x BN623); black and red colored numbers of the F2 individual are matched and mismatched lines, respectively as early- and late-flowering lines. M = 100 bp DNA marker. Figure S4. Regression and correlation coefficient between marker dosage and phenotypes as days to flowering after sowing (DAS) explained by the F7R7 marker in 141 F2 individual. ** indicates p < 0.01. (PPT 2295 kb)

**Additional file 5:** Sequence alignment of the total sequences of the gene BoFLC3.C3 and BoFLC3.C3 cloned from early-flowering line (BN623) and late-flowering line (BN3848) aligned with reference sequence of BoFLC3.C3 and BoFLC3.C3 genes, respectively. Red highlighted letter indicate SNPs variations in the early-flowering line. Forward and reverse arrows indicate forward and reverse primer sets used for cloning and sequencing. (DOC 747 kb)

Abbreviations

COI: CONSTANS LIKE 1; DAS: Days after sowing; FLC: FLOWERING LOCUS C; FLM: FLOWERING LOCUS M; FT: FLOWERING LOCUS T; GOC: Gene order conservation; Indel: Insertion/deletion; LFY: LEAFY; MAF: MADS AFFECTING FLOWERING; PAW: Presence/absence variants; PCR: Polymerase Chain Reaction; SD: Standard deviation; SOCI: SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1; SPL: SQUAMOSA PROMOTER BINDING LIKE; SVP: SHORT VEGETATIVE PHASE; UTR: Untranslated region; VRN: VERNALIZATION

Acknowledgments

We thank the Asia Seed Co., Ltd., Republic of Korea for providing *B. oleracea* seeds.

Funding

This research was supported by the Golden Seed Project of the Center for Horticultural Seed Development (grant number 213007–05–3-3B110), the Ministry of Agriculture, Food and Rural Affairs, the Ministry of Oceans and Fisheries, the Rural Development Administration, and the Korea Forest Service, Korea. The funding body provided monthly salary for the first two authors and who were directly involved in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials

We declare that the dataset(s) supporting the conclusions of this article are included within the article (and its additional file(s)).

Authors’ contributions

The work presented here was carried out in collaboration with all authors. MA carried out the experiments, performed in silico analyses and cloning, and prepared the tables and figures and first manuscript draft. UKN collected primary gene data and wrote, edited, and finalized the manuscript. JIP formulated the experimental concept and provided the plant materials. HTK conducted greenhouse experiments and took care of the plant populations. MJI helped to extract RNA and synthesize cdDNA. ISN designed and participated in all experiments, and assisted in improving the technical aspects of the project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher’s Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

1Department of Horticulture, Sunchon National University, 255 Jungang-ro, Sunchon, Jeonnam 57922, Republic of Korea. 2Department of Agronomy, Patuakhali Science and Technology University, Patuakhali 8362, Bangladesh. 3Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensing 2202, Bangladesh. 4University–Industry Cooperation Foundation, Sunchon National University, 255 Jungang-ro, Sunchon, Jeonnam 57922, Republic of Korea.

Received: 7 September 2018 Accepted: 18 March 2019

Published online: 27 April 2019

References

1. Hébrard C, Trap-Gentil M-V, Lafon-Placette C, Delaunay A, Joseph C, Lefèbvre M, Barnes S, Maury S. Identification of differentially methylated regions during vernalization revealed a role for RNA methyltransferases in bolting. J Exp Bot. 2013;64(2):651–63.
2. Xia G, He Q, Zhao S. Physiological and biochemical properties analysis of late-bolting transgenic Chinese cabbage (*Brassica rapa* ssp. *pekinessis*). J Anim Plant Sci. 2015;25(Suppl):1:152–7.
3. Mao Y, Wu F, Yu X, Bai J, Zhong W, He Y, MicroRNA319a-targeted *Brassica rapa* ssp. *pekinessis* TCP genes modulate head shape in Chinese cabbage by differential cell division arrest in leaf regions. Plant Physiol. 2014;164(2):710–20.
4. Wang Y, Wu F, Bai J, He Y. BspSPL9 (*Brassica rapa* ssp. *pekinessis* SPL9) controls the earliness of heading time in Chinese cabbage. Plant Biotechnol J. 2014;12(3):312–21.
5. Ausin I, Alonso-Blanco C, Martinez-Zapater J-M. Environmental regulation of flowering. Int J Dev Biol. 2004;48(3–4):6189–705.
6. Fornara F, de Montaigu A, Coupland G. SnapShot: control of flowering in Arabidopsis. Cell. 2010;141(3):550 e552.
7. Blázquez MA, Green R, Nilsson O, Suisman MR, Weigel D, Gibberellins promote flowering of Arabidopsis by activating the LEAFY promoter. Plant Cell. 1998;10(5):791–800.
8. Nilsson O, Lee I, Blázquez MA, Weigel D. Flowering-time genes modulate the response to LEAFY activity. Genetics. 1998;150(1):403–10.
9. Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G. Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science. 2000;288(5471):1613–6.
10. Andrés F, Coupland G. The genetic basis of flowering responses to seasonal cues. Nat Rev Genet. 2012;13(9):627–39.
11. Liu C, Zhou J, Bracha-Drori K, Yalovsky S, Ito T, Yu H. Specification of Arabidopsis floral meristem identity by repression of flowering time genes. Development. 2007;134(10):1901–10.
12. Melzer S, Lins F, Gennien I, Vanneste S, Rohde A, Beeckman T. Flowering-time genes modulate meristem determinacy and growth form in Arabidopsis thaliana. Nat Genet. 2008;40(12):1489.
13. Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM. LEAFY controls floral meristem identity in Arabidopsis. Cell. 1992;59(5):843–59.
59. Ishizawa M, Kobayashi Y, Miyamura T, Matsuura S. Simple procedure of DNA isolation from human serum. Nucleic Acids Res. 1991;19(20):5792.

60. Okazaki K, Sakamoto K, Kkuchi R, Saito A, Togashi E, Kuginuki Y, Matsumoto S, Hirai M. Mapping and characterization of FLC homologs and QTL analysis of flowering time in Brassica oleracea. Theor Appl Genet. 2007;114(4):595–608.

61. https://www.ebi.ac.uk/Tools/msa/clustalo/ accessed on 20.01.2018.

62. Zhang B, Hu Z, Zhang Y, Li Y, Zhou S, Chen G. A putative functional MYB transcription factor induced by low temperature regulates anthocyanin biosynthesis in purple kale (Brassica oleracea var. acephala f. tricolor). Plant Cell Rep. 2012;31(2):281–9.

63. Nawaz I, Iqbal M, Hakvoort HH, Blik M, de Boer B, Schat H. Expression levels and promoter activities of candidate salt tolerance genes in halophytic and glycophytic Brassicaceae. Environ Exp Bot. 2014;99:59–66.

64. Lee J, Yang K, Lee M, Kim S, Kim J, Lim S, Kang G-H, Min SR, Kim S-J, Park SU. Differentiated cuticular wax content and expression patterns of cuticular wax biosynthetic genes in bloomed and bloomless broccoli (Brassica oleracea var. italica). Process Biochem. 2015;50(3):456–62.