Research Paper

The production and characterization of a *BoFLC2* introgressed *Brassica rapa* by repeated backcrossing to an F₁

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Flowering time is an important agronomic trait for *Brassica rapa* crops, and previous breeding work in *Brassica* has successfully transmitted other important agronomic traits from donor species. However, there has been no previous attempts to produce hybrids replacing the original *Brassica FLC* alleles with alien *FLC* alleles. In this paper, we introduce the creation of a chromosome substitution line (CSSL) containing a homozygous introgression of Flowering Locus C from *Brassica oleracea* (*BoFLC2*) into a *B. rapa* genomic background, and characterize the CSSL line with respect to the parental cultivars. The preferential transmission of alien chromosome inheritance and the pattern of transmission observed during the production of the CSSLs are also discussed.

Key Words: *Brassica oleracea*, *Brassica rapa*, flowering time, introgression, vernalization, chromosome segment substitution line.

Introduction

The *Brassica rapa* and *B. oleracea* species each contain several agronomically important crops. Varieties of *B. rapa* includes turnip, mizuna, napa cabbage, bok choy, and leafy vegetables grown as both food and feed crops; and *B. oleracea* contains many common food and feed crops, such as cabbage, broccoli, cauliflower, kale, savoy, and kohlrabi. *B. rapa*, *B. nigra*, and *B. oleracea* are diploid organisms capable of interspecific hybridization. This relationship was first identified by Nagaharu U in 1935 and is known as the triangle of U (U 1935). The respective genomes are referred to as the A, B, and C genomes. The three species are closely related, sharing a common ancestor that experienced a whole genome triplication (WGT) event approximately 15.9 million years ago (MYA) after its divergence from *Arabidopsis*

thaliana (Cheng *et al.* 2014). The speciation events that led to the A, B, and C genomes is estimated to have occurred approximately 4.6 MYA, identified by interspecific genome comparisons (Liu *et al.* 2014). The *B. rapa* (AA) and *B. oleracea* (CC) species serve as the parental genomes for the agronomically important allopolyploid *Brassica napus* (AACC); a seed oil-crop species that produces rapeseed and canola. Thus, *B. napus* is an amphidiploid species that is derived from the interspecific hybridization of *B. rapa* and *B. oleracea*.

Both *B. rapa* and *B. oleracea* possess a broad range of vernalization requirements amongst their respective varieties, ranging from near-obligate to a complete lack of the vernalization requirement. However, both species contain biennial varieties possessing near-obligate vernalization requirements, meaning exposure to a prolonged cold period is necessary for the transition from the vegetative growth state to inflorescence (Friend 1985). Vernalization maximizes reproductive success by preventing premature flowering prior to the winter season (Andrés and Coupland 2012). *B. rapa* varieties with a vernalization requirement generally require four to eight weeks of low temperatures (5°C) to flower

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(Kim *et al.* 2007). *B. oleracea* generally requires a longer cold period of six or more weeks of low temperatures (5°C) in order to flower (Friend 1985). In contrast to *B. rapa*, the onset of cold must take place after the plant has reached the mature vegetative growth stage of development for successful vernalization to occur in *B. oleracea* (Ito *et al.* 1966).

Vernalization in both species is controlled by the MADS domain protein FLOWERING LOCUS C (FLC), first identified in the model organism for plant genetic research *Arabidopsis thaliana* (Michaels and Amasino 1999). The expression of the floral integrators *FLOWERING LOCUS T* (*FT*) and *SOC1* are both regulated by *FLC* in a dose-dependent manner (Lee *et al.* 2000, Onouchi *et al.* 2000). Thus, *FLC* functions as the central suppressor of flowering. Subsequently, QTL studies conducted on flowering time in *B. napus*, *B. rapa*, and *B. oleracea* have identified various paralogs of *FLC* as strong contributors (Axelsson *et al.* 2001, Kakizaki *et al.* 2011, Okazaki *et al.* 2007, Razi *et al.* 2008, Schranz *et al.* 2002, Tadege *et al.* 2001, Zhao *et al.* 2010). Further examination of the genetic sequences of *FLC* alleles has revealed that allelic differences in the non-coding region of intron one are responsible for differences in flowering times between varieties in all three species (Kitamoto *et al.* 2013, Lin *et al.* 2005, Razi *et al.* 2008, Schiessl *et al.* 2014, Wu *et al.* 2012, Yuan *et al.* 2009). Other studies have identified the number of the functional copies of *FLC* paralogs (Golicz *et al.* 2016). While geographic climatic variation may partially account for the differences seen in flowering times due to the differing length and severity of the winter season at different latitudes (Irwin *et al.* 2016), other studies attribute the *FLC* sequence variations in Brassica species to the selective pressures exerted by agricultural breeding (Golicz *et al.* 2016, Martynov and Khavkin 2005).

In *B. rapa* cultivars, stable year-round production is compulsory because of their importance as a vegetable. To accomplish this, the development of late-bolting cultivars with a winter cropping pattern is required. Because *FLC* acts as a key regulatory gene in the vernalization pathway of both *B. rapa* and *B. oleracea*, and allelic differences in the *FLC* genes present in Brassica affects flowering time, we reasoned that selection for a cabbage-like strong vernalization requirement would be of agricultural benefit to *B. rapa* cultivars such as Chinese cabbage. We thus thought it useful to attempt to assign species-specific vernalization-response phenotypes to chromosomes using chromosome segment substitution lines (CSSL) derived from the interspecific crossing between both *B. rapa* and *B. oleracea*. Previous breeding work in Brassica using monosomic alien addition lines (MAALs) has successfully transmitted important agronomic traits such as disease resistance (Akaba *et al.* 2009, Chèvre *et al.* 1997a, Kaneko *et al.* 1996, Peterka *et al.* 2004, Tsunoda *et al.* 1980), and yellow seed color (Heneen *et al.* 2012) from donor species possessing the respective desired trait. Additionally, when making MAALs and CSSLs derived from different Brassica species, the use of chromosome specific markers identified each chromosome in the A,

B, and C genomes was very effective (Chèvre *et al.* 1997a, 1997b, Heneen *et al.* 2012, Li *et al.* 2013). However, the production and application of MAALs and CSSLs has been scarcely reported in Brassica, and to our knowledge there have been no previous attempts reported on the production of MAALs or CSSLs replacing the original Brassica *FLC* alleles with alien *FLC* alleles.

The objectives of this study were two-fold: 1) to analyze additional alien chromosome inheritance patterns during the process of the construction of a CSSL of *B. rapa* possessing an introgression of the *FLC* paralog *BoFLC2*, located on the upper-arm of Chromosome 2 in the *B. oleracea* genome; and 2) to characterize the vernalization-response phenotype and flowering time in a winter cropping pattern in relation to the parental lines. *BoFLC2* was selected as our target *FLC* allele for introgression, because it was shown to possess a strong contribution to the flowering time and reproductive development of cabbage (Okazaki *et al.* 2007, Ridge *et al.* 2014).

Materials and Methods

Plant materials and crossing scheme

A commercial cultivar, CR Kanki (Nihon Norin Seed Co. Ltd., Japan) of Chinese cabbage (*B. rapa* var. *pekinensis*) and a DH line (P01) of *B. oleracea* var. *capitata* cv. Reiho (Ishi seed company, Japan) were used as female and male parents, respectively. Flower buds were emasculated and immediately pollinated with fresh pollen grains collected from the male parent. Pollinated flowers were covered with thin paper bags. The ovaries were collected 4–20 days after pollination (DAP) for F₁ embryo rescue. Ovary culture was carried out according to the method reported by Inomata (1977). The surface-sterilized ovaries were placed on MS (Murashige and Skoog 1962) medium aseptically supplied with 3% sucrose and 0.8% agar, adjusted to pH 5.8. Plastic petri dishes 90 × 15 mm were used for the cultures and placed in a growth chamber maintained at 24°C with a 16 h photoperiod. Ovaries were kept on the medium until embryos were fully germinated and rooted. The seedlings were transplanted into 3.5-inch pots containing vermiculite soil:regular soil (1:1) and covered with transparent polyethylene sheet. The plants were then placed in a growth chamber maintained at 22°C with a 16 h photoperiod at light intensity of 300 μmol·m⁻²·s⁻¹. After proper hardening, the plants were transferred to a greenhouse. Twenty-Five F₁ hybrids were obtained from the culture of ovules derived from the crossing between *B. rapa* cv. CR Kanki and *B. oleracea* DH line Reiho P01. After adequate vernalization of F₁ plants, in order to restore seed fertility by doubling the chromosome number, colchicine solution 0.05% was applied on leaf axils of each F₁ plant as per the previous report (Chen *et al.* 1988). The flowers with pollen in the treated F₁ plants were self-pollinated to produce amphidiploids. Simultaneously, in order to produce BC₁ plants, the fertile pollen of the F₁ hybrids were backcrossed to *B. rapa*. The embryos

Fig. 1. Schematic diagram showing the development process of *BoFLC2* introgressed *B. rapa* via successive backcrossing to an F₁ (AACC) of *B. rapa* and *B. oleracea*.

obtained from the backcross were excised from the ovule at 20–30 days after crossing and cultured on MS medium. As a result, more than 60 BC₁ plants were obtained. Additionally, amphidiploids were used as the pollen parent and backcrossed to CR Kanki to produce BC₁F₁ plants. Ploidy level of BC₁F₁ plants was measured by flow cytometry, as described previously (Okazaki *et al.* 2005).

Then the BC₁F₁ plants were used as pollen parents and backcrossed to *B. rapa* resulting in 264 BC₂F₁ plants, from which 160 plants were genotyped by the C chromosome specific DNA markers and days-to-bolting after vernalization was examined (Fig. 1). To shorten the breeding cycle from the BC₁ to BC₃ generations, embryos obtained in the backcrossing were excised from the ovules 20–30 days after pollination and cultured on MS medium. The resultant plants were flowered by adequate vernalization (later mentioned). Among the BC₂F₁ plants, #81 and #174 plants, plants containing the *BoFLC2* genomic region were identified by genotyping, selected as female parents, and backcrossed to CR Kanki. In the resulting BC₃F₁ population, the #174-12 and #174-22 plants were selected from the #174 line and self-pollinated to produce BC₃F₂ lines. In the #174-12 line of BC₃F₂, three plants (#174-12-18, -26, and -28) homozygous for the *BoFLC2* allele were selected and self-pollinated. The obtained seedlings were characterized for vernalization requirement and genotyped by DNA marker analysis.

Phenotyping of the obtained progenies

Vernalization was given to the plants cultured in the test

tube for the BC₂F₁ and BC₃F₁, seedlings in Petri dish culture for BC₃F₂, and seedlings in soil for BC₃F₃, as follows. When the BC₂F₁ and BC₃F₁ seedlings formed roots in ovule culture, they were transferred to MS medium in test tubes, and treated in a growth chamber at 4°C for 8 weeks. After vernalization, they were planted in commercial soil suitable for growth. For the BC₃F₂ lines (#174-12 and #174-22), the seeds were sown on MS medium containing 1% sucrose and then grown at 4°C for 8 weeks. After vernalization, they were then planted in commercial soil.

For the BC₃F₃ generation, two lines, #174-12-18 and #174-12-28 were characterized to assess bolting and flowering under three different cropping patterns in field trials conducted in Ogata Ami-machi Inashiki-gun, Ibaraki-ken, Japan (35.9819516 Latitude, 140.2668901 Longitude). Comparison to the parental early-heading *B. rapa* cv. CR Kanki No. 100 and three late-heading commercial cultivars, *B. rapa* cv. Haruhinata, *B. rapa* cv. Kigaku No. 70, and *B. rapa* cv. Harurisu was tested using five to ten plants from each lineage ($n = 5$ to 10). For cropping pattern one, seeds were sown in commercial soil in a greenhouse environment on January 14th, 2017 using a hotbed for one month and then the seedlings were grown for 2 weeks without heating for acclimatization to the cold. After that, the seedlings were transplanted outdoors at the end of February and grown under field conditions (Supplemental Fig. 1). For cropping pattern two, seeds were sown in commercial soil in a greenhouse environment on January 14th, 2017, using a hotbed for one month. Then the seedlings were transplanted outdoors in the middle of February and grown under field conditions. For cropping pattern three, seeds were sown in commercial soil in a greenhouse environment on February 1st, 2017 using a hotbed for one month and then seedlings were transplanted outdoors at the beginning of March and grown under field conditions. For all three cropping patterns, plants were grown in the field under cover using vinyl tunnels (Type 9245) and 20 kg/a of nitrogen based fertilizer was applied to the soil. Flowering was assessed by the presence or absence of inflorescence (flower buds or flowers) and bolting stem length was measured, in centimeters, from the crown to the tip of the apical stem. For plants that had already flowered at the time of measurement, a stem bolting length of 100.0 cm was recorded. The flowering and bolting data was collected for cropping patterns one, two, and three on May 5th, 2017; May 16th, 2017; and May 19th, 2017; respectively. The data was then imported into R (Ihaka and Gentleman 1996) and statistical analysis performed for both flowering and bolting stem length in response to cropping pattern and lineage by linear regression, using factorial analysis of variance (ANOVA) with an interaction term for cropping pattern and lineage. For a significant interaction term, *post-hoc* pairwise comparison of least-square means with Tukey-method correction was used (Lenth 2016).

Genotyping and chromosome observation of the obtained progenies

For collection of genotype data, total genomic DNA was extracted from the young leaves of backcrossed progenies using the CTAB (cetyltrimethyl-ammonium bromide) method (Murray and Thompson 1980). The primer pairs identified in each C genome chromosome were selected from the *B. oleracea* linkage map, and their map positions were confirmed by mapping to a *B. oleracea* linkage map (Nagaoka *et al.* 2010) and by the Bolbase genome database. Markers were amplified by PCR in 5 µl reaction solution mixture (EmeraldAmp Max PCR Master Mix, Takara Bio. Inc., Japan) with the following parameters: 1 cycle of 94°C for 5 min, 35 cycle of 94°C for 1 min, optimized annealing temperature (50 to 56°C) for 30 s, and 72°C for 90 s followed by a final extension 72°C for 7 min. Electrophoresis was conducted using an 8–13% polyacrylamide gel (Kikuchi *et al.* 1999) for separation of amplified products. The gel was stained with a Gelstar solution (0.1 µl/10 ml; Takara Biomedicals, Japan). The chromosome number of seedlings of BC₃F₃ was determined from root tips according to the method reported by Karim *et al.* (2016).

Results

Characterization of BC₂F₁ population

The random assortment of C genome chromosomes during the meiosis of BC₁F₁ is thought to cause 50% of the transmission rate for each chromosome in the BC₂F₁ plant. To assess this theoretical ratio, two DNA loci whose positions are already determined in the linkage map (Nagaoka *et al.* 2010) were assigned to each C chromosome (**Supplemental Fig. 2**). Since the A and C genomes share nucleotide sequence similarity, we compared PCR profiles among CR Kanki, Reiho P01, and their hybrids to examine whether our designed DNA markers can distinguish between homologous sequences of the A and C genomes; consequently, 19 DNA markers were chosen (**Supplemental Fig. 2, Supplemental Table 1**). The screening of 169 BC₂F₁ plants using the chromosome specific DNA markers revealed that in most cases both loci were simultaneously transmitted, while in some plants a single locus of each chromosome was separately transmitted to a plant (**Fig. 2**). The transmission rate of C chromosome specific DNA loci ranged from 14.2% to 69.2%, depending on the chromosome. Each C chromosome was transmitted as a whole chromosome from 11.8% to 47.3% of the plants (32.5% on average), as indicated by the horizontal lines between the measurements of each loci on a given chromosome, and as an incomplete chromosome from 0% to 27.2% of the plants (9.24% on average).

The expected number of extra C genome chromosomes transmitted to the BC₂F₁ population ranges from 0 to 9. To assess chromosome inheritance in the BC₂F₁ plants, we used two C-chromosome specific DNA loci per chromosome. When at least one of the two C-chromosome specific loci is detectable in a BC₂ plant, the plant is regarded as an

Fig. 2. Transmission rate of DNA loci of each C chromosome in the BC₂F₁ plants (n = 169); recurrent parent, CR kanki (*B. rapa*); donor parent, Reiho (*B. oleracea*). Dotted line indicates the theoretical value of loci transmission. A and B refer to the two markers used to examine loci and are listed in **Supplemental Table 1**. Asterisks indicate deviation from the 1:1 ratio of loci transmission by distorted C chromosome assortment; * P < 0.05, ** P < 0.01, ***P < 0.001.

Fig. 3. Number of extra C chromosomes of 169 BC₂F₁ plants, predicted using the specific C genome chromosome markers, and their flowering response after 8w vernalization at the seedling stage. CR kanki (*B. rapa*) was used as recurrent parent, Reiho (*B. oleracea*) as donor parent.

aneuploid possessing the corresponding C-chromosome segment (**Fig. 3**). The marker assay revealed that the number of extra C genome fragments/chromosomes of the BC₂F₁ population ranged from 0 to 9 (4.59 on average), and was skewed towards both the smaller (n = 10–12) and the larger numbers (n = 16–19), as compared to the binomial theoretical distribution (**Fig. 3**). The number of days to bolting in the BC₂F₁ plants was observed along with CR Kanki and Reiho P01 after 8 weeks of vernalization, as mentioned in Materials and Methods. CR Kanki flowered up to 29–49 days after 8 weeks of vernalization, but Reiho P01 had no flowering. In the BC₂F₁ population (n = 169), 28% of the plants bloomed within 60 days after 8 weeks of vernalization and the remaining 72% of the plants did not. The groups containing more C genome fragments/chromosomes

tended not to bloom after 8 weeks of vernalization, indicating that C genome elements added to A genome made the plants resistant to vernalization.

To examine the effect of each C genome chromosome on the flowering response of the BC₂ plants, the plants were categorized with or without the respective chromosome, regardless of the number of extra C genome chromosomes added in each plant (Fig. 4). As a result, the plants with the C2A chromosome region, detected by the C2A DNA marker, significantly had no flowering after 8 weeks of vernalization, while the plants with the C6 chromosome had a higher flowering rate at the significant level ($p < 0.01$) as detected by binomial analysis (Fig. 4a). In contrast, the plants without the C2A and C6 chromosome regions revealed the promotion and the delay of flowering after 8 weeks of vernalization, respectively (Fig. 4b). It was previously reported that the *BoFLC2* allele that delays flowering in *B. oleracea* was found on the C2A chromosome region (Okazaki *et al.* 2007). Based upon these results, thereafter, we focused on C2A chromosome region.

Characterization of BC₃F₁ population

Among BC₂F₁ population, #81 and #174 plants, which

were confirmed to contain the C2A genomic region by the C2A DNA marker (designated as the C2A chromosome region), were selected as female parents and backcrossed with CR Kanki. #81 had only C2A and C2B chromosome regions, and #174 had C2A and C9A chromosome regions (data not shown).

The screening of 76 plants of the BC₃F₁ #81 line, using the C2 chromosome specific DNA markers, revealed that the transmission rates were 0.38, 0.43, and 0.45 in *BoFLC2*, C2A, and C2B markers, respectively (Fig. 5a). The DNA marker analysis indicated that #174 line contained a more limited region of the C2 chromosome (*BoFLC2* to C2A marker position), compared to the #81 line (*BoFLC2* to C2B marker position). The transmission rate of the #174

Fig. 4. Flowering rate of plants with (a) or without (b) each C chromosome marker in the BC₂ plants (n = 169); recurrent parent, CR kanki (*B. rapa*); donor parent, Reiho (*B. oleracea*). Dotted line indicates the mean value of flowering rate. Asterisks indicate significant differences by binomial analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 5. Transmission rate of C2 chromosome markers in the BC₃F₁ plants derived from #81 and #174 plant of BC₂ containing C2 chromosome segments that detected by the respective markers (a). Segregation of flowering time in the BC₃F₁ plants derived from #81 (b) and #174 (c) plant of BC₂ containing *BoFLC2* chromosomal segments, and its relevance to introgression of *BoFLC2*. NF indicates non-flowered plants.

line was the same as that of the #81 line. We found a segregation of flowering time in both the #81 and #174 BC₃F₁ lines after cold treatment at 4–9°C for 8 weeks (Fig. 5b, 5c). The plants with a C2A chromosome region, detected by the *BoFLC2* marker, significantly tended to have no flowering after vernalization in both lines. Counterintuitively, some plants not possessing the *BoFLC2* region revealed no flowering or delayed flowering after vernalization, while all of the control plants (CR Kanki) flowered within 28 days after vernalization. The #174 line contained a C9A chromosome region, however we did not check the segregation of the C9A chromosome region in this generation, but evidence of its transmission and segregation was present in the #174-12-26 fixed line (as discussed below).

Characterization of BC₃F₂ population

From the BC₃F₁ population, #81-4, #81-54, #174-12, and #174-22 plants were selected from the #81 and #174 lines and self-pollinated to produce BC₃F₂ lines. #81-4 was selected from the non-flowered plants having the *BrFLC2* homozygotes (lacking *BoFLC2*) and the other 3 lines from the heterozygote (*BrFLC2/BoFLC2*) BC₂ plants. We found segregation of the *BoFLC2* allele, including heterozygotes containing *BrFLC2* and *BoFLC2* alleles and homozygotes of both the *BoFLC2* and *BrFLC2* alleles, respectively (Fig. 6b–6d). The plants with a *BoFLC2* allele tended to show no flowering after vernalization. All of the control plants (CR Kanki) flowered within 28 days after vernali-

zation. We observed plants homozygous for the *BoFLC2* allele in the #174-12 line, but not in the #81-54 and #174-22 lines. Then, several plants (including #174-12-18, -26, and -28) homozygous for the *BoFLC2* allele were self-pollinated to develop the BC₃F₃ lines. We then examined the chromosome number in the root tips of the #174-12-18, #174-12-26, and #174-12-28 seedlings, and confirmed the euploidy (2n = 20; Data not shown).

On the other hand, we have the unexpected results that the #81-4 plants where the *BoFLC2* gene was not detected exhibited no flowering or delayed flowering (Fig. 6a), while all the control plants (CR Kanki) flowered within 28 days after vernalization. Therefore, we made a subsequent BC₃F₂ generation of the #81-4 lacking the *BoFLC2* gene (Supplemental Fig. 4). These subsequent plants (#81-4-18 and #81-4-19) revealed a flowering response like that of the *B. rapa* parent, indicating a failure to maintain tolerance against vernalization in the #81-4 line lacking the *BoFLC2* region.

The BC₃F₃ #174-12-18 and #174-12-28 populations show reduced flowering and bolting in a winter cropping pattern

A field trial was conducted on two BC₃F₃ populations, #174-12-18 and #174-12-28, homozygous for *BoFLC2*, along with CR Kanki No. 100 (recurrent parent) and three late-heading varieties of Chinese cabbage. Weather data recorded during the course of the experiment shows that daily mean temperatures were adequate for vernalization to occur (Supplemental Fig. 5). Results of the field trial showed that the C2 introgressed populations responded more like the late-heading varieties of *B. rapa* than that of the parental *B. rapa* cv. CR Kanki No. 100 (Supplemental Fig. 6, Supplemental Table 2). ANOVA on flowering in response to lineage and cropping pattern revealed that both main effects were statistically significant with respect to flowering, with p-values $p < 2.2 \cdot 10^{-16}$ and $p = 1.74 \cdot 10^{-9}$ for cropping pattern and lineage, respectively. The interaction between cropping pattern and lineage (cropping pattern $\times$ lineage) was also found to be statistically significant, with a p-value of $p = 5.30 \cdot 10^{-10}$. Post-hoc analysis of the least-square means of the interaction term revealed that the parental CR Kanki flowers similarly under all cropping patterns (Supplemental Fig. 7, Supplemental Table 3). However, the #174-12-18 and #174-12-28 lines show a marked difference in inflorescence under cropping pattern one, with respect to the early-heading parent CR Kanki, grouping with the late-heading cultivars Hinata, Kigaku No. 70, and Harurisu (Supplemental Fig. 8).

The ANOVA conducted for stem bolting length in response to cropping pattern and lineage revealed significant effects for both cropping pattern and lineage, with p-values of $p = 1.01 \cdot 10^{-6}$ and $p < 2.2 \cdot 10^{-16}$, respectively. The mean bolting length for CR Kanki No. 100 was recorded as 100.00 cm under all three cropping patterns, as all the plants had already flowered at the time of measurement (Supplemental Fig. 9). The interaction effect was also significant, with a p-value of $8.23 \cdot 10^{-5}$. The post-hoc least-square

Fig. 6. Segregation of flowering time in the BC₃F₂ plants, #81-4 (a), #81-54 (b), #174-12 (c) and #174-22 (d) derived from self-pollination of #81 and #174 plants of BC₃F₁. #81-4 was selected from the non-flowered plants having the *BrFLC2* homozygotes (lacking *BoFLC2*) and the other 3 lines from the heterozygotes (*BrFLC2/BoFLC2*) of BC₂ plants. NF indicates non-flowered plants.

means analysis of the interaction term revealed that the two introgressed populations grouped together under cropping pattern one, with a least-squares mean bolting lengths of 2.76 cm and 4.94 cm for #174-12-28 and #174-12-18, respectively (Supplemental Fig. 10, Supplemental Table 4).

Discussion

Chromosome transmission in the BC₂ population shows preferential rates of transmission

Several chromosome addition lines, including monosomic alien addition lines (MAALs) and disomic addition lines, have been developed from different cross-combinations in the Brassicaceae (Heneen *et al.* 2012, Kaneko and Bang 2014). The aims of those studies were to assign species-specific characteristics to particular chromosomes, dissect genome homology by monitoring chromosome pairing/recombination through meiosis, and transfer desirable agronomic traits between species. Backcrossing to recurrent parents via interspecific crossing is the essential approach to produce MAALs.

In this study, we found that the transmission rates of the C chromosomes varied. Leflon *et al.* (2006) reported 23.3–46.8% for the whole C chromosome transmission and 1.3–13% for the fragmented C chromosome transmission in the cross of AAC × AA (CD × C in their designation). Leflon *et al.* (2006) also found that N12 (DY2 in their designation, corresponding to C2) and N15 (DY18, corresponding to C5) was frequently transmitted via female gametes, and N18 (DY8, corresponding to C8) was less frequently transmitted. In contrast, in this study, an AAC sesquiploid was crossed as the male parent to *B. rapa* (AA); consequently, the C5, C6, C7, C8, and C9 chromosomes were less frequently transmitted, and two fragments (C1A and C2B) of the C1 and C2 chromosomes were transmitted to the subsequent progeny with a significantly high frequency. The discrepancy between Leflon *et al.* (2006) and this study may be due to the difference of the crossing direction and/or the parental materials used in the two studies. It has been reported that in the 4x × 2x crosses of tulips, additional chromosomes tend to be transmitted more frequently by the female gametes than by the male gamete (Mizuochi *et al.* 2009). Chèvre *et al.* (1997b) reported a very low male transmission rate of chromosome 3 (1.3%) from the B genome in *B. napus*-*B. nigra* MAALs, while the remaining chromosomes (1, 2, 4 and 5) in their respective MAALs were frequently transmitted (18.1–28.8%). Thus, the distorted chromosome transmission rates, especially via male gametes, has been generally reported in several species.

We found that the number of extra C genome chromosomes transmitted to the BC₂F₁ population ranged from 0 to 9 (Fig. 3). This frequency distribution was skewed towards the tails of the binomial distribution, resulting in both smaller (n = 10–12) and larger (n = 16–19) numbers of transmitted chromosomes. The data shown in Fig. 3 is represented from plants with two DNA markers, and either of the two DNA

marker of the respective chromosome. We therefore overestimate the number of extra C chromosomes in BC₂F₁ plants from the point of view of counting fragmented chromosomes. However, since the transmitted rate of the fragmented chromosomes was not high (Fig. 2), it is likely that the unbalanced gametes (n = 13–15) are less frequently transferred to the BC₂F₁ population from the sesquiploid (AAC); and it is also likely that other relatively balanced gametes, with lower and higher numbers of C genome chromosomes, are frequently transmitted. This is in agreement with previous studies (Lee and Namai 1992, Leflon *et al.* 2006, Lu *et al.* 2001).

Transmission of C2 in the BC₃ population indicates a homozygous transmission of BoFLC2

In the subsequent BC₃F₁ progenies of this study, #81 and #174 BC₂F₁ plants containing the C2A genomic region were subjected to analysis to examine the patterns of alien chromosome inheritance. The transmission rate of C2 chromosome specific loci was around 40%, indicating the transmission rate was sufficient for development of a BoFLC2 introgression line (Fig. 5). McGrath and Quiros (1990) and Heneen *et al.* (2012) studied alien chromosome inheritance patterns using the backcrossed or selfed progenies of MAALs derived from the cross of *B. rapa* (AA) × *B. napus* (AACC). They reported the following transmission rates of C genome chromosome loci: 30.4% for *6Pdg-1* and 38.0% for *Pgm1* (McGrath and Quiros 1990), and 30.3–36.7% for C1–C9 chromosome (Heneen *et al.* 2012). These transmission rates were like that of the BC₃F₁ population in our study. In the subsequent BC₃F₂ progenies developed from the selfing of plants heterozygous for the BoFLC2/BrFLC2 markers, we found a segregation of the BoFLC2 allele. Consequently, we identified seven plants homozygous for the BoFLC2 allele in the #174-12 lines; where BrFLC2 homo-, BoFLC2 homo-, and heterozygotes segregated at a ratio of 7:7:15, fitting the theoretical 1:1:2 ratio (p = 0.98); the BoFLC2 homozygous plants lacked the BrFLC2 locus.

Marker based analysis of A2 in the #174-12-26 population indicates that the BrFLC2 allele was homozygously replaced with BoFLC2 in the A genome background, via homologous recombination of the target chromosomal region, with a region of homozygous introgression derived from C2 approximately 6.5 Mbp in size and corresponding to the top of the A2 chromosome (Shea *et al.* 2017). The size of the introgression was calculated as the distance between the top of C2 and the locus of the BnGMS239 marker using coordinates with respect to the Bolbase reference genome for *B. oleracea* var. *capitata*. It was also estimated by the newly developed algorithm (designated as IntroMap, Shea *et al.* 2017) by scanning a re-sequenced #174-12-26 genome and identifying introgression regions derived from alien species hybridization. The scan using IntroMap confirmed the #174-12-26 line contains the BoFLC2 region and a small region encompassing no flowering related genes of the C9 chromosome.

Flowering rates in the BC₃F₁ population were possibly affected by genome shock

In the BC₃F₁ populations of the #81 and #174 lines, the segregation of non-flowering and flowering plants after vernalization was observed, and the plants with the C2A chromosome region detected by the *BoFLC2* marker significantly tended to have no flowering after vernalization. In analyses of the BC₂ and BC₃F₁ populations using C2 chromosome specific DNA markers, the #81 lines contained only the C2 chromosome. Similarly, a whole genome scan using IntroMap confirmed the #174-12-26 line contains the *BoFLC2* region and a small C9 chromosome region encompassing no flowering related genes (Shea *et al.* 2017). This suggests that *BoFLC2* acts as a repressor of flowering, consistent with previous reports (Okazaki *et al.* 2007, Ridge *et al.* 2014). On the other hand, we have the unexpected results that some plants in the BC₃F₁ and BC₃F₂ generations, where the *BoFLC2* gene was not detected (*BrFLC2* homozygous plants), exhibited no flowering or delayed flowering (Figs. 5, 6) but the subsequent progeny of these plants failed to maintain tolerance to vernalization such that they revealed a flowering response like that of the *B. rapa* parent (Fig. 6, Supplemental Fig. 4). This restoration of the *B. rapa* vernalization type, in the subsequent generations of the two lines homozygous for the *BrFLC2* gene, is not caused by deleting other introgressed C genomic regions from the lines, as the previously mentioned genome-wide analysis revealed there are no additional C genome regions. Therefore, the failure of the *BrFLC2* homozygous plants to flower after vernalization in the BC₃F₁ and the subsequent populations may possibly be explained by genome shock (McClintock 1984). Either via the perturbation in genome-wide expression that results from either monosomy or nullisomy for an A genome chromosome, similar to C genomic results reported in *B. napus* (Zhu *et al.* 2015), or through the loss of transgenerational DNA methylation marks at the introgressed locus. Introgression resulting in a disruption of epigenetic regulatory activity via loss of the transgenerational inheritance of DNA methylation was reported in *Arabidopsis ddm1* mutants, and recovered by complementation with *DDM1* (Ito *et al.* 2015, Kakutani *et al.* 1998). This would explain the phenotypic instability observed in the earlier generations, followed by stabilization in subsequent generations (Comai *et al.* 2003). However, analysis of genome-wide DNA methylation levels and gene expression in the flowering and non-flowering BC₃F₁ and BC₃F₂ populations is required to confirm this idea.

The BC₃F₃ population exhibits several interesting traits that require further investigation

During observation of the growth of the BC₃F₃ C2 introgression lines, the plants exhibited several interesting traits that merit further investigation. Notably, in most cases, the *BoFLC2* introgressed plants exhibited a perennial growth habit (Supplemental Fig. 8). This is in contrast to CR Kanki which after flowering and setting seed rapidly senesces.

After vernalization and the flowering of the main stem, the main stem transitioned to a woody stem, whereas the axial shoots remained vegetative and later formed heads. However, the perennality of this line did not appear to persist, unlike *B. oleracea*. Plants exhibiting perennial growth characteristics appear to do so because of a failure to silence *BoFLC2* production via vernalization, with cold treatment resulting in the shoot apical meristem proceeding to bolt, followed by flowering. Axial shoots that maintained vegetative growth in these plants may be influenced by continued expression of *BoFLC2* in the root tissues or in the remaining aerial parts, due to their physical proximity to the crown.

The results obtained from the field trials showed that the proportion of flowering plants increased for all cultivars under cropping patterns two and three, with the exception of CR Kanki which always flowered in all three cropping patterns (Supplemental Table 2). This data shows that the C2 introgressed region alters the flowering response of CR Kanki from an early-heading type, to a late-heading type. This delay in flowering may possibly be attributed to the introduction of the alien *FLC* allele *BoFLC2*. Previous research in both *B. rapa* and *B. oleracea* identified *FLC2* as a key functional regulator of flowering time (Irwin *et al.* 2016, Kitamoto *et al.* 2013, Ridge *et al.* 2014). Allelic variation, derived from nucleotide variations present in the first intron, appears to be at least partially responsible for such flowering time variations in both *B. rapa* (Kitamoto *et al.* 2013) and *B. oleracea* (Irwin *et al.* 2016). However, the location of the intron one nucleotide variations appears to differ between *BrFLC2* and *BoFLC2*, and furthermore, the underlying molecular mechanism ultimately responsible for the observed variation to flowering time as a result of these intron one polymorphisms is currently unknown. Thus, our developed line may be a useful material for further research into such variations. However, a near isogenic line (NIL) containing only the *BoFLC2* gene is required, because other genes within the introgressed C2 segment may also affect flowering time.

In this study, we clarified alien chromosome inheritance patterns from BC₂ to BC₃ populations during the process of the construction of a CSSL of *B. rapa*, with phenotypic evaluation of the vernalization requirement. In addition, we show that *B. oleracea* provides an additional source of germplasm useful for the production of vernalization tolerant *B. rapa* cultivars of agricultural significance, allowing for the creation of a new winter season cropping-pattern Chinese cabbage.

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