DNA Research **16**, 311–323, (2009) doi:10.1093/dnares/dsp020

A *Brassica rapa* Linkage Map of EST-based SNP Markers for Identification of Candidate Genes Controlling Flowering Time and Leaf Morphological Traits

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(Received 30 June 2009; accepted 20 September 2009; published online 2 November 2009)

Abstract

For identification of genes responsible for varietal differences in flowering time and leaf morphological traits, we constructed a linkage map of *Brassica rapa* DNA markers including 170 EST-based markers, 12 SSR markers, and 59 BAC sequence-based markers, of which 151 are single nucleotide polymorphism (SNP) markers. By BLASTN, 223 markers were shown to have homologous regions in *Arabidopsis thaliana*, and these homologous loci covered nearly the whole genome of *A. thaliana*. Synteny analysis between *B. rapa* and *A. thaliana* revealed 33 large syntenic regions. Three quantitative trait loci (QTLs) for flowering time were detected. *BrFLC1* and *BrFLC2* were linked to the QTLs for bolting time, budding time, and flowering time. Three SNPs in the promoter, which may be the cause of low expression of *BrFLC2* in the early-flowering parental line, were identified. For leaf lobe depth and leaf hairiness, one major QTL corresponding to a syntenic region containing *GIBBERELLIN 20 OXIDASE 3* and one major QTL containing *BrGL1*, respectively, were detected. Analysis of nucleotide sequences and expression of these genes suggested possible involvement of these genes in leaf morphological traits.

Key words: DNA markers; synteny; bolting time; leaf lobe; leaf hairiness

1. Introduction

The *Brassica* genus comprises a number of important vegetables and condiment crops and is also a source of oil seed and fodder. Among the *Brassica* species, *B. rapa* (AA, $2n = 20$), *B. nigra* (BB, $2n = 16$), and *B. oleracea* (CC, $2n = 18$) are diploid species, and *B. juncea* (AABB, $2n = 36$), *B. napus* (AACC, $2n = 38$), and *B. carinata* (BBCC, $2n = 34$) are amphidiploids having combinations of the genomes of these diploids. Genetic maps with molecular markers are necessary to understand the origin and relationship among the genomes of the *Brassica* species and can be utilized in applied genetics and breeding of *Brassica* crops. In past decades, genetic maps of *Brassica* have been constructed with a range of marker types, including RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), RFLP (Restriction Fragment Length Polymorphism), and SSR (Simple Sequence Repeats) markers, and these maps have contributed to genetic analysis of quantitative traits. RFLP and SSR markers anchored in genetic maps have also been used for comparison of the chromosome organization among *Brassica* species, even between *Brassica* and its related model plant *Arabidopsis thaliana*.[1–3] The numbers of currently available mapped RFLP and SSR markers in *Brassica* are both more than 1000 in total.[3–6]

Edited by Satoshi Tabata

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Brassica rapa includes a variety of vegetables such as Chinese cabbage, Pakchoi, and turnip as well as oilseed crops such as turnip rape and sarson. More than 20 genetic linkage maps have been generated in *B. rapa*, some of which have been applied to quantitative trait locus (QTL) analysis of morphological traits,[7,8] and agronomical traits including fatty acid content,[9] glucosinolate accumulation,[10] phytate and phosphate concentrations,[11] and disease resistance.[12,13] Because *Brassica* belongs to the same family as *A. thaliana*, in which the complete genome sequence has been determined and functions of many genes have been well characterized, a clear synteny map between them can offer a powerful tool to find candidate genes after QTL analysis. By synteny analysis of QTL regions of *B. rapa* with the *A. thaliana* genome, Saito *et al.*[12] have fine mapped the clubroot resistance gene, *Crr3*, and Zhang *et al.*[14] have successfully cloned a gene controlling hairiness and seed coat color traits. However, AFLP and RAPD markers are anonymous markers, which make it difficult to compare and combine linkage maps. Some RFLP probes give rise to hybridization with multiple loci because of genomic segment replication,[1,15] and SSR markers are usually located in non-coding sequences, which are less conserved between the replication blocks than coding regions.[5] These shortcomings may limit their application in genome analysis. Other marker types such as SCAR (Sequence Characterized Amplified Region) and CAPS (Cleaved Amplified Polymorphic Sequence) have also been produced and used to construct genetic maps,[5,14] but the limited number of markers with polymorphism between different lines in a species does not meet the need of fine mapping. At present, in most detailed linkage map of *B. rapa*, which comprises 556 markers, only 120 markers have been detected in homologous regions of *A. thaliana*.[5]

Single nucleotide polymorphisms (SNPs) are the most common types of DNA polymorphism in genomes. In plants, SNP frequencies vary widely: 1 SNP/124 bp in coding region and 1 SNP/31 bp in the non-coding region of 36 inbred lines of maize;[16] 1 SNP/72 bp in expressed genes and 1 SNP/58 bp in non-coding sequences among a panel of 13 lines of sugar beet (*Beta vulgaris* L.);[17] and 1 SNP/2.1 kb to 1 SNP/1.2 kb between two cultivars of *B. napus*, Tapidor and Ningyou 7.[18] For genotyping of SNPs, gel electrophoresis, fluorometry, DNA microarrays, MALDI-TOF mass spectrometry, and labeled oligonucleotide hybridization are the most commonly used techniques.[19,20] Among them, for a small-scale operation in common laboratories, the dot-blot-SNP technique[20,21] based on labeled oligonucleotide hybridization is considered to be a low-cost, labor-saving technique for analysis of a large number of individuals. SNPs have been extensively applied to genetic studies, including association analysis of candidate genes with phenotypic variation,[22] fine mapping of QTLs,[14] EST-mapping,[17] linkage disequilibrium-based association mapping,[23] and genetic diversity assessment.[24] Whereas, in *Brassica*, very limited SNP markers, ~50, have been developed,[14,18] and no SNP linkage map has been published.

As ESTs are highly conserved in *Brassica* and *A. thaliana*, the linkage map based on ESTs can eventually provide a direct genomic comparison of macro- and micro-collinearity across these species. In the present study, we mainly used EST-based SNP markers to construct a *B. rapa* linkage map, which was then compared with *A. thaliana* linkage map based on homologous loci and revealed a fine synteny relationship. Flowering time and leaf morphological traits are important characteristics as vegetables, and therefore ones of breeding objectives. It has been indicated that multiple loci are involved in the variation of these traits,[7,8,25,26] but limited gene information has been obtained. We analyzed QTLs for these traits and inferred some candidate genes from the corresponding syntenic regions of *A. thaliana*.

2. Materials and methods

2.1. Plant materials and growth conditions

Brassica rapa cv. 'Yellow Sarson' C634, an early-flowering Indian oilseed rape, has been maintained by self-pollination for more than five generations. A doubled haploid line P11 of *B. rapa* cv. 'Osome', a Japanese commercial variety of late flowering leafy vegetable, was provided by Dr Kuginuki of Asahi Noen Seed Co. Ltd. The two lines were crossed to produce an F_2 population. As temperature is an important factor for flowering, the F_2 population was divided into two subpopulations of 132 and 134 individuals, which were cultivated using 24-cm-diameter pots in an unheated greenhouse and a heated greenhouse, respectively, in Sendai, Japan (38°16′N, 140°52′E), from October 2007 to April 2008. The average highest and lowest temperature of each month in Sendai is listed in Supplementary Table S1. The room temperature of the heated greenhouse was maintained above 10°C. Genomic DNA was prepared from leaves by a modified CTAB (cetyltrimethylammonium bromide) method.[27]

2.2. Investigation of phenotypes

The leaf characteristics were scored 60 days after sowing using the largest leaf of each plant. Lamina width (LW) and sinus width (SW) were measured as

illustrated in Fig. 1, and lobe depth ratio (LDR) was calculated as (LW − SW)/LW. Leaf hairiness (LH) was scored with 0–5 scale: 0, hairless; 1, some hairs along the leaf edge; 2, some hairs on the margin of abaxial surface of leaf; 3, dense hairs on the margin of abaxial surface of leaf; 4, dense hairs covering ca. 1/3 of abaxial surface of leaf and some hairs on the adaxial veins; 5, dense hairs covering more than 1/3 of abaxial surface of leaf and dense hairs on the adaxial veins. Days to bolting (DBO), budding (DBU), and flowering (DFL) were recorded as the number of days from sowing to achievement of a 3-cm-high main flower stalk, emergence of buds, and opening of the first flower, respectively. Days from bolting to bud formation (DBF) was the difference between DBU and DBO.

2.3. Primer design

In total, 533 sequences of genes or hypothetical genes in *Brassica* from BrassicaDB (http://brassica.bbsrc.ac.uk/BrassicaDB), 80 BAC sequences from the *B. rapa* Genome Project (http://www.brassica-rapa.org/BRGP/chromosomeSequence.jsp), 6 sequences of *A. thaliana* genes related to leaf shape from NCBI (http://www.ncbi.nlm.nih.gov/), and 308 EST sequences from the *Raphanus sativus* database (http://radish.plantbiology.msu.edu/index.php/Sequences:All) were selected to design PCR primers using the Primer3 software (http://frodo.wi.mit.edu/), expected sizes of amplified fragments being between 600 and 1500 bp.

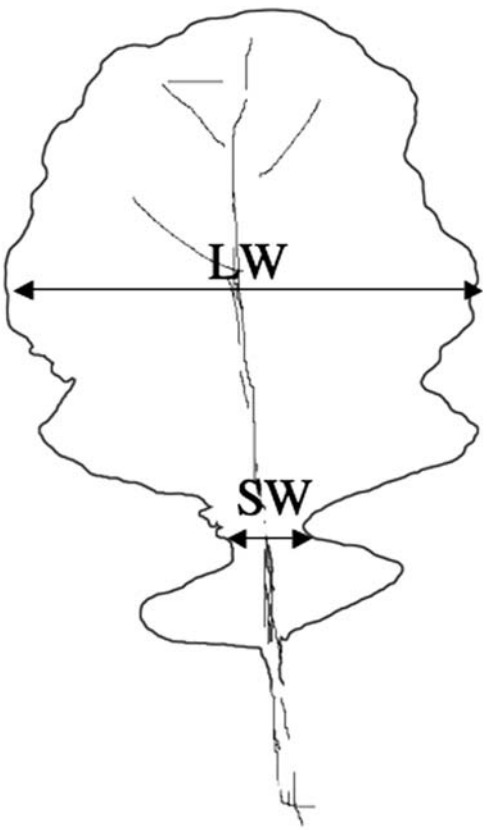

Figure 1. Diagram of traits of fully expanded leaves. LW and SW indicate lamina width and sinus width, respectively.

2.4. SCAR and CAPS analyses

PCR was performed in a reaction mixture of 20 μl, consisting of 40 ng genomic DNA as a PCR template, 0.5 μM of primers, 1 U of *Taq* DNA polymerase (*TaKaRa Ex Taq*® or *TaKaRa Taq*™, Takara Biomedicals, Japan), 1 × Ex *Taq* buffer or 1 × PCR buffer, and 200 μM of dNTPs. The thermal cycle of PCR was set to be as follows: 1 min denaturation at 94°C, 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C, and 1 min 30 s extension at 72°C, and 1 min 30 s final extension at 72°C. PCR products amplified by the primers of the *Brassica* sequence were separated on 0.8% agarose gel for SCAR analysis. For CAPS analysis, PCR products were digested by a restriction enzyme *MboI* or *MspI* and then were separated by 6% polyacrylamide gel in 1 × TBE buffer. The resulting DNA bands of SCAR and CAPS were stained with ethidium bromide.

2.5. SSR analysis

From *Brassica* microsatellite information exchange (http://www.brassica.info/resource/markers/ssr-exchange.php), 80 SSRs were selected to analyze polymorphism between the two parental genotypes. The PCR products were separated by 8% polyacrylamide gel in 1 × TBE buffer.

2.6. Nucleotide sequencing and dot-blot-SNP analysis

When SCAR and CAPS analyses did not differentiate the two parental genotypes, the amplification products were sequenced with a DNA analyzer (CEQ2000, Beckman Coulter, http://www.beckmancoulter.com/Default.asp?bhfv=6) and aligned using SEQUENCHER version 4.7 to identify SNPs. PCR products amplified by the primers of the *R. sativus* EST sequence were directly sequenced to identify SNPs without SCAR and CAPS analyses, and the SNP frequency between P11 and C634 was estimated.

5′-Biotin-labeled probes and bridge probes[21] were designed for dot-blot-SNP analysis (Supplementary Table S2). DNA fragments containing SNPs were amplified by PCR using the primers listed in Supplementary Table S2. The PCR products were mixed with an equal volume of a denaturation solution containing 0.4 N NaOH and 10 mM EDTA and dot-blotted onto a nylon membrane by Multi-pin Blotter (ATTO, Japan). After UV crosslinking using GS Gene Linker UV Chamber (Bio-Rad Laboratories, USA), the membrane was hybridized for 3 h or overnight with the probes and washed with washing

buffer (Supplementary Table S2). Digoxigenin and biotin were reacted with anti-DIG-IgG alkaline phosphatase conjugate (Roche Diagnostics, Switzerland) and streptavidin-alkaline phosphatase conjugate (Promega Corp., Madison, WI, USA), respectively, and alkaline phosphatase activity was detected using CSPD (Roche Diagnostics).

2.7. Linkage analysis and map construction

All 241 DNA markers were scored in the unheated-greenhouse subpopulation. To investigate the environmental effect on QTLs, about half of the markers covering the whole genome of *B. rapa* were scored in the heated-greenhouse subpopulation. Linkage analysis and map construction were performed using Antmap version 1.2.[28] Linkage groups were identified in the LOD (logarithm of odds) threshold range of 3.0, and the Kosambi mapping function was used to convert recombination frequencies into map distances (cM).

QTL analysis was performed using a composite interval-mapping analysis with Windows QTL Cartographer v2.5.[29] A permutation test was applied to each data set (1000 repetitions) to determine the LOD thresholds ($P = 0.05$). LOD values of 3.6 for the unheated-greenhouse subpopulation and 3.8 for the heated-greenhouse subpopulation were used as significant thresholds for the presence of a candidate QTL.

2.8. Locus nomenclature

The markers based on the genes or hypothetical genes were named
<Name 2-6 letter code>, and those based on ESTs of *R. sativus* were named
<EST name>. The marker assay types were designated with single letters, following the recommendations of De Vicente *et al*.[30] as follows: m, SSR; p, CAPS; c, SCAR; s, SNP.

2.9. Comparison with the A. thaliana genome sequence

The sequences of sequence-tagged SNP, SCAR, and CAPS loci in the map were aligned with *A. thaliana* genome sequences using the BLASTN program of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). We regarded the sequences as homologous loci of *A. thaliana* genome with a threshold value of $E < 10^{-20}$, and the homologous loci of *A. thaliana* were located on a physical map of *A. thaliana*, according to the TAIR database (http://www.arabidopsis.org/). The regions having conserved collinearity with *A. thaliana* were regarded as homologous syntenic regions. In the *A. thaliana* syntenic regions corresponding to QTLs in the present study, loci defined as transcribed units (e.g. AT2G03340) in the *A. thaliana* genome were searched in the TAIR database (http://www.arabidopsis.org/) to find the related candidate genes.

2.10. Sequence and expression analysis of candidate genes

Young leaves were collected from 30-day-old plants in the greenhouse at 20°C for RNA extraction. Total RNA was extracted from 30 mg of leaf using the SV Total RNA Isolation System (Promega Corp.). First-strand cDNA was synthesized from 1 μg of total RNA by reverse-transcription using a GE Healthcare first-strand cDNA Synthesis kit (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK). RT–PCR was performed by specific primers of *BrFLC*s, listed in Supplementary Table S3, by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Real-time PCR was performed to semi-quantify mRNAs of *BrTTG1* and *BrGA20OX3* using a LightCycler (Roche Diagnostics) with SYBR Premix Ex Taq (Takara Biomedicals) according to Fujimoto *et al*.[31] Specific primers of *BrTTG1* and *BrGA20OX3* are listed in Supplementary Table S3.

Nucleotide sequences of three BAC clones, KBrH080A08 (accession number in NCBI: AC155344), KBrH004D11 (accession number in NCBI: AC155341), and KBrB004B12 (accession number in NCBI: AC189195), which carry *BrFLC1*, *BrFLC2*, and *BrGA20OX3*, respectively, and that of the gene *BnTTG1* (accession number in NCBI: EF175930) were used to design primer sequences for nucleotide sequencing.

3. Results and discussion

3.1. Production of DNA markers and mapping

Of the 533 primer pairs designed from *Brassica* EST and BAC sequences and *A. thaliana* EST sequences, single DNA fragments were amplified by 383, in which 8 showed polymorphism between the parents of F_2 population, i.e. C634 and P11, by SCAR analysis and 70 showed such polymorphism by CAPS analysis (Supplementary Table S4). Of the remaining 305 primers, 160 were used for PCR amplification and the PCR products of C634 and P11 were sequenced and aligned. As a result, 95 SNP markers were produced (Supplementary Table S5). Among the 288 primer pairs from ESTs of *R. sativus*, 132 amplified single DNA fragments. Of these, 100 PCR amplicons covering ~34 kb were sequenced, and 71 showed nucleotide variation with a total of 427 SNPs (12.6 SNPs/kb) between the parents, 56 of which were used as SNP markers. Surveying 80 SSR primers, 12 revealed polymorphism between the parents. In total, 241 markers, including 170 EST-based markers (120 being SNP markers), 59 BAC sequence-based

markers (31 being SNP markers), and 12 SSR markers, were generated (Supplementary Table S6).

A total of 241 markers were assigned to 10 linkage groups (Fig. 2), designated as A01–A10, corresponding to the linkage groups of the JWF3p reference map (http://www.brassica-rapa.org/BRGP/chromosomeSequence.jsp), based on the SSR markers and the BAC sequence-based markers. The linkage group names A01–A10 correspond to R01–R10, a previously determined classification.[2,5,15,32] The linkage map had a total length of 1396 cM, and the average distance between ordered adjacent markers was 5.79 cM. The largest linkage group consisted of 38 markers and had a length of 176.7 cM, and the smallest consisted of 12 markers with a length of 73.2 cM. From the physical length of *B. rapa*, i.e. 717 Mb,[33] the relationship between the physical and genetic distances was 1 cM = ca. 514 kb.

Nucleotide sequences of ESTs for primer design in the present study were mainly derived from *B. rapa*, *B. oleracea*, and *B. napus*. Most of these ESTs have known or hypothetical functions (Supplementary Table S4). The nucleotides sequences of ESTs of *R. sativus* were also used to design primers and the sequencing results showed that PCR products of *B. rapa* and *R. sativus* had high nucleotide similarity, suggesting the feasibility of genome comparison between the two species using markers generated from the PCR products amplified by the same primers. The linkage map also contains 59 BAC sequence-based markers, which are located across all 10 linkage groups, and the order of these markers in each linkage group was almost the same as that of the JWF3p reference map (http://www.brassica-rapa.org/BRGP/chromosomeSequence.jsp).

A conspicuous characteristic of the present map is that more than half of the markers, 151 markers, are SNP markers, which greatly contribute to increasing the number of sequence-based markers. The SNP frequency between the parents was estimated to be 12.6 SNPs/kb. Such high frequencies of SNPs have also been reported in other species such as maize,[16] sugar beet,[17] and forage grass species *Lolium perenne*.[34] Therefore, almost all of the EST markers mapped in the present study can be applied to segregating populations of other parental lines in *B. rapa*, even in different species of *Brassica* or *Raphanus*, if they are used as SNP markers.

3.2. Synteny between B. rapa and A. thaliana

All the markers except the SSR markers in the present map were sequence-tagged markers. BLASTN was used to test homology between the *B. rapa* sequence and the *A. thaliana* genome sequence, the results being shown in Supplementary Table S7. By a significance threshold of $E < 10^{-20}$, 223 markers had homologous regions in *A. thaliana*, and these homologous loci covered nearly the whole genome of *A. thaliana*, except that only one homolog was located on the short arm of chromosome 2 (Supplementary Fig. S1). The five chromosomes of *A. thaliana* were divided into many segments and distributed to various regions of the *B. rapa* map (Fig. 2). There were 33 large syntenic regions containing at least three markers and having conserved collinearity with the homologues in *A. thaliana*. By comparing the syntenic regions from the same chromosome of *A. thaliana*, doubled and triplicated regions were identified. The region from 3.5 to 6.0 Mb in chromosome 1 was triplicated in A06, A08, and A09 of *B. rapa*, and that from 3.0 to 5.5 Mb in chromosome 5 was triplicated in A02, A03, and A10. The region between 12.9 and 14.9 Mb in chromosome 2 was doubled in A04 and A05, that between 2.8 and 7.5 Mb in chromosome 3 was doubled in A03 and A05, and that between 8.5 and 13.2 Mb in chromosome 4 was doubled in A01 and A08. Furthermore, these doubled regions also appeared to be partly conserved in other chromosomes of *B. rapa*. Segment duplications within the same chromosome were shown in A01, A02, A03, A05, A07, and A09. Insertion and rearrangement were the most apparent events and occurred in every chromosome of *B. rapa*.

Brassica and *A. thaliana* are considered to have originated from a common ancestor and diverged 14.5–20.4 million years ago.[35] *Arabidopsis thaliana* has a small genome (~146 Mb) with relatively little repetitive DNA and a high gene density,[36] whereas the diploid *Brassica* genome is significantly larger and has a lower gene density (*B. rapa*, 717 Mb).[33,37] Comparative genetic mapping between the diploid *Brassica* species and *A. thaliana* using RFLP probes has identified genome duplication and triplication in the *Brassica* genome and collinearity disrupted by multiple rearrangements.[3,38,39] Suwabe *et al.*[2] and Choi *et al.*[5] using 74 and 120 sequence-tagged markers of *B. rapa*, which have corresponding homologous regions in *A. thaliana*, have also identified some syntenic regions and illustrated the complex nature of the chromosomal rearrangements that may have occurred before/after differentiation of *A. thaliana* and *B. rapa*. Physical mapping and microsynteny analysis by nucleotide sequencing have further corroborated these findings.[37,40] The present comparative map of *B. rapa* and *A. thaliana* using 223 sequence-based markers revealed 33 large syntenic regions, which comprise the previously reported syntenic regions, and shows consistency.[2,5] Calculating physical distances of the syntenic regions in *A. thaliana* provided genome-wide synteny and

Figure 2. Linkage map of *B. rapa* with comparative maps of *A. thaliana*. Ten linkage groups are represented by vertical bars. Linkage group numbers correspond to the *Brassica* A genome linkage. Map distances are given in centimorgans (cM) on the left side of the linkage groups, and the locus designations are given on the right side. Loci tested for homology with *A. thaliana* using BLAST are shown to the right of the *B. rapa* linkage groups as colored vertical bars, which represent different chromosomes of *A. thaliana*. The locus names of the homologous regions of *A. thaliana* are given on the right side of the colored vertical bars and the megabase distances are given to the left. Groups of two or more markers showing homology with *A. thaliana* in collinearity are regarded as synteny regions. Except for both ends of each synteny region, the names and megabase distances of identified homologous loci are not shown, but are shown in the Supplementary Table S5 and Fig. S1. The *A. thaliana* chromosomes are colored as the bottom right of the figure.

Table 1. QTL analyses of flowering and leaf morphological traits in *B. rapa* under two treatments

QTL	Marker interval	Linage group	Growth condition	LOD	Exp%[a]	Additive effect[b]
DBOQTL1	BrRS2CL1605-BrBELLs	A02	Unheated	6.0	16.0	19.0
			Heated	6.7	14.0	13.3
DBOQTL2	BrSTs-BRGGRp	A07	Unheated	11.5	27.1	22.1
			Heated	8.4	19.1	14.3
DBOQTL3	KBrH080A08p-BrA9	A10	Unheated	—	—	—
			Heated	9.2	19.9	13.9
DBUQTL1	BrRS2CL1605-BrBELLs	A02	Unheated	11.7	27.9	12.6
			Heated	10.6	21.7	13.3
DBUQTL2	KBrH080A08p-BrA9	A10	Unheated	—	—	—
			Heated	12.7	26.4	12.6
DFLQTL1	BrRS2CL1605-BrBELLs	A02	Unheated	5.4	18.4	6.9
			Heated	8.5	18.1	14.5
DFLQTL2	KBrH080A08p-BrA9	A10	Unheated	—	—	—
			Heated	11.4	26.1	15.7
DBFQTL1	BrSTs-BRGGRp	A07	Unheated	9.1	29.3	−20.0
			Heated	9.8	30.4	−14.3
LDRQTL1	KBrH080A08p-BrRS2CL2387s	A10	Unheated	13.0	35.8	−0.3
			Heated	19.8	43.7	−0.3
LDRQTL2	BrADs-BrRS2CL1965s	A03	Unheated	3.9	8.2	−0.1
			Heated	—	—	—
LHQTL1	KBrB021P11s-BrTTG1s	A06	Unheated	40.9	66.8	−1.7
			Heated	23.0	50.2	−1.4
LHQTL2	BrADs-BrRS2CL1965s	A03	Unheated	4.5	5.6	−0.6
			Heated	—	—	—

[a]Exp: phenotypic variation explained.
[b]Effect of P11 alle.

micro-synteny, enabling detailed identification of duplicated and triplicated regions in every chromosome of *A. thaliana*. This increased detail will contribute to further study of genome structures and identification of candidate genes in *B. rapa*.

3.3. *Analysis of QTLs and candidate genes for flowering time*

There were significant differences in flowering traits, i.e. DBO, DBU, and DFL, between P11 and C634. Budding time of P11 was almost equal to bolting time; however, bolting time of C634 was significantly earlier than budding time. Flowering traits of F_1 were similar to those of P11, indicating that late flowering trait was dominant over early-flowering trait. The traits including DBO, DBU, DFL, and DBF showed continuous phenotypic distribution in the F_2 population under both heated and unheated conditions (Supplementary Fig. S2).

Under the two conditions, three QTLs of bolting time (DBOQTL1−3) were identified on A02, A07, and A10 (Table 1, Fig. 3). DBOQTL1 and DBOQTL2 were detected in both heated and unheated conditions, and the explained phenotypic variance was from 14.0% to 27.1%. DBOQTL3 was detected only in the heated condition with an explained phenotypic variance of 19.9%. Two QTLs (DBUQTL1/DFLQTL1 and DBUQTL2/DFLQTL2) for budding time and flowering time were detected on the same regions in A02 and A10 as DBOQTL1 and DBOQTL3 in two different conditions (Table 1, Fig. 3). One QTL for DBF (DBFQTL1) was detected in both conditions (Table 1, Fig. 3), and this QTL was co-located with DBOQTL2 on A07, but with an opposite effect, i.e. a P11 allele of this locus delayed the bolting time and accelerated bud formation so that the bolting time and the budding time were almost equal. Bolting time and budding time of *B. rapa* have also been analyzed previously, and some QTLs have been identified.[25,26] However, these linkage groups have not been assigned to the reference linkage groups and therefore it is not possible to compare these QTLs with the three QTLs detected in the present study.

BrFLC2 and *BrFLC1*[41] were located in the regions of DBOQTL1/DBUQTL1/DFLQTL1 and DBOQTL3/DBUQTL2/DFLQTL2, respectively. These two loci have also been detected in previous study on QTL of flowering time.[8,41,42] In *A. thaliana*, *FLC* encodes a MADS box protein that acts as a dosage-dependent repressor of flowering.[43,44] Evidence has been presented that the *FLC* homologues in *Brassica* species act similar to *AtFLC* and play a central role in the repression of flowering.[8,41,42,45] In the present study, QTL with *BrFLC2* was

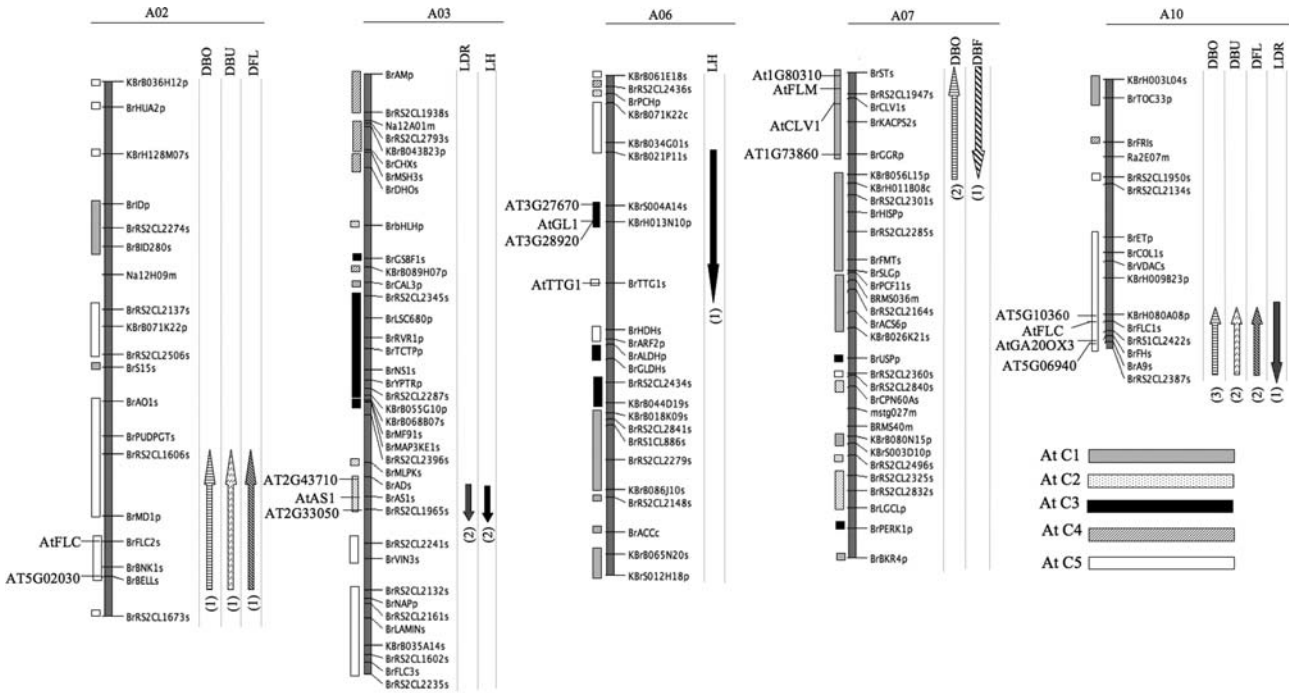

Figure 3. The genetic locations of QTL for days to bolting (DBO), budding (DBU), flowering (DFL), days from bolting to bud formation (DBF), lobe depth ratio (LDR), and leaf hairiness (LH). Syntenic regions of *A. thaliana* are shown to the left of the *B. rapa* linkage groups as patterned vertical bars, which represent different chromosomes of *A. thaliana*. Candidate genes and locus names of both ends of each syntenic region corresponding to each QTL are shown on the left side of the patterned vertical bars. The lengths of the arrows indicate the 2-LOD support intervals. The traits are indicated above each column. The direction of the arrowheads indicates the allelic effect: upward, P11 increases and C634 decreases for the F_2 population; downward: P11 decreases and C634 increases for the F_2 population. The numbers in the brackets under the arrows indicate the order numbers of the QTLs for each trait shown in Table 1.

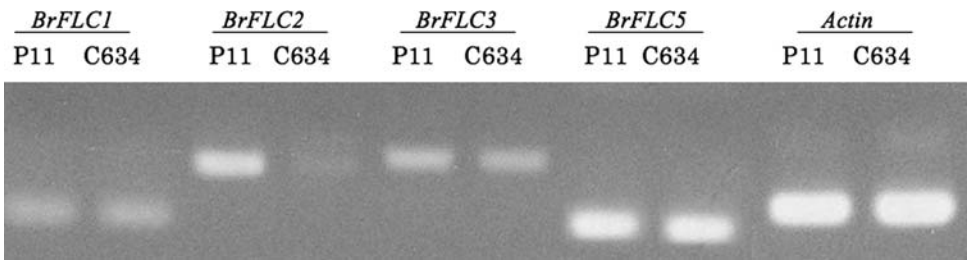

Figure 4. RT–PCR analysis of *BrFLC*s transcripts in *B. rapa* lines, P11 and C634, using primer pairs of exons 1–4. Leaf samples were collected from plants grown for 30 days in the green house at 20°C. Actin was used as a control to demonstrate equal RNA loading.

detected in both heated and unheated conditions, and that of *BrFLC1* was sensitive to environment, which is in accord with previous findings.[8,42,46]

Expressions of *BrFLC1, BrFLC3*, and *BrFLC5* were all detected in the leaves of P11 and C634 (Fig. 4) when the RT–PCR was performed for 30 cycles with the primer pairs of exons 1–4. In contrast, *BrFLC2* expression in P11 was strong, whereas very faint in C634. Sequence analysis of *BrFLC1* in C634 and P11 revealed three nucleotide polymorphisms (Fig. 5A). One was a nonsynonymous SNP in the first exon, one was in the second intron, and the other was at the 5′ splicing site of the sixth intron. RT–PCR using a primer pair of exons 4–7 revealed two different transcripts of *BrFLC1* in C634 (Supplementary Fig. S4), suggesting abnormal splicing of the C634 allele. This splicing site polymorphism has recently been reported to contribute greatly to flowering-time variation in *B. rapa*.[47] For *BrFLC2*, eight SNP sites were identified between C634 and P11 (Fig. 5B). Four of these SNPs were in the first and sixth introns, one was a synonymous SNP in the third exon, and the remaining three occurred in the region −800 bp upstream of the translation start site of *BrFLC2*, which might cause the difference of *BrFLC2* expression between C634 and P11.

One QTL (DBFQTL1) for DBF with minus effect co-located with DBOQTL2. In DBFQTL1/DBOQTL2, a

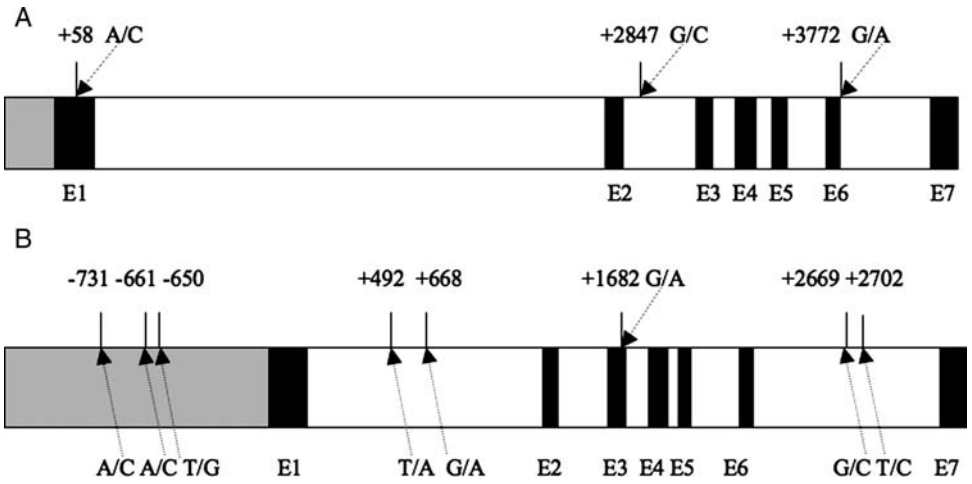

Figure 5. Nucleotide polymorphisms of *BrFLC1* (**A**) and *BrFLC2* (**B**) between P11 and C634. Exons (E), introns, and 5′ sequences upstream of translational start site (+1) are represented by black, white, and gray boxes, respectively. Numbers above the boxes indicate SNP sites connected by dotted arrows showing nucleotides of P11 and C634 at the left and right of the slashes, respectively.

Brassica ortholog of *CLAVATA1* (*BrCLV1*)[48] was located. In *A. thaliana*, the *CLV1* gene is involved in controlling shoot apical and floral meristem size and contributes to the establishment and maintenance of floral meristem identity.[49,50] Opposite effects between DBFQTL1 and DBOQTL2 indicated that at least two related genes located and were responsible for bolting and bud formation, respectively. As the region of DBFQTL1/DBOQTL2 showed a fine syntenic relationship with *A. thaliana*, we searched the genes in the syntenic region from 27.7 to 30.2 Mb of chromosome 1 in *A. thaliana* (http://www.arabidopsis.org/) and revealed 705 genes, among which, besides *CLV1*, a gene involved in the control of flowering time *FLOWERING LOCUS M* (*FLM*) (AT1G77080)[51] was found. *FLM*, also called *MAF1* (MADS AFFECTING FLOWERING1), encodes a floral repressor closely related to *FLC*.[51] Repression of *FLM* or non-functional *FLM* contribute to acceleration of flowering.[52] A BAC clone KBrH80C09, containing a tandem array of three *MAF* genes[53] and mapped on the bottom of A02, has been suggested to correspond to the QTL 'VFR1', which has been identified by Osborn *et al*.[42] The speculated homolog of *MAF1* in A07 of *B. rapa* needs to be cloned and characterized.

3.4. Analysis of QTLs and candidate genes for leaf morphological traits

3.4.1. Lobe depth ratio Two QTLs for LDR (LDRQTL1 and 2) were detected. A large percentage of phenotypic variance of 35.8% and 43.7% in the unheated-greenhouse subpopulation and the heated-greenhouse subpopulation, respectively, were explained by LDRQTL1 on A10. A minor QTL LDRQTL2, linked with a *Brassica* ortholog of *ASYMETRIC LEAVES 1* (*BrAS1*) on A03, was only detected in the unheated-greenhouse subpopulation. In *A. thaliana*, expression of the *KNOTTED*-like homeobox (*KNOX*) genes in the shoot apical meristem is required for maintenance of a functional meristem, but suppressed in cells destined for leaf primordia.[54,55] *AS1* maintains the down-regulation of *KNOX* expression in leaf primordia, and the *as1* mutation leads to lobed leaves.[56] In *Brassica*, QTLs in A02 and A03[8] and an AFLP maker on A08[57] have been indicated to be associated with leaf edge shape (LES). In the present study, we analyzed the trait of LDR calculated by (LW − SW)/LW. A similar trait, LES, scored as 1–4, has been analyzed by Lou *et al*.,[8] and a major QTL has been detected on the bottom of A03, where is seemed to be near to the LDRQTL2 in the present study.

We searched the genes in the syntenic region of LDRQTL1 from 2.1 to 3.2 Mb of chromosome 5 in *A. thaliana* (http://www.arabidopsis.org/) and found 348 genes, among which *AtGA20OX3* (AT5G07200) encoding a gibberellin 20-oxidase was noted. It has been reported that reduction in GA signaling or biosynthesis increases the number of lobes per leaf of *as1* mutation, and conversely, exogenous GA application or constitutive GA signaling suppresses ectopic expression of *KNOX* and both the number and depth of lobes.[58] Blast analysis showed a *B. rapa* homologue of *AtGA20OX3* (*BrGA20OX3*) in a *B. rapa* BAC clone KBrB004B12, which contains the sequence of the BrA9s marker located in LDRQTL1. Identities of nucleotide sequences and deduced amino acid sequences between *BrGA20OX* and *AtGA20OX3* were 92% and 86%, respectively (Supplementary Fig. S3). Semi-quantitative RT–PCR analysis showed that *BrGA20OX3* expression in leaves of P11 was more than six times as high as that in leaves of C634 (Fig. 6A). The lobed leaves of

C634 may be caused by the low expression of *BrGA20OX3*. Nucleotide sequence analysis of *BrGA20OX3* revealed four SNPs in the promoter region of *BrGA20OX3* between P11 and C634 (Fig. 6B), which might be the cause of different expression levels of *BrGA20OX3* between P11 and C634.

3.4.2. Leaf hairiness A major QTL for LH (LHQTL1) was detected on A06, which explained the phenotypic variances of 66.8% and 50.2% in the unheated-greenhouse subpopulation and the heated-greenhouse subpopulation, respectively. A minor QTL, LHQTL2, co-located with LDRQTL2, was detected only in the unheated-greenhouse subpopulation. The LH trait has been reported to be conditioned by a major QTL and a minor QTL in *B. rapa*.[7] Association mapping of leaf trichome detected an associated AFLP marker in A05.[57] *BrTTG1*, encoding a WD40 repeat protein, TRANSPARENT TESTA GLABRA01, which has been recently cloned from A06 and verified to simultaneously control both hairiness and seed coat color,[14] was located in LHQTL1. However, P11 has hairless leaves and brown seeds, whereas C634 has hairy leaves and yellow seeds.

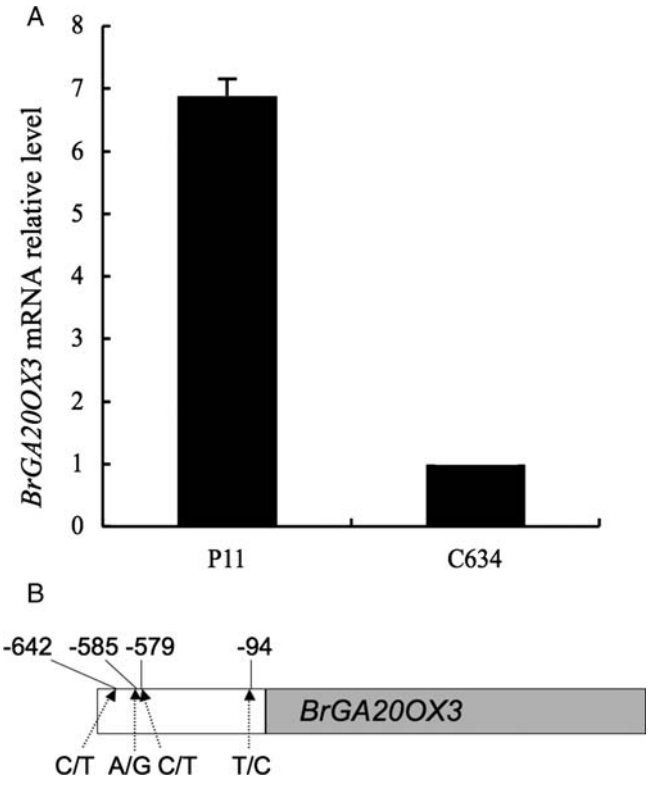

Figure 6. Gene expression and SNPs of *BrGA20OX3*. (**A**) Semi-quantitative RT–PCR analysis of *BrGA20OX3* in the leaves of P11 and C634. Data were normalized to C634; mean ± SE ($n = 3$). (**B**) Nucleotide polymorphisms of a promoter region of *BrGA20OX3* between P11 and C634. The 5′ sequence upstream of translational start site (+1) is represented by a white box. Numbers above the box indicate SNP sites connected by dotted arrows showing nucleotides of P11 and C634 at the left and right of the slashes, respectively.

BrTTG1 expression in leaves of P11 was over twice of that in leaves of C634 (Supplementary Fig. S5), which is not in accord with phenotypic difference that P11 was hairless and C634 was hairy. Nucleotide sequences of the coding region of *BrTTG1* in P11 and C634 were determined and aligned together with the sequences of hairy DH lines 'Y177-12' and 'P-8'.[14] There were 11 nucleotide polymorphisms between P11 and C634, which were not specific to P11 in multiple alignments (Supplementary Fig. S6). It can be inferred that *BrTTG1* of P11 is not responsible for the leaf hairless trait of P11.

Synteny analysis revealed that *GLABRA01* (*GL1*) (AT3G27920), which is a well-known gene controlling *A. thaliana* trichome development,[59,60] was located just in the syntenic region of a central site of LHQTL1. Blast analysis of *GL1* showed that a homologue of *GL1* with a maximum nucleotide identity of 90% was present in the *B. rapa* BAC clone KBrS004A14, which was located in LHQTL1. We determined nucleotide sequences of *GL1* alleles of C634 and P11 and found a 5-bp deletion in exon 3 of a P11 allele (Fig. 7). This deletion is considered to be a frameshift mutation causing the loss of the function of the P11 allele.

3.5. Conclusion

As the protein-coding regions of the genomes of *Brassica* species show high sequence conservation with those of *A. thaliana*, and the SNPs constitute

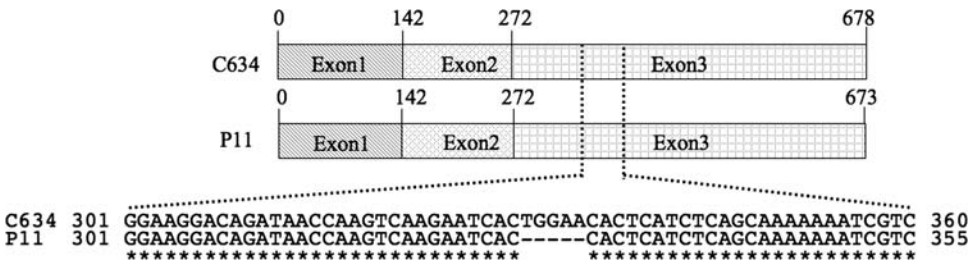

Figure 7. Identification of a 5-bp deletion in exon 3 of *GL1* in P11. Exons 1–3 are indicated by different boxes, and nucleotide sequences around the deletion are shown on the bottom.

the most common DNA sequence variations found in genomes of most organisms, EST-based SNP markers contribute to fine comparative mapping of *Brassica* species and *A. thaliana*. The present study shows that complex rearrangements and overlaps of the *A. thaliana* genome exist in the genome of *B. rapa*; however, genomic collinearity is conserved in many segments of *B. rapa*, which implies that we can use the positional information from the *A. thaliana* genome. After QTL mapping in *B. rapa*, we inferred some candidate genes from the corresponding region in *A. thaliana*. However, these candidate genes need further analyses, such as complementation or association studies.

Acknowledgements: We are grateful to Dr Kuginuki for providing a plant material.

Supplementary data: Supplementary Material is available at www.dnaresearch.oxfordjournals.org.

Funding

This work was supported in part by a Grant-in-Aid for Scientific Research (A) (19208001) from the Japan Society for the Promotion of Science (JSPS) and the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN). F.L. is a recipient of the Monbukagakusho fellowship from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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