Molecular Cytogenetic Characterization of the *Antirrhinum majus* Genome

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ABSTRACT

As a model system in classical plant genetics, the genus Antirrhinum has been well studied, especially in gametophytic self-incompatibility, flower development biology, and transposon-induced mutation. In contrast to the advances in genetic and molecular studies, little is known about Antirrhinum cytogenetics. In this study, we isolated two tandem repetitive sequences, CentA1 and CentA2, from the centromeric regions of Antirrhinum chromosomes. A standard karyotype has been established by anchoring these centromeric repeats on meiotic pachytene chromosome using FISH. An ideogram based on the DAPI-staining pattern of pachytene chromosomes was developed to depict the distribution of heterochromatin in the *Antirrhinum majus* genome. To integrate the genetic and chromosomal maps, we selected one or two molecular markers from each linkage group to screen an Antirrhinum transformation-competent artificial chromosome (TAC) library. These genetically anchored TAC clones were labeled as FISH probes to hybridize to pachytene chromosomes of *A. majus*. As a result, the relationship between chromosomes and the linkage groups (LGs) in Antirrhinum has been established.

THE genus Antirrhinum ($2n = 16$) has >20 species, most found around the Mediterranean Sea and in North America (STUBBE 1966). Among them, only *Antirrhinum majus* has been domesticated as an ornamental. Most species of the genus Antirrhinum are characterized by gametophytic self-incompatibility, regulated by a single multialleleic *S* locus (EAST 1940), which has been used as a model system for studying self-incompatibility (XUE *et al.* 1996; LAI *et al.* 2002; MA *et al.* 2002; ZHOU *et al.* 2003; QIAO *et al.* 2004).

Mutation instability and variegation have been well documented in *A. majus*, which led to the isolation of the first transposon in plants and its further application in gene tagging (reviewed by SCHWARZ-SOMMER *et al.* 2003a). In the first decade of the twentieth century, Baur and his colleagues isolated some unstable mutations in *A. majus*. During the 1950s to the 1970s, Harrison's group began to work with the unstable mutants related to two genes, *NIVEA* (*NIV*) and *PALLIDA* (*PAL*; STICKLAND and HARRISON 1974; HARRISON and CARPENTER 1979). By cloning *NIV* from an unstable *niv* allele, Hans Sommer's group was the first to isolate an autonomous transposon, *Tam1*, in plants (BONAS *et al.* 1984). After-

ward, several other transposons were isolated from the *NIV* locus including *Tam3*, which was in turn used as a molecular tag to isolate *PAL* (MARTIN *et al.* 1985; SOMMER *et al.* 1985). The accumulated information on various transposons in Antirrhinum provided the basis for a better understanding of the function and structure of transposons in maize (SCHWARZ-SOMMER and SAEDLER 1985). Moreover, several genes, especially those related to flower development, have been cloned in *A. majus* using the transposon tagging system (COEN *et al.* 1990; SOMMER *et al.* 1990; BRADLEY *et al.* 1996).

The first classical genetic map of *A. majus* covered a genetic distance of ∼420 cM, including 57 morphological markers scattered on eight linkage groups (STUBBE 1966). A molecular marker-based genetic linkage map has been constructed recently on the basis of an F_2 population of 92 individuals derived from an interspecific hybrid *A. majus* × *A. molle* (SCHWARZ-SOMMER *et al.* 2003b). These maps, together with its relatively small genome size, make positional cloning feasible in Antirrhinum (LAI *et al.* 2002).

In contrast to the significant advances in the genetic and molecular study of *A. majus*, very limited work has been done on the karyotype and cytogenetic structure of the *A. majus* genome. The genetic linkage groups have not been integrated into individual chromosomes. In recent years, extensive molecular and genomic resources have been established in *A. majus* (SCHWARZ-SOMMER *et al.* 2003a). Bacterial artificial chromosome (BAC) and transformation-competent artificial chromosome (TAC) libraries representing the Antirrhinum ge-
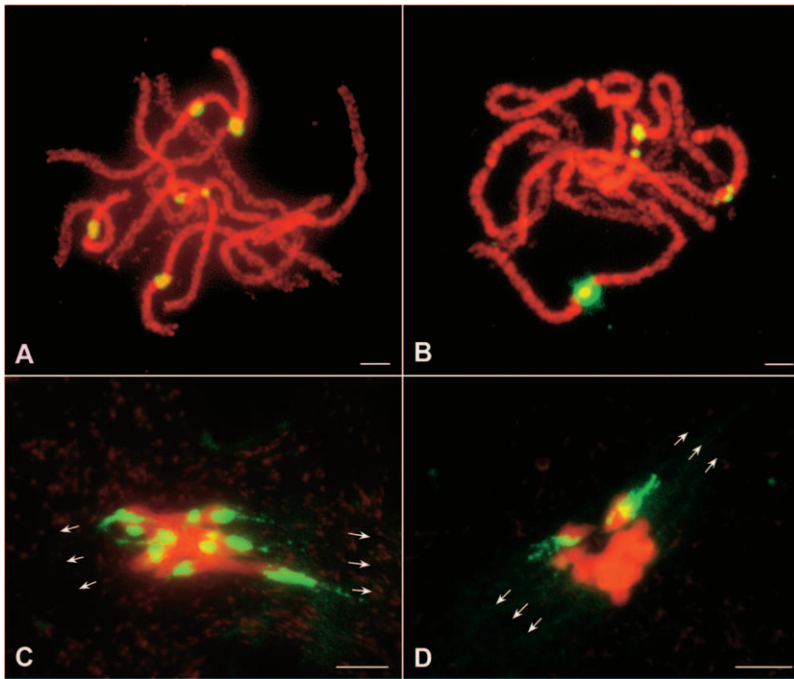
FIGURE 1.—FISH analysis of the BAC clones related to centromeres in Antirrhinum. (A) Chromosomes in a pachytene cell of *A. majus* probed by 36D21 (green signals). (B) Chromosomes in a pachytene cell of *A. majus* probed by 5E10 (green signals). (C) The metaphase I chromosomes probed by 36D21 (green signals), showing the signals are located in stretched regions of the bivalent. The arrows point to both spindle poles. (D) The metaphase I chromosomes probed by 5E10 (green signals), showing only the strongest signals of 5E10 are located at both stretched end regions of one bivalent. All chromosomes are stained with DAPI and pseudocolored as red. The arrows point to both spindle poles. Bars, 5 μm.

nome are available now. An expressed sequence tag (EST) database has been created in this species containing ∼12,000 unique sequences. These resources provide us with an opportunity to investigate the *A. majus* genome using a cytogenetic approach that has been established in the model cytogenetic species *Arabidopsis thaliana* (FRANSZ *et al.* 1998) and rice (CHENG *et al.* 2001a).

We isolated two tandem repeats from centromeric regions of Antirrhinum chromosomes. With the aid of these two centromeric repeats, the positions of centromeres of Antirrhinum chromosomes can be unambiguously located, and the molecular structure of the major heterochromatin domains of *A. majus* pachytene chromosomes can be extensively investigated. We also isolated 14 chromosome-specific TAC clones that are anchored by genetically mapped DNA markers. FISH mapping of these chromosome-specific TAC clones on pachytene chromosomes enabled us to establish the relationship between chromosomes and the linkage groups of Antirrhinum.

MATERIALS AND METHODS

A. majus (stock 75) plants were grown in a greenhouse environment as described by XUE *et al.* (1996) and LAI *et al.* (2002). Young buds were collected for meiotic chromosome preparation and leaf tissue was harvested for genomic DNA isolation. The BAC library (LAI *et al.* 2002), constructed from a self-incompatible line with S_2S_4 alleles, was screened to isolate the centromere-specific DNA sequence. A total of 14 molecular markers mapped on eight linkage groups (SCHWARZ-SOMMER *et al.* 2003b), including 9 RFLPs, 4 cleaved amplified polymorphic sequences (CAPS), and 1 PCR marker, were used

to screen the TAC library (ZHOU *et al.* 2003) and the positive clones were labeled as FISH probes.

Chromosome preparation: Immature *A. majus* flower buds at the length of 1.5–3.0 mm were harvested and fixed in Carnoy's solution (ethanol:glacial acetic acid, 3:1). Microsporocytes at meiosis stage were squashed in an acetocarmine solution according to WU (1967). Slides were frozen in liquid nitrogen. After coverslip removal, slides were dehydrated through an ethanol series (70, 90, and 100%) prior to use in FISH.

Fluorescence *in situ* hybridization: The FISH procedure used for chromosomes was according to JIANG *et al.* (1995). BAC/TAC DNAs were isolated by using a standard alkaline extraction procedure (SAMBROOK *et al.* 1989) and labeled with either biotin-11-dUTP or digoxigenin-16-dUTP (Roche Diagnostics, Indianapolis) by nick translation. Slides bearing meiotic chromosomes were denatured on an 80° heating plate for 2 min with 70% formamide in 2× SSC and then immediately immersed in a set of precooled ethanol (70, 90, and 100% for 5 min each). Denatured probe mixture (20 μl containing 50–100 ng labeled probe, 2× SSC, 50% deionized formamide, and 10% dextran sulfate) was applied to each slide and covered with a coverslip. Hybridization was carried out at 37° overnight in a moist chamber. After removing the coverslips, slides were washed in 2× SSC for 10 min at 42°, 2× SSC for 5 min each at room temperature. Probes labeled with biotin were detected with Texas red-conjugated avidin (Vector Laboratories, Burlingame, CA), whereas probes labeled with digoxigenin were examined with fluorescein isothiocyanate (FITC)-conjugated sheep-antidigoxigenin (Roche). Chromosomes were counterstained with 4′,6-diamidino-phenylindole (DAPI) in an antifade solution (Vector).

Chromosomes and FISH signal images were captured with an Olympus BX61 fluorescence microscope conjunct with a microCCD camera. Grayscale images were captured for each color channel and then merged using Image-Pro Plus (IPP) software. The pachytene chromosome lengths were measured using IPP software.

DNA sequencing and assembly: BAC clones were purified by cesium-chloride gradient. For a shotgun approach, sheared

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Consensus   1  C T T C G C C A T G A G C T G T G T C G T A T T T T G G G A T G C A G T C G A G A A A G C G T T A G C G T T T T T G A A   60
A           1  . . . . . . . . . . . . . . . t . . . . . . . . . . . . . . . . . . . . a . . . . . . t . . . . . . . . a . . . . . . . . t   60
B           1  . . . . . . . . . . . . . g . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . g . . . . . . . . . . . t   60
C           1  . . . . . . . . . t . . . . . . . . . . . t . . . . . . . . . . . . . . . . . . . . . . . . . . . g . . . . . . c . . . t   60
D           1  . . . . . . . . . . . g . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . t . . g . . . . . . . . . t   60
E           1  . . . . . . . . . . t . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   60
F           1  . . . . . . . . . . . . . . . . . a . . . . . . . . . . . . . . . . . . . . t . . . . . . . . . . . . . . . . . . .   60
G           1  . . t . . . . t . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . t . . . . . . . . . . . . c . . . .   60
H           1  . . a . t . . . . t . . . . a . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . a . . . . g . . g   60
I           1  . . . . . . . . . t . . . . . . . . . . . . . . . . . . . . . t . . . . t . . . . . . . . . . . . . . c . . . .   60
X67294      1  . . . . . . . . . . . . . . . . . . . . . . . t . c . . . . . . . . . ~ . . . . t . . a . . . . . t . .   59

Consensus  61  G A A A T A T G A T T T A T G C A G G A A T A A A G G C T A A A T C T ~ G G A G T G G T C C G T T T T T G A C A A A T C  119
A          61  . . . . . . . g . . . . . . . . . . . . . . . . . . . . . . . . . a . . c . . . . . . . . a . . . . . . . . . .  119
B          61  a . . ~ . . . . . . . . . . . . . . . . . . . . . . . . . . . . a a . . . . . . . . . . . . . . . .  118
C          61  . . . . . . . . . . . . . . . . . . . . . . . c t . . t c . . . . . . . . . . . . . . . c . . . . . . . . . .  119
D          61  a . . ~ . g . . . . . . . . . . . . . . . . . . . . . . . . . a a . . . . . . . . . . . . . . . . . . .  118
E          61  . . . . . . . . . . . c . . . a a . . . . c . a . . . . . . . . . . . . a . . . . . . . . . . . . . c .  119
F          61  . . . . . . . . . . . a . . . . . . . c . a ~ . . . . . . . . . . t . c . a . . . . . . . . . . . . .  118
G          61  . . . . . . . . . . . . . . . . . . . . . c g . . t c . . . . a . . . . . . . . . . . . . . . . . . . .  119
H          61  . . . ~ . . . . . . . . . a . . c . . . . . . . . . t c . . . . . . a . . . . . . . . . . . . . . . .  118
I          61  . . . . . . . . . . . . . . . . . . . . . c g . . t c . . . . . . . . . . . . . t . . . . . . . . . . .  119
X67294     60  . . . c . . . . . . . c . . . . . . . . . . . . . . . a . t a . . . . . . t . . . . . c . . . . g . . a .  119

Consensus 120  G G T A G G C T A A T T T C G T C A G A A A C G G G T C A A G G C A C A A T A G T C A A N G  165
A         120  . . . . . . . . . . . . . . . . . t t . . . . . . . . . . . . c . . . . . . . . . c .  165
B         119  . . . . . . . . . . . . . . . . . . t . . . . . . . . . . . . . . . c . . . . . . c .  164
C         120  . . . . . . . . . . . a . . . . . t . . . . . . . . a g . . . ~ . . . . t t .  164
D         119  . . . . . . . . . . . . . . . . . t t . . . . . . . . . . . . . . . . . . . . . c .  164
E         120  . . . . . . . . . . . . . . . a . . . . . . . . . . . . . . . . t . . . . t .  165
F         119  . a . . . . . . . . . g . . . . . . . . a . . . . . . . . . . . . . . . . c .  164
G         120  . . . . . . . . . . . . a a . . . . t . . . . . . a g . . . . a . . . t a .  165
H         119  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . a . . . . t .  164
I         120  . . . . . . . . . . . . a . . . t . . t . . . a . . . a g . . . . . . . t a .  165
X67294    120  a . . . . . . . . . . t . . . . . . . . . . . . . . . . a g . . . . . . . . t a .  165
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Figure 2.—Alignment of nine 165-bp repeats and X67294. A–I, nine randomly selected CentA1 repeats from 36D21.

BAC DNA (2–3 kb) was ligated into a pBluescript vector and transformed into *Escherichia coli* DH5α. The shotgun subclones were sequenced from both ends by the dideoxy chain termination method using either BigDye Terminator Cycle Sequencing V2.0 Ready Reaction (Applied Biosystems) or DYEnamic ET Dye Terminator kit (MegaBACE; Amersham Pharmacia Biotech). The shotgun sequences were assembled using the PHRED and PHRAP programs first, and primary assembly results were refined by careful manual checking to overcome the misalignments caused by repeats.

RESULTS

Isolation of the DNA sequence related to *A. majus* centromeres: Repetitive DNA elements often provide characteristic cytological landmarks for karyotyping. To isolate repetitive DNA elements, we screened an Antirrhinum BAC library (Lai *et al.* 2002) using sheared *A. majus* genomic DNA as a probe. A total of 50 positive clones showing strong hybridization signals were selected and labeled as FISH probes to hybridize to *A. majus* pachytene chromosomes. Those clones can be divided into four groups according to the FISH signal patterns. The first group, containing 17 clones, hybridizes to the centromere regions of six chromosomes (Figure 1A). The second group, consisting of only 1 BAC clone, hybridizes to the centromeric regions of three chromosomes (Figure 1B). The third group, consisting of 6 clones, uniformly hybridizes to all chromosomes, showing stronger signals in the heterochromatic regions than in euchromatic regions. This result indicates that these 6 clones contain dispersed repeat sequences, most

likely transposons or retrotransposon-related sequences. The fourth group had 26 clones, which hybridized only to specific locations on pachytene chromosomes.

To pinpoint cytological locations of the BAC clones derived from the centromeric regions, two BAC clones, 36D21 (group 1) and 5E10 (group 2), were selected and labeled as FISH probes to hybridize to the metaphase I chromosomes of *A. majus*. FISH signals from 36D21 were consistently detected at the most poleward positions on the bivalent chromosomes (Figure 1C), suggesting that the 36D21-related DNA sequences are located at chromosomal regions associated with the kinetochore complex. However, for BAC 5E10, the strongest FISH signal from the three chromosomes with hybridization signals was always pulled out of the bivalent chromosome (Figure 1D), indicating that 5E10 contains centromeric sequences of this chromosome.

BACs 36D21 and 5E10 were sequenced using the shotgun approach. Both clones are mainly composed of tandem repetitive DNA sequences, thereby making complete assembly difficult. The largest contigs for 36D21 and 5E10 are 33.237 and 25.313 kb in size, respectively, which are far smaller than the estimated insert sizes (∼70 kb) based on fingerprinting data (data not shown). Tandem repeat finder software (version 3.21; Benson 1999) was used to define the repeat unit. The 36D21 clone is composed exclusively of a 165-bp tandem repeat, named as CentA1. The CentA1 repeat is homologous to the *A. majus* repeat family described previously (Schmidt and Kudla 1996; accession no. X67294).

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CentA2-I   1   ~~~~~~TTGTCGTGATATGATTTTTAATTTCAAAATTTTTACATATTTATTTTTGT~~~~   50
CentA2-II  1   TTTACCACGTCCTGATAGCTTTTTTGATTCAAAAAGTTTCATATGTTTGATTTTTTAGAT   60
A.t 5S     1   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~    1

CentA2-I   50  ~TAAAAGTGGACGTTGCGA~~~TAGATTGCACAATGACTTTAGTTTGTG~~~~~~~~~~~   95
CentA2-II  61  GTGAACGATGTGAATTCGACACTAAATTGCGTTACGACTTTTGGTTGTGATGATAC~ACC  119
A.t 5S     1   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GGATGCGATCATACCAGC     18

CentA2-I   95  ~~~~~~~~~~~~~~~~~~~ATCACTAACTTCAGTGTTAAGCGTGCTTGAGGGAGAATAGTA  137
CentA2-II  120 ACAAATTCTTCAGATACCATCAC~AACTCCAAAGTTAAGCGCGTTTGGGTGAGACTAGCA  178
A.t 5S     19  ACTAATGCACCGGATCCCATCAG~AACTCCGCAGTTAAGCGTGCTTGGGCGAGAGTAGTA   77

CentA2-I   138 CTATGATTTGTGATCCTAAGGATA~CTTCGGGTTGCACCCATTTTTTGTATTCACAATTT  196
CentA2-II  179 GTGATCTGGATGACCGTAACGATGTCCTCTTGTAACATCC~TTTTTTGTATTTGCGTTTT  237
A.t 5S     78  CTAGGATGGGTGACC~~~~~~~~~~~TCCTGGGAA~GTCCTCGTGTTGCATCCCT      120

CentA2-I   197 TGTTTTTATCGATTTAATTTAAGCGTTTCTCTCGTGTTCTGCGGTGTCATAT~T~ATTTT  254
CentA2-II  238 TAATTTCGTCGATTTTTATTTATGAAATACTCTTGAGTTGTGCA~TGCCAAATGTTATTTT  296
A.t 5S

CentA2-I   255 TGTCAATTT~ATTTTAAT~TCAAGAGACACGTAGCTTGGAGCGAAGCGCCTCAGTCCGAC  312
CentA2-II  297 TGTCATTTTTATTTTAAAATCATCAGTC~~~~~~~~~~~~~~~~~GCGCCTCGCTTCG~C  338
A.t 5S

CentA2-I   313 TCTTC~TTTGGAATACATG~~~~~~GTCTTGTTTTTCATAAACTTTTTGTATACAAATAT  365
CentA2-II  339 CATTCCTTCCCAATACTTGATTCTAGTTTTGTTTTTTATCAACTATTTGTTTACACATAT  398
A.t 5S

CentA2-I   366 ATAGA~ATATATATAGATATGTATAATTTAA~TTATGATTTTTCATTATATTTTTTAAG~  422
CentA2-II  399 TAATATATATATATATATAACATAAATGTAACTTTTGATATTTTATTTTATTTTTTGTGT  458
A.t 5S

CentA2-I   422 ~~~~~~GCGCAAAGCTT   433
CentA2-II  459 ATTGTTGCTCAATTCTT   475
A.t 5S
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FIGURE 3.—Alignment of CentA2-I, CentA2-II, and *A. thaliana* 5S rRNA. CentA2-I, CentA2-II, the two subunits of the 921-bp repeat from 5E10. A.t5s, 5S rDNA of *A. thaliana* (accession no. AF330998); high similarity portion is underlined.

Pairwise alignment of nine randomly selected CentA1 monomers with X67294 shows 80–97% sequence identity (Figure 2).

The 5E10 clone is composed of a 921-bp tandem array sequence, named as CentA2. The similarity between each unit is ~89%. The CentA2 monomers include two subrepeats, CentA2-I and CentA2-II, each with ~450 bp (Figure 3). The similarity between the two subrepeats, excluding a few small insertion/deletion regions, is ~59%. These results suggest that the 921-bp repeat is a high order repeat derived from a 450-bp repeat. Further sequence analysis revealed homology between the CentA2 sequence and 5S rRNA gene sequences, suggesting a possible evolutionary link between these sequences. The sequence similarity between bases 1–92 of *A. thaliana* 5S rRNA (AF330998) and the corresponding region on CentA2-II is as high as 84% (Figure 3).

Karyotype of the *A. majus* genome and pachytene chromosome identification: To develop a pachytene chromosome-based karyotype, we analyzed 51 pollen mother cells at the pachytene stage of meiosis. FISH signals derived from CentA1 and CentA2 were used to anchor the centromere position of individual pachytene chromosomes. The absolute length, relative length, and arm ratio of each chromosome were measured using IPP software (Table 1). The nomenclature of *A. majus* chromosomes was according to the descending order of the pachytene chromosome lengths. Chromosome 1 is the longest with an average length of 63.90 μm, accounting for 18.73% of the total length of the chromosome complement. Chromosome 8 is the shortest one,

~33.05 μm in length, which accounts for 9.69% of the total chromosome length. *t*-Tests for chromosome length were conducted on each contiguous chromosome pair. Five chromosome pairs, *i.e.*, chromosomes 1 and 2, chromosomes 2 and 3, chromosomes 5 and 6, chromosomes 6 and 7, and chromosomes 7 and 8, were detected with significant difference in chromosome length ($P < 0.01$). The lengths of the other chromosome pairs were not significantly different and cannot be easily distinguished. Within a single cell, only chromosomes 1 and 2 could be unambiguously identified solely on the basis of chromosome length.

Arm ratio is an important feature that identifies pachytene chromosomes. In *A. majus,* arm ratios of the pachytene chromosomes range from 1.08 to 2.99. Among them, chromosomes 2 and 5 are the two medium centromeric chromosomes with arm ratios of 1.58 and 1.08, respectively. The other six chromosomes are all submedium centromeric chromosomes according to the criterion recommended by LEVAN *et al.* (1964). Chromosome 5 is the only chromosome that can be easily distinguished from the chromosome complement on the basis of its arm ratio.

Besides chromosomes 1, 2, and 5, it is difficult to distinguish the rest of the pachytene chromosome according to chromosome length and arm ratio. However, when the pachytene chromosomes are probed with CentA1, they can be divided into three groups. Group 1 chromosomes, consisting of chromosomes 1 and 7, do not hybridize to CentA1. Within group 1, chromosomes 1 and 7 can be distinguished by length; the longer

The karyotype analysis of the pachytene chromosomes in Antirrhinum

Chromosome no.	Short arm (μm)	Long arm (μm)	n^a	Total length (μm)	Relative length[b] (%)	Arm ratio[c]
1	19.22 ± 3.40	44.68 ± 6.93	51	63.90 ± 9.18	18.73 ± 1.24	2.32 ± 0.37
2	19.64 ± 2.90	31.00 ± 4.10	51	50.64 ± 6.60	14.85 ± 0.83	1.58 ± 0.20
3	12.16 ± 2.39	29.44 ± 4.29	51	41.61 ± 5.87	12.20 ± 0.92	2.42 ± 0.40
4	13.47 ± 1.62	27.36 ± 4.30	51	40.82 ± 5.43	11.97 ± 0.75	2.03 ± 0.26
5	19.00 ± 2.60	20.53 ± 3.11	51	39.53 ± 5.30	11.59 ± 0.86	1.08 ± 0.12
6	9.41 ± 1.30	27.57 ± 3.85	51	36.98 ± 4.63	10.84 ± 0.74	2.93 ± 0.39
7	11.73 ± 2.19	22.85 ± 3.17	51	34.58 ± 4.45	10.14 ± 0.62	1.95 ± 0.37
8	8.28 ± 1.08	24.78 ± 3.70	51	33.05 ± 4.46	9.69 ± 0.79	2.99 ± 0.36

[a] Number of measurements.

[b] Relative chromosome length is given as a percentage of the total complement length.

[c] Long arm/short arm.

is chromosome 1 and the shorter is chromosome 7. Group 2 chromosomes, consisting of chromosomes 2 and 5, show weak CentA1 signals. These two chromosomes can be distinguished from one another by centromere position. Chromosome 5 has metacentric signals whereas chromosome 2 has submetacentric signals. The remaining four chromosomes, chromosomes 3, 4, 6, and 8, are in group 3. Among this group, chromosomes 4 and 6 have only one CentA1 domain in the centromere region; the longer is chromosome 4 and the shorter is chromosome 6. Chromosomes 3 and 8 have two or three CentA1 domains. These two chromosomes can be distinguished from one another by length; the longer is chromosome 3 and the shorter is chromosome 8. Therefore, all eight Antirrhinum pachytene chromosomes could be distinguished according to CentA1 FISH signal patterns.

Mapping of the major heterochromatin domains in the Antirrhinum genome: When *A. majus* pachytene chromosomes are stained with DAPI, the brightly stained regions correspond to the heterochromatic domains. The DAPI-staining pattern of a given pachytene chromosome is highly consistent among different cells. We analyzed the DAPI-staining patterns in 51 *A. majus* pachytene cells. In general, heterochromatin is mainly located in the pericentric regions. This heterochromatin distribution pattern is similar to that reported in *A. thaliana* (Fransz *et al.* 1998). In addition to this general distribution pattern, chromosome 1 has only a few small heterochromatin domains, mainly located on the short arm close to the centromere. The long arm of chromosome 1 (the longest arm in the genome) contains no clearly visible heterochromatin, which could be used as the most important characteristic to distinguish chromosome 1 (Figure 4, A and B). The short arms of chromosomes 3 and 8 are highly heterochromatic. Moreover, major heterochromatin domains are located at the ends of the short arms of chromosomes 3, 4, and 6. The heterochromatic domain on the short arm of chromosome 6 is the most pronounced of the telomeric heterochromatin.

An ideogram map based on the DAPI-staining pattern of pachytene chromosomes was developed to depict the distribution of heterochromatin in the *A. majus* genome (Figure 5). The ideogram is generalized on the basis of the same 51 pachytene cells used in karyotype analysis. The consistently bright DAPI-staining regions were drawn as solid circles, which represent the heterochromatin along the pachytene chromosomes. In early pachytene, the adjacent heterochromatin domains are better separated and easily identified. However, in late pachytene, the closely adjacent heterochromatin domains tend to merge. Therefore, early pachytene is the most appropriate stage to visualize the distribution of heterochromatin (Figure 4, A and B).

Integration of the chromosome map and its genetic map in Antirrhinum: A molecular linkage map was constructed on the basis of an interspecific hybrid *A. majus* × *A. molle* F_2 population with 243 individual loci. This map included 164 protein-coding loci and a similar number of unknown sequences such as AFLP, RFLP, inverse sequence tagged repeat, and intersimple sequence repeat (ISSR) markers with a total length of 613 cM (Schwarz-Sommer *et al.* 2003b). However, the relationship between the linkage groups and their corresponding chromosomes is not known. We used FISH to integrate the genetic linkage maps to individual *A. majus* chromosomes. TAC clones that are anchored by genetically mapped DNA markers were used as FISH probes and were thus hybridized to specific chromosomes. One or two markers on each linkage group were selected by screening a TAC library constructed from a self-incompatible *A. majus* × *A. hispanicum* line (Zhou *et al.* 2003). Using this strategy, a total of 14 molecular markers, consisting of 9 RFLPs, 4 CAPS, and 1 PCR marker, were physically mapped on eight *A. majus* pachytene chromosomes through their corresponding TAC clone FISH (Figure 4C; Table 2). Five linkage groups are physically anchored by 2 markers, and the remaining three linkage groups are anchored by 1 marker close to the chromosomal end. The short/long arm orientation of all eight
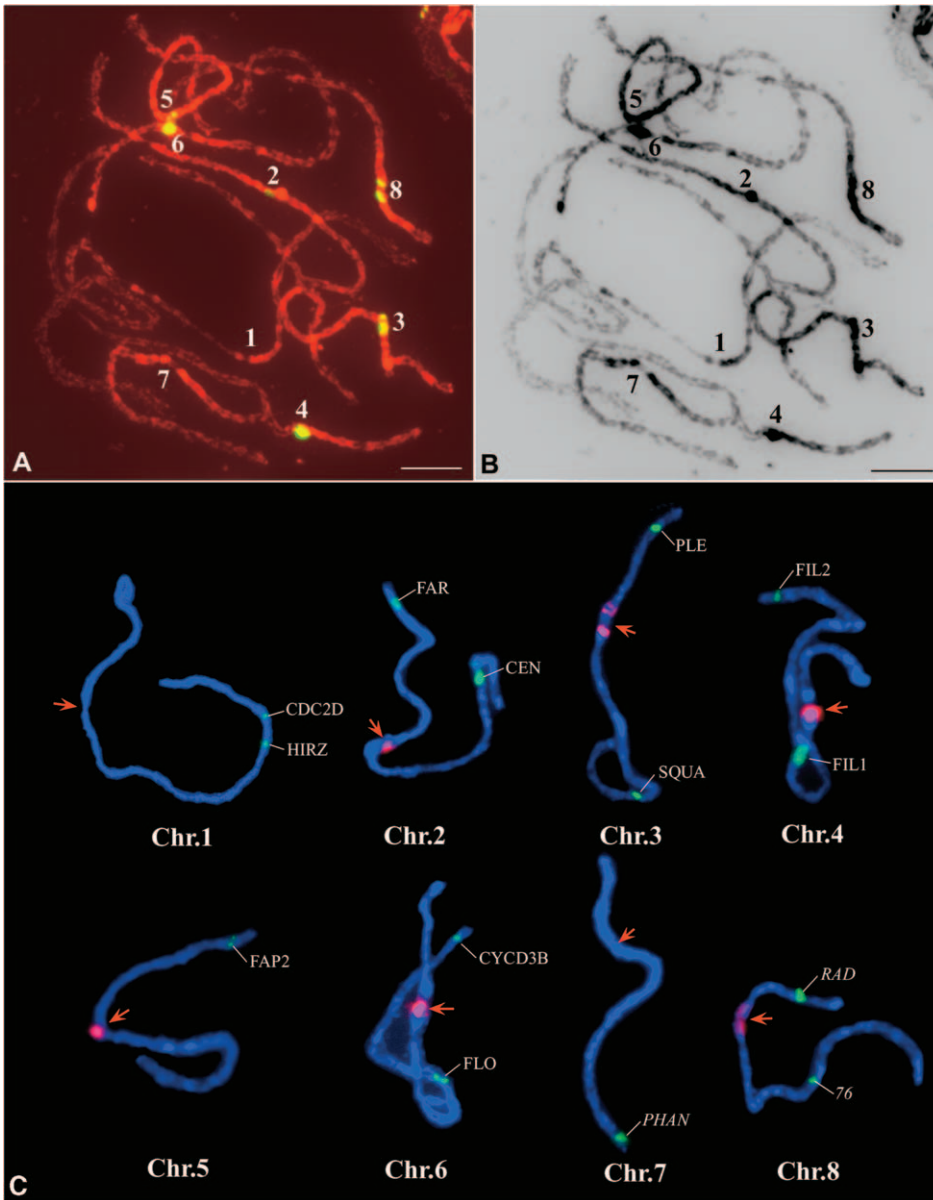
FIGURE 4.—Mapping of heterochromatin regions and integration of the chromosome map with the linkage group in Antirrhinum. (A) Pachytene chromosomes of *A. majus* were probed with CentA1. Chromosomes are stained with DAPI and pseudocolored as red. Bar, 5 μm. (B) DAPI-stained chromosomes in A were converted to a black-and-white image to enhance the visualization of distribution of euchromatin and heterochromatin regions along the pachytene chromosomes. (C) The pachytene chromosomes of *A. majus* were probed by different TAC clones (green signals) anchored by different markers as well as CentA1 (red signals). Arrows show the centromere positions.

Antirrhinum linkage groups has been established on the basis of the TAC clone FISH signal positions. A new linkage map adapted from the original molecular map constructed by SCHWARZ-SOMMER *et al.* (2003b) was developed (Figure 6) on the basis of the descending order of pachytene chromosome lengths and the corresponding relationship between the chromosomes and the linkage map. In the adapted linkage groups, we have also switched the directions of four original linkage groups, including LG2, LG3, LG4, and LG6, and reorientated all the ends of the short arms to the north side of the linkage maps and the long arm to the south side. These four linkage groups have been computationally redrawn on the basis of the linkage groups that were previously reported (SCHWARZ-SOMMER *et al.* 2003b).

Our FISH mapping effort revealed varied recombination values in different chromosomal regions in the *A. majus* genome. In general, physical distance is less than genetic distance when markers are located close to the end of a chromosome. For example, the two markers *HIRZ* and *CDC2D* have a genetic distance of 33.13% of the whole chromosome 1. However, the physical distance between these markers is only 5.56% of the total length of chromosome 1. Markers near the centromere region display the opposite relationship. In these cases, the physical distance is always longer than the corresponding genetic distance, as observed for the markers on chromosomes 2, 3, and 8. This discrepancy between genetic distance and physical distance is probably due to the reduced recombination rate in the pericentromeric regions of *A. majus* chromosomes. This skewed relationship between genetic and physical distances may also
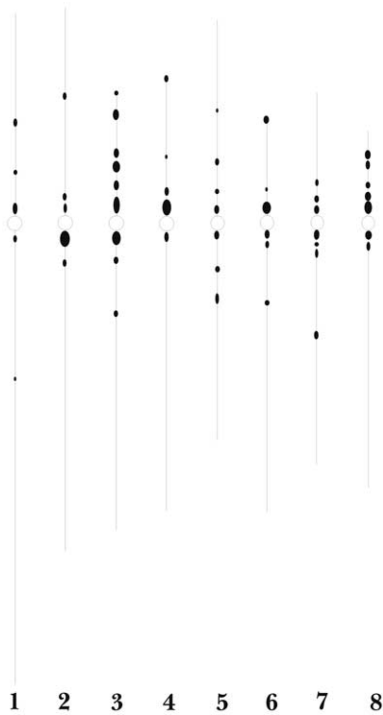
FIGURE 5.—Ideogram of the distribution of DAPI-bright regions on *A. majus* pachytene chromosomes. The ideogram was generalized on the basis of observations of 51 pachytene cells. The solid circles represent a distinct DAPI-bright region that is consistently observed in the majority of the pachytene chromosomes. Open circles represent the location of the centromeres. The relative length and arm ratio of each chromosome were drawn on the basis of Table 1 data.

be affected by the relatively low density of markers on the current genetic map. In addition, our FISH mapping data also showed that the genetic distance of different chromosomes does not always correspond to their physical distance, indicating that the current Antirrhinum genetic map may not fully cover the whole genome.

DISCUSSION

Centromeric sequences in Antirrhinum: Centromeres are the essential structure of eukaryotic chromosomes and are responsible for precise segregation of sister chromatids at both mitosis and meiosis. The DNA structure and associated proteins have been well studied in yeast and human. In plants, different kinds of repetitive DNA sequences related to centromeres have been isolated and characterized (BIRCHLER 1997; JIANG *et al.* 2003). In monocot grass species, centromeric specific sequences have been isolated in rice, wheat, barley, maize, sorghum, rye, and sugar cane (ARAGÓN-ALCAIDE *et al.* 1996; DONG *et al.* 1998; MILLER *et al.* 1998; NAGAKI *et al.* 1998; PRESTING *et al.* 1998; KISHII *et al.* 2001; PAGE *et al.* 2001; CHENG and MURATA 2003; NAGAKI *et al.* 2003). All the centromeres of these species are composed of two kinds of repetitive sequences, tandem re-

peats and retrotransposons. Centromere-specific tandem repeats are often species specific, while the retrotransposons are much more conserved, with a high similarity among related species. The centromere structure of the model dicot plant, *A. thaliana*, has also been well characterized (BRANDES *et al.* 1997). The 180-bp repeat family, pAL1, is the major centromere-specific element, which is organized into long tandem arrays and may be interrupted by the 106B repeat, a diverged copy of the long terminal repeat of the *A. thaliana* retrotransposon. Apart from *A. thaliana*, the centromere-specific repeats have been identified in two more dicot plants, Beta and Brassica. In Beta species, several types of tandem repeats and retrotransposons related to the centromere were identified (GINDULLIS *et al.* 2001). For example, the 326–327-bp tandem repeat, pBV1, and 417-bp Ty3-*gypsy*-like retrotransposon, pBv26, are the major centromeric elements in *B. vulgaris*, while the 158–160-bp tandem repeat, pTS5, 312-bp tandem repeat, pTS4.1, and 417-bp Ty3-*gypsy*-like retrotransposon, pBb10, are the major centromeric elements in *B. procumbens*.

In this study, two centromeric tandem repeats, CentA1 and CentA2, were isolated from *A. majus.* The CentA1 is a 165-bp tandem repeat that hybridized to six centromeres of eight *A. majus* chromosomes, while the CentA2 is a 921-bp tandem repeat that hybridized to only three of those six centromeres with CentA1 signals. Among the six centromeres, CentA2 is the centromeric element only for chromosome 2, but CentA1 acts as the centromeric element for the other five centromeres according to the FISH signal pattern on metaphase I (Figure 1, C and D). We still have not found centromere-specific repetitive sequences for two chromosomes in the *A. majus* genome, chromosomes 1 and 7. Sequence analysis showed that CentA2 is composed of two subrepeats, each with a length around 450 bp. CentA2 is highly similar to *A. thaliana* 5S rRNA bases 1–92. It has also been reported that rice satellite sequence RC48 shows homology to 5S rRNA (WU and WU 1987), but the degree of similarity (42.3 and 44.8%) is lower than that of CentA2. It seems that 5S rDNA could act as an active agent in producing various tandem repeats by an uncharacterized mechanism.

Antirrhinum chromosome identification: Identification of individual chromosomes is essential for cytogenetic research. In classical cytogenetic analysis, somatic metaphase chromosomes or meiotic pachytene chromosomes are stained with acetocarmine or Giemsa. Individual chromosomes are then distinguished on the basis of their length, arm ratio, staining pattern, and additional physical characteristics. Since chromosome morphology varies among different cells, the identification of individual chromosomes is usually time consuming and the results from different labs or experiments often do not agree with one another, especially for species with small chromosomes. Modern cytogenetic approaches using fluorescent microscopy have been developed in part to

TABLE 2

Comparison of genetic distances of DNA markers and cytogenetic distances of corresponding TAC clones

Chromosome	BAC clones	Linkage group (LG)	n^a	Genetic location (cM) Old ^{d}	New ^{e}	Relative genetic location ^{b}	Physical location ^{c}
1L	*HIRZ*	8	10	44.8	44.8	66.87	72.93 ± 5.80
1L	*CDC2D*	8	10	67.0	67.0	100	78.49 ± 6.07
2S	*FAR*	4	10	89.1	0.00	0.00	4.58 ± 0.94
2L	*CEN*	4	10	60.9	35.2	39.51	69.82 ± 5.30
3S	*PLE*	2	10	16.1	17.4	51.94	8.51 ± 0.81
3L	*SQUA*	2	10	9.7	23.8	71.04	75.42 ± 2.58
4L	*FIL1*	1	10	20.4	20.4	24.64	29.08 ± 2.10
4L	*FIL2*	1	10	55.6	55.6	67.15	96.06 ± 1.23
5S	*FAP2*	7	10	15.1	15.1	16.78	6.65 ± 0.73
6L	*FLO*	3	10	33.4	38.6	53.61	58.48 ± 3.90
6L	*CYCD3B*	3	10	2.0	70.0	97.22	95.76 ± 1.02
7L	*PHAN*	5	10	82.8	82.8	100	95.80 ± 0.79
8S	*RAD*	6	10	82.1	13.8	14.39	10.24 ± 1.76
8L	76	6	10	74.4	21.5	22.42	70.40 ± 2.69

[a] The number of measurements.

[b] Relative genetic position is calculated as $(cmX \div cmT) \times 100$, where cmX is the cM value of the RFLP marker on the linkage map and cmT is the total cM value of the same linkage map (Schwarz-Sommer *et al.* 2003b). For example, *HIRZ* was mapped to 44.8 cM on linkage map 8 that totals 82.8 cM. The relative genetic position of *HIRZ* is $(44.8 \div 67.0) \times 100 = 66.87$.

[c] Physical location is calculated as $(S \div T) \times 100$, where S is the distance (in micrometers) from the FISH hybridization site to the end of the short arm of the chromosome and T is the total length of the chromosome in micrometers.

[d] The genetic distance of the BAC clones on the linkage map (Schwarz-Sommer *et al.* 2003b).

[e] The genetic distance of the BAC clone on the adapted linkage map.

address this problem. Staining of chromosomes with fluorescent dyes, particularly with DAPI, offers several major advantages compared to classical methods: (1) only chromosomes are stained; all the other parts of a cell, *i.e.*, cell wall, cytoplasm, nucleolus, etc., are not stained; (2) the euchromatic regions and heterochromatic regions have different staining patterns, which are consistent with those of Giemsa staining; and (3) it is more convenient to be combined with fluorescence *in situ* hybridization. Thus DAPI has become the most routine dye for chromosome staining in cytogenetic labs.

Although *A. majus* has only eight chromosome pairs at pachytene stage, unambiguous identification of individual pachytene chromosomes based on their morphology is almost impossible. But when they are probed with CentA1, the pachytene chromosomes can be readily distinguished. Moreover, a new strategy for chromosome identification using chromosome-specific BAC clones was recently demonstrated in several plants, such as potato, rice, etc. (Cheng *et al.* 2001a; Dong *et al.* 2001). Using the same strategy, in this study we have developed a set of chromosome-specific or chromosome arm-specific markers, which are very useful molecular cytological markers to easily identify specific *A. majus* chromosomes.

Recombination in the *A. majus* genome: The recombination rate is variable among different chromosomal regions. Generally, the pericentromeric region has a lower recombination rate than the distal region, especially for grasses with large genomes and high repetitive DNA content, such as wheat, barley, and sorghum. In wheat, the recombination rate around the centromeric region was highly decreased. The distal one-third of any wheat chromosome arm shows 8–15 times the recombination compared to the proximal one-third (Hohmann *et al.* 1994; Gill *et al.* 1996). On the short arm of chromosome 1, recombination near the centromere is negligible. Ninety-nine percent of the recombination occurs in the distal 60% of the arm (Sandhu *et al.* 2001). The same phenomenon was observed in barley by FISH or by translocation breakpoints analysis (Pedersen *et al.* 1996; Kunzel *et al.* 2000). In sorghum, the relationship between genetic distance and physical distance was demonstrated in detail by probing a 14-BAC probe cocktail to pachytene chromosome 1. Only ~1.7 of the 242.9 map units of total chromosome 1 were found to span ~60% of the physical length of somatic metaphase chromosome 1. This ~60% was located in the proximal chromosomal region, indicating that the recombination rate in the pericentromeric region is much less than that in the distal end region (Islam-Faridi *et al.* 2002).
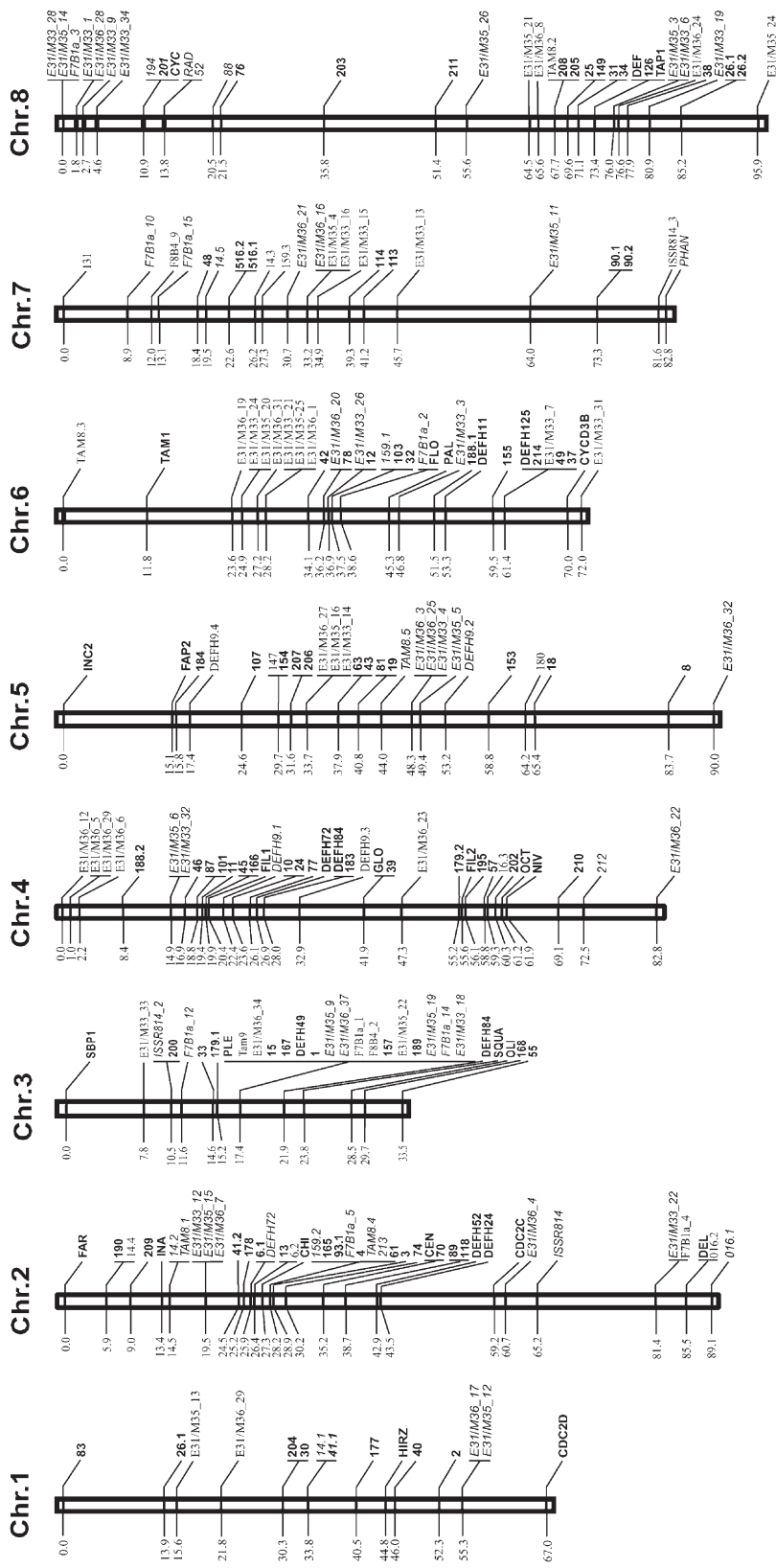
FIGURE 6.—The adapted linkage map of Antirrhinum based on the descending order of pachytene chromosome length.

But for genomes with small chromosomes, such as rice and Arabidopsis, the decreased recombination rate in the pericentromeric region is relatively low (Mayer *et al.* 1999; Cheng *et al.* 2001b; Wu *et al.* 2003). For instance, rice genetic markers R2174 and C148 are nearby the chromosome 10 centromere and genetically span ∼4.8% of the total chromosome. Cytogenetic analyses indicate that these markers physically span 17.1% of the entire chromosome (Cheng *et al.* 2001b). Similar rates of recombination were found on Arabidopsis chromosome 4 (Schmidt *et al.* 1995). In *A. majus*, we also found the recombination rate in the pericentromeric regions is less than that in the distal end region. In addition, our detailed cytological characterization of the Antirrhinum genome will greatly help advance genomic studies of these species.

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