The Korea Brassica Genome Project: A glimpse of the Brassica genome based on comparative genome analysis with Arabidopsis

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Received: 21 January 2005
Accepted: 2 February 2005

Abstract

A complete genome sequence provides unlimited information in the sequenced organism as well as in related taxa. According to the guidance of the Multinational Brassica Genome Project (MBGP), the Korea Brassica Genome Project (KBGP) is sequencing chromosome 1 (cytogenetically oriented chromosome #1) of Brassica rapa. We have selected 48 seed BACs on chromosome 1 using EST genetic markers and FISH analyses. Among them, 30 BAC clones have been sequenced and 18 are on the way. Comparative genome analyses of the EST sequences and sequenced BAC clones from Brassica chromosome 1 revealed their homeologous partner regions on the Arabidopsis genome and a syntenic comparative map between Brassica chromosome 1 and Arabidopsis chromosomes. In silico chromosome walking and clone validation have been successfully applied to extending sequence contigs based on the comparative map and BAC end sequences. In addition, we have defined the (peri)centromeric heterochromatin blocks with centromeric tandem repeats, rDNA and centromeric retrotransposons. In-depth sequence analyses of five homeologous BAC clones and an Arabidopsis chromosomal region reveal overall co-linearity, with 82% sequence similarity. The data indicate that the Brassica genome has undergone triplication and subsequent gene losses after the divergence of Arabidopsis and Brassica. Based on in-depth comparative genome analyses, we propose a comparative genomics approach for conquering the Brassica genome. In 2005 we intend to construct an integrated physical map, including sequence information from 500 BAC clones and integration of fingerprinting data and end sequence data of more than 100 000 BAC clones. The sequences have been submitted to GenBank with accession numbers: 10 204 BAC ends of the KBrH library (CW978640–CW988843); KBrH138P04, AC155338; KBrH117N09, AC155337; KBrH097M21, AC155348; KBrH093K03, AC155347; KBrH081N08, AC155346; KBrH080L24, AC155345; KBrH077A05, AC155343; KBrH020D15, AC155340; KBrH015H17, AC155339; KBrH011H24, AC155335; KBrH080A08, AC155344; KBrH004D11, AC155341; KBrH117M18, AC146875; KBrH052008, AC155342. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: KBGP; Brassica rapa; genome sequencing; integrated physical mapping; comparative genomics; in silico; BAC end sequences
Introduction

The Arabidopsis genome has been sequenced completely by an international consortium (the Arabidopsis Genome Initiative, 2000). Arabidopsis and Brassica diverged 14.5–20.4 million years ago from a common ancestor (Bowers et al., 2003). Comparative genetic mapping has revealed co-linear chromosome segments (Kowalski et al., 1994; Lagercrantz et al., 1996; Paterson et al., 2000, 2001; Schmidt et al., 2001) in the family Brassicaceae and linkage arrangements between Arabidopsis and B. oleracea (Lukens et al., 2003)

The genomes of Brassica species have duplicated, perhaps triplicated, counterparts of the corresponding homeologous segments of Arabidopsis (O’Neill and Bancroft, 2000; Rana et al., 2004).

Brassica is one of the core genera in the family Brassicaceae. Six Brassica species are cultivated worldwide; three diploids: B. rapa (AA, 2n = 20), B. nigra (BB, 2n = 16) and B. oleracea (CC, 2n = 18), and three amphidiploids (allotetraploids): B. juncea (AABB, 2n = 36), B. napus (AACC, 2n = 38) and B. carinata (BBCC, 2n = 34) (U. 1935). The species B. rapa (syn. campestris), with 529 Mb per haploid genome equivalent (Johnston et al., 2005), was prioritized for sequencing by a multinational collaboration. The Multinational Brassica Genome Project (MBGP) and Brassica rapa Genome Sequencing Project (BrGSP) are aiming to completely sequence the genome of Brassica rapa inbred line ‘Chiifu’ (http://www.brassicagenome.org; http://www.brassicarapa.org).

Korea launched the Korea Brassica Genome Project (KBGP) for complete sequencing of the cytogenetic chromosome 1 using BAC-by-BAC shotgun sequencing. In-depth comparative sequence analyses of the sequenced B. rapa BAC clones revealed overall co-linearity with a homeologous region of the Arabidopsis genome. Comparative sequence analyses suggest that we can use the Arabidopsis genome as a backbone for in silico clone validation of seed BAC clones and physical mapping as in the report of Love et al., 2004.

Here we propose an efficient clone validation method for selecting chromosome-specific seed BACs using comparative physical mapping and BAC end sequences. In 2005, KBGP aims to sequence 500 BAC clones that correspond to the majority of Arabidopsis euchromatin regions. The 500 BACs will be distributed and mapped on B. rapa chromosomes through sequence tagged site (STS) or simple sequence repeat (SSR) markers. BAC end sequences of 100,000 BACs (STC) and fingerprinting polymorphism-based BAC contigs (FPC) will be available soon. Hence, the sequence and map information of 500 BACs can be integrated with STCs and FPCs, resulting in an integrated physical map. The integrated physical map will provide a high resolution genome wide comparative map with Arabidopsis and will be supplied to MBGP to accelerate the Brassica genome sequencing.

Materials and methods

DNA sequencing

Shotgun sequencing libraries were constructed in pCU1blu31 for average insert size of 3 kb (Kim et al., 2004; Yang et al., 2004; Yang et al., 2005). BigDye terminators chemistry v3.0 (ABI) was used for the reactions. The sequences were analysed using ABI3730 automatic DNA sequencers (ABI). Base-calling was performed automatically using phred, and vector sequences were removed by CROSS_MATCH (Ewing and Green 1998; Ewing et al., 1998). High quality, vector-trimmed sequences were thus used for the sequence assembly of each BAC clone, using phrap and consed (Gordon et al., 1998).

Sequence analysis

Pairwise sequence comparison was conducted using PipMaker (Schwartz et al., 2000) and BLAST2 analysis (http://www.ncbi.nlm.nih.gov/BLAST/). MegaBLAST against the Arabidopsis chromosome database and BLAST-nr were used as needed (http://www.ncbi.nlm.nih.gov/BLAST/). Gene annotation was achieved using several web based gene prediction programs, e.g. FGENE-SH Arabidopsis (http://www.softberry.com/berry.phtml) and GeneMark Arabidopsis (http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi).

Repeats were identified using Repeatm masking (http://ftp. genome.washington.edu/RM/webrepeatmaskerhelp.html).
Fluorescence in situ hybridization

Our FISH protocol was adapted from Lim et al., (2001, 2005a) with minor modifications. FISH signals were pseudo-coloured and further improved for optimal brightness and contrast with Adobe Photoshop image processing software.

Results and discussion

Overview of Brassica rapa genome structure

A genetic map of Brassica rapa, using segregating doubled haploid lines of Chifu and Kenshin, covering 1046 cM with 494 markers on 10 linkage groups, was constructed with 895 DNA markers, AFLP, PCR-RFLP, ESTP, CAPS and SSR (http://www.brassicagenome.org). We have constructed another EST-RFLP genetic map of B. rapa using 478 tissue-specific cDNA clones consisting of 176 cDNAs from immature flowers, 252 cDNAs from anthers and 50 from dark-grown seedlings of B. rapa ssp. pekinensis cv. Jangwon. This molecular map covered 3412 cM on 10 linkage groups. Aligning RFLP marker sequences on the counterpart Arabidopsis chromosomes shows syntenic colinearity, resulting in a highly informative comparative genetic map (Kim, 2001). The karyotypes of B. rapa chromosomes were studied previously (Fukui et al., 1998; Snowdon et al., 2002; Koo et al, 2004). We further characterized chromosomes in detail using fluorescence in situ hybridization (FISH) using repetitive DNAs, such as 45S rDNA, 5S rDNA, centromeric repeats (CentBr) and centromere-specific retrotransposons (Lim et al., 2005a). The cytogenetic chromosomes were integrated with genetic maps by painting with chromosome-specific BAC clones identified by unique EST clones from each linkage group (LG1–LG10) (Lim et al., 2005b). The cytogenetic chromosome numbers, our linkage groups (LG1–LG10) and the international standard linkage numbers (R1–R10) (Lombard and Delourme, 2001) will be integrated soon.

We have sequenced four BAC clones that form the counterpart of an Arabidopsis chromosomal region (chromosome 5: 3.1–3.2 Mb) containing flowering locus C (FLC). Comparisons of the sequenced Brassica BAC clones with the homologous regions of Arabidopsis showed overall colinearity with 81% sequence similarity. The average sequence similarity between Brassica BACs is 82% with exceptionally high similarity (97%) of two clones, 117M18 and 52O08, representing two regions that have recently been duplicated. The co-linear 125 kb Arabidopsis sequence was reduced by up to 40% by deletions of DNA segments in Brassica BAC clones (Table 1). Among 36 genes in the 125 kb of Arabidopsis sequence, only 24, 17, 13, and 13 homologues remained in the common sequence of each BAC clone, 80A08, 4D11, 52O08 and 117M18, respectively. Only four genes remain in all four BAC clones, with 77–96% similarity in amino acid sequences. Newly emerged (or inserted)

Table 1. Comparison of four homologous Brassica BAC clones and its counterpart Arabidopsis sequence

| Subject | Homologous Brassica BAC clones | Arabidopsis chrom. 5 |
|---------|-----------------------------|------------------|
|         | 80A08 | 4D11 | 52O08 | 117M18 |           |
| Insert size (bp) | 110219 | 106476 | 153587 | 132883 | — |
| Common sequence (begin) | 1 | 15001 | 58254 | 18227 | 3134987 |
| Common sequence (end) | 110219 | 89318 | 115292 | 70502 | 3258842 |
| Total length of common sequence (bp) | 110217 | 74316 | 57037 | 52274 | 123855b |
| Aligned nucleotide (bp)a | 67412 | 47325 | 33870 | 31161 | — |
| Internal deletion or substitution (bp) | 42795 | 26991 | 23167 | 21113 | — |
| Co-linearity indexb | 0.9 | 0.6 | 0.5 | 0.4 | 1.0 |
| Alignment indexc | 0.61 | 0.64 | 0.59 | 0.60 | — |
| Homology (Arabidopsis vs. Others) (%) | 81.0 | 81.1 | 81.7 | 81.3 | 100.0 |
| Homology (117M18 vs. Others) (%) | 82.3 | 81.8 | 98.1 | 100.0 | 82.3 |

a A total of nucleotides that show significant sequence similarity with co-linear Arabidopsis sequence.

b Represents genome expanding or reducing in Brassica BAC clones compared to the co-linear Arabidopsis sequence (= Total length of common sequence of Brassica/Common sequence of Arabidopsis).

c Represents the significantly homeologous region in the common sequence (= Aligned nucleotide/Total length of common sequence.)
genes including transposons are detected six, three, two and one times in each BAC clone, respectively. The data support previous reports (O’Neill and Bancroft, 2000; Rana et al., 2004) and provide in depth information about howtriplicated Brassica genome sequences are modified after divergence with Arabidopsis at around 20 million years ago (Bowers et al., 2003).

Pericentromeric heterochromatin blocks in the Brassica rapa genome

The centromeric region of Brassica is occupied by 176 bp tandem repeats (Harrison and Heslop-Harrison, 1995). The 176 bp centromeric repeat of Brassica (named CentBr) occurred in 30% of our BAC end sequences (10 204 BAC ends of the KBrH library; GenBank accession numbers CW978 640–CW988 843) as tandem arrays, indicating that the CentBr is a major component of the B. rapa centromere. The CentBr sequences are subdivided into two classes, named CentBr1 and CentBr2, based on sequence similarity (82–84% between two classes and over 92% between members in each class). CentBr1 and CentBr2 occupy the centromeres of eight and two chromosomes, respectively (Lim et al., 2005a).

Figure 1. Comparative map of B. rapa chromosome 1 and Arabidopsis chromosomes based on sequence similarity of EST markers and sequenced BAC clones. The far left of the figure represents the features of chromosome 1, BAC clones were selected by filter hybridization using mapped EST markers and their actual chromosomal locations were confirmed by FISH analyses, using metaphase or pachytenephase chromosomes (left). The cartoon at the left of pachytenephase chromosome represents the features of chromosome 1, showing pericentromeric heterochromatin and heterochromatin (brown and purple boxes, respectively), based on numerous inspections by DAPI staining and FISH analyses using repetitive elements. The linkage groups containing 46 markers and corresponding elements of chromosome 1 are represented and the syntenic regions are represented on Arabidopsis chromosomes (right).
We have sequenced two centromeric BAC clones, KBrH015B20 (102 kb) and KBrH001P13 (17 kb), containing centromeric tandem repeats for increased understanding of major elements in the (peri)centromeric region of the *Brassica* genome. Careful sequence analysis revealed several families of centromere-specific retrotransposons of *Brassica* (CRB). Among these, two long terminal

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**Figure 2.** Dot-plot analysis of three contiguous sequenced BAC clones from *B. rapa* chromosome 1 and the counterpart region of *Arabidopsis* (chromosome 3, 22.9–23.3 Mb). The region is marked as a green circle in Figure 1. The beginning and ending nucleotide of the counterpart *Arabidopsis* sequence for the three *Brassica* BAC clones are represented as numerals under the figure. The co-linear index (= The length of co-linear *Arabidopsis* sequence/Co-linear *Brassica* BAC sequence which is in accordance with the slope) of each BAC clone is represented in the dot plot.

**Table 2.** *Brassica* BAC clones sequenced and the counterpart *Arabidopsis* sequence

| *Brassica* BAC Clone | Length (bp) | Co-linear Index *Arabidopsis/Brassica* | Length (bp) | Begin | End | Chrom. No |
|----------------------|-------------|---------------------------------------|-------------|-------|-----|-----------|
| 01H24                | 118 144     | 0.9                                   | 103 420     | 23 156 540 | 23 259 960 | 3         |
| 15H17                | 110 885     | 0.8                                   | 85 302      | 23 224 319 | 23 309 621 | 3         |
| 20D15                | 143 633     | 0.8                                   | 119 117     | 80 474 979 | 8 166 614  | 1         |
| 77A05                | 113 253     | 1.1                                   | 125 641     | 19 236 565 | 19 362 206 | 3         |
| 80L24                | 115 119     | 4.0                                   | 459 225     | 17 701 122 | 18 160 347 | 1         |
| 97P21                | 131 063     | 2.4                                   | 314 999     | 10 677 001 | 10 992 000 | 3         |
| 117N09               | 125 390     | 1.7                                   | 218 929     | 17 478 001 | 17 696 930 | 2         |
| 138P04               | 137 697     | 1.3                                   | 173 013     | 2 128 977  | 2 301 990  | 1         |
| 4D11                 | 106 476     | 1.7                                   | 184 272     | 3 092 144  | 3 276 416  | 5         |
| 80A08                | 110 038     | 1.1                                   | 121 376     | 3 137 466  | 3 258 842  | 5         |
| **Average insert size (bp)** | **121 170** | **1.6**                                   | **190 529** |       |     |           |
| **STD**              | **±12 682** | **1.0**                                   | **116 235** |       |     |           |

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Comp Funct Genom 2005; 6: 138–146.
repeat (LTR) retrotransposons, a Ty3-gypsy-like one (PCRB; 9135 bp with 2047bp LTR) and a Ty1-copia-like one (CRB; 6010 bp with 597 bp LTR) predominantly occupied each BAC sequence. FISH analyses revealed that the CRB is a major component of the centromere of all chromosomes and the PCRB is a major component of the large pericentromeric heterochromatin regions of three chromosomes. Based on the BAC end sequence information and FISH analyses, we assume these four (peri)centromeric repeats occupy just over 40% of the Brassica genome. Since heterochromatin blocks are hard to sequence, we will focus on sequencing euchromatin regions, which probably constitute less than 60% of the Brassica rapa genome.

Progress in sequencing chromosome 1 in Korea

The Korea Brassica Genome Project (KBGP) is aiming to complete the sequencing of cytogenetic chromosome 1 using three BAC libraries, KBrH, KBrB, and KBrS (Sau3AI), of B. rapa ssp. pekinensis inbred line ‘Chiifu’. Physical mapping is on-going by fingerprinting of the KBrH and KBrB libraries (http://www.brassicagenome.org; http://www.brassica-rapa.org). Anchoring the fingerprint polymorphism contigs (FPC) on the chromosome remains an obstacle to overcome for physical mapping and clone validation. We have selected 48 seed BACs on chromosome 1 through screening with EST markers and confirmation by FISH analyses. Among them, 30 BAC clones were sequenced and they show co-linearity with the counterpart homeologous region of Arabidopsis, with about 82% sequence similarity (Table 1).

The comparative analyses of the EST sequences mapped on chromosome 1 with their homeologous partner regions of Arabidopsis revealed counterparts in the Arabidopsis genome (Figure 1). The sequenced BAC clones show overall co-linearity with a counterpart Arabidopsis chromosomal region which was expected, based on the

![Figure 3. In silico allocation of Brassica BAC clones on Arabidopsis chromosomes. The beginning part of Arabidopsis chromosome 1 is represented. BAC clones are aligned on Arabidopsis chromosomes based on significant and directional matches of both ends within a 30–500 kb interval. The forward and reverse ends are marked as grey bars (left). An example of the minimum tiling path of the three BAC clones are boxed.](http://www.brassicagenome.org)
comparative map (Figure 2). Based on the comparative physical map and micro-co-linearity between the *Brassica* and *Arabidopsis* sequences, we have proposed an efficient and novel clone validation method for sequencing in advance of the complete physical map. The *Brassica* BAC clones were allocated to *Arabidopsis* chromosomes by *in silico* allocation based on unique, significant (<1E-6), and directional matches: one BAC end is forward and the other end is the reverse, with a complement match within a 30–500 kb interval. BAC-FISH and STS mapping using BAC end sequences on the counterpart *Arabidopsis* chromosomal region showed the real locations of the BAC clones on the chromosomes. At least one in three BAC clones is mapped onto the expected region of chromosome 1 due to the triplicated nature of the *Brassica* genome. All the sequenced BAC clones provide a further starting point for selection of seed BAC clones for extending the sequence.

**Integrated physical mapping**

Successful clone validation based on *in silico* allocation to counterparts of chromosome 1 suggests a novel strategy for integrated physical mapping, using comparative mapping of BAC ends onto *Arabidopsis* chromosomes. The integrated physical mapping strategy encompasses *in silico* allocation of *B. rapa* BAC clones to the counterpart locations of *Arabidopsis* chromosomes, based on significant BLAST matches. A *Brassica* BAC clone (average size 120 kb) covers an average of 190 kb *Arabidopsis* sequence based on a co-linearity index of 1.6 (= co-linear *Arabidopsis* sequence/*Brassica* nucleotide) (Table 2). We have analysed 91 000 BAC end sequences (Table 3). Among them, a total of 45 232 BAC end sequences (50%) show significant sequence similarity with unique *Arabidopsis* sequences, and a total of 4317 BAC clones (9.5%) are allocated on *Arabidopsis* chromosomes by significant matching with both ends within 30–500 kb interval (Table 3). These 4317 clones span 93 Mb of *Arabidopsis* euchromatin regions, representing 78.2% of the total *Arabidopsis* genome. A total of 26 Mb remain as unconvered gaps: among these 9.4 Mb (3.1 Mb, 1.8 Mb, 2.4 Mb, and 1.0 Mb from *Arabidopsis* chromosomes 1, 2, 3, 4, and 5, respectively) might be from euchromatin gaps at 116 sites ranged from over 20–585 498 bp, except for the 16.6 Mb of pericentromeric heterochromatin gaps. A single *Brassica* BAC clone spans an average of 147 kb (±74 kb) *Arabidopsis* sequence (Figure 3). A total of 500

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**Table 3. Brassica rapa BAC end sequence and the results of blast analyses against Arabidopsis chromosomes**

| Library | Unique hits (<1E-6) | BAC clones with both ends |
|---------|---------------------|--------------------------|
|         | Query No. | Hit No. | % | Pairhit No. | Clone No. | % |
| KBrH (HindIII) | 10 204 | 4 195 | 41.1 | 1 162 | 581 | 11.4 |
| KBrB (BamHII) | 72 343 | 36 833 | 50.9 | 6 906 | 3 454 | 9.5 |
| KBrS (Sau3AI) | 8632 | 4 204 | 48.7 | 5 646 | 2 828 | 6.5 |
| Total | 91 179 | 45 232 | 49.6 | 8 634 | 4 317 | 9.5 |

*100* (Pairhit No./Query No.). Clone numbers represent the numbers of BAC clones allocated on *Arabidopsis* chromosome by directional hitting with both ends within 30–500 kb interval.
BACs with an average 120 kb of insert will cover around 80 Mb of the euchromatin regions of the *Arabidopsis* genome (almost all of the euchromatin). The 500 BACs will be scattered into the triuplicated regions on *Brassica* chromosomes (e.g. Figure 4). The actual chromosomal location of a sequenced BAC can be mapped on the genetic map through SSR or STS-PCR using its sequence information. Recently, we have selected the minimum tiled 629 *Brassica* BAC clones spanning 86 Mb of *Arabidopsis* from the *in silico* allocation (data is available at our website: www.brassica-rapa.org). Each BAC clone will be mapped on the *Brassica* chromosomes by STS mapping and FISH analyses. About 75 Mb from gene rich euchromatin regions of *Brassica* will be obtained from sequencing of the 629 BACs (average insert 120 kb) that may be distributed into 10 *B. rapa* chromosomes (average 60 BACs for each chromosome) with an average 240 kb gap (Figure 4). All the sequenced BAC clones will be provided to MBGP and used as a starting point for the selection of seed BAC clones extending to the flanking sides with minimum overlap based on sequence tagged connectors (STC). The results will provide in depth information about the comparative genomics between *Brassica* and *Arabidopsis*.

Complete sequencing of *Brassica rapa* will give great opportunities to increase our understanding of the evolution of the polyploidized genome and of agricultural aspects, especially for breeding and molecular farming, through finding novel or useful genes, not only in *B. rapa* but also in other important crops in the genus *Brassica*.

**Acknowledgements**

This work was supported by BioGreen 21 Program, RDA, and NIAB, Korea. We are grateful to Macrogen (http://www.macrogen.com/english/index.html) for the sequencing.

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