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Reciprocal and Multi-Species Chromosome BAC Painting in Crucifers (Brassicaceae)

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Key Words

Arabidopsis halleri · *Arabidopsis thaliana* · BAC FISH · Brassicaceae · Collinearity · Comparative chromosome painting · Plant cytogenetics

Abstract

In crucifer cytogenomics, BAC contigs of *Arabidopsis thaliana* have been used as probes for comparative chromosome painting among species. Here we successfully tested chromosome-specific BAC contigs of *A. thaliana* (n = 5) and *A. halleri* (n = 8) as probes for reciprocal BAC painting. Furthermore, BAC contigs of both *Arabidopsis* species were applied as multi-species painting probes to a third crucifer species, *Noccaea caerulescens* (n = 7), revealing their shared chromosome homeology. Specifically, we found homeology across portions of chromosomes At2, Ah4, and Nc4, which reflects their shared common origin with chromosome AK4 of the Ancestral Crucifer Karyotype (n = 8). We argue that multi-species and multi-directional painting will significantly expedite comparative cytogenomics in Brassicaceae and other plant families.

Identification of plant chromosomes by chromosome-specific painting probes is hampered by a wide spectrum of dispersed repetitive elements equally distributed over chromosomes of a given complement. Hence, all flow-sorted or microdissected, and DOP-PCR amplified, DNA probes yielded unspecific cross-hybridization signals [reviewed by Schubert et al., 2001]. An alternative strategy to paint chromosomes of plants has been found in the application of contigs and supercontigs of chromosome-specific BAC clones. This approach relies on the availability of chromosome-specific BAC libraries and a low amount of repetitive elements in the donor and target genomes.

Arabidopsis thaliana (n = 5) has become the first plant species with all chromosomes painted using chromosome-specific BAC supercontigs [Lysak et al., 2001; Pecinka et al., 2004]. Later the very same BAC contigs were applied as painting probes for comparative chromosome painting (CCP) across the mustard family (Brassicaceae) [e.g. Lysak et al., 2003, 2006; Mandáková and Lysak, 2008]. Recently, cross-species BAC FISH was successfully developed for sorghum and maize (Poaceae) [Amarillo and Bass, 2007], tomato and potato (Solanaceae) [Iovene et al., 2008; Tang et al., 2008] as well as for cucurbit species (Cucurbitaceae) [Han et al., 2009].

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In Brassicaceae, BAC clones of *A. thaliana* were exclusively used as painting probes for cross-species CCP as chromosome-specific BAC libraries for other crucifer species were not developed. This is changing as genomes of an increasing number of cruciferous species are being sequenced and genomic resources become available. Chromosome-specific BAC contigs were assembled for *Brassica rapa* [Mun et al., 2008] or *A. halleri*, a model species of heavy metal accumulation [Lacombe et al., 2008]. In upcoming years we will witness a steadily increasing number of whole-genome sequencing projects including important crop and model crucifer species such as *A. lyrata*, *Boechera* spp., *Capsella rubella*, *Eutrema* (*Thellungiella*) *halophila* (DOE Joint Genome Institute; http://www.jgi.doe.gov), and *B. rapa* (http://www.brassica.info). These sequencing efforts will generate new cytogenomic resources and significantly facilitate family-wide comparative research.

In anticipation of newly available genomic resources for crucifer cytogenomics, we have tested chromosome-specific BAC contigs of *A. halleri* (n = 8) and *A. thaliana* as probes for reciprocal CCP. Furthermore, the BAC contigs of both *Arabidopsis* species were applied simultaneously as multi-species painting probes to reveal shared chromosome homeology in a third species.

Material and Methods

Plant Material

Inflorescences of *A. halleri* subsp. *tatrica* (Pawł.) Kolník (2n = 16) were collected from a single wild population (Slovakia, Belianske Tatry Mts., Tatranská Javorina, the Zadné Meďodoly valley). *A. thaliana* accession C24 (2n = 10) was used in the present study. Flower buds of *Noccaea caerulescens* (2n = 14) were collected from a population in the village of Kořenec, Czech Republic [Mandáková and Lysak, 2008].

Preparation of Pachytene Chromosomes

Entire inflorescences were fixed in freshly prepared ethanol:acetic acid (3:1) fixative overnight and stored in 70% ethanol at –20°C until use. Selected inflorescences were rinsed in distilled water and citrate buffer (10 mM sodium citrate, pH 4.8) and subsequently incubated in the 0.3% enzyme mix (cellulase, cytohelicase and pectolyase; all Sigma-Aldrich) in citrate buffer at 37°C for 3–4 h. After digestion, individual flower buds were disintegrated by a needle in a small drop of citrate buffer and the material spread in 20 μl of 60% acetic acid on a hot plate (50°C). The chromosomes were fixed using 100 μl of ice-cold ethanol:acetic acid fixative, then the slides were dried using a hair dryer. The preparations were staged using a phase contrast microscope and suitable slides were post-fixed in 4% formaldehyde in distilled water for 10 min and air-dried.

Painting Probes

A. thaliana BAC clones were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Columbus, OH). The 3 BAC contigs used [F3K23 (AC006841) – F8N16 (AC005727), F16P2 (AC004561) – F7F1 (AC004669), and F13P17 (AC004481) – T8I13 (AC002337)] represent an almost complete BAC tiling path of *A. thaliana* chromosome At2, corresponding to the collinear region on the ancestral chromosome AK4 of the Ancestral Crucifer Karyotype [Schranz et al., 2006]. From this tiling path each third BAC was isolated and labeled.

A. halleri BAC clones were selected from the genomic BAC library [Lacombe et al., 2008] and 2 BAC contigs were built based on the chromosome collinearity existing between *A. thaliana* and *A. lyrata* [Kuittinen et al., 2004]. The A contig (from AT2G31130 to AT2G32520) comprises BAC clones XIII-P-6, II-F-23, XIV-I-23, II-K-14, V-A-2, I-E-5, I-K-8, and I-I-19. The B contig (from AT2G32840 to AT2G33510) is composed of BAC clones IV-K-16, III-M-8, and II-F-13. Furthermore, 2 repeat-rich BAC clones IV-E-9 and IX-B-21 [Lacombe et al., 2008] were tested (fig. 1A, D).

DNA of individual BAC clones was isolated using a standard alkaline extraction omitting the phenol:chloroform purification step. BAC DNA was labeled by biotin-, digoxigenin-, and Cy3-dUTP via nick translation as follows: 1 μg of BAC DNA diluted in distilled water to 29 μl, 5 μl of nucleotide mix (2 mM dATP, dCTP, and dGTP, 400 μM dTTP; all Roche), 5 μl of 10× NT buffer (0.5 M Tris-HCl, pH 7.5; 50 mM $MgCl_2$, 0.05% BSA), 4 μl of 1 mM x-dUTP (in which x was biotin, digoxigenin or Cy3), 5 μl of 0.1 M b-mercaptoethanol, 1 μl of DNase I (Roche), and 1 μl of DNA polymerase I (Fermentas). The nick translation mixture was incubated at 15°C for 90 min (or longer) to obtain fragments of ~200 to 500 bp. The reaction was stopped by adding 1 μl of 0.5 M EDTA, pH 8.0, followed by incubation at 65°C for 10 min. Labeled DNA of individual BAC clones was stored at –20°C until use.

Chromosome Painting: BAC FISH

The selected slides were treated by RNase (AppliChem; 100 μg/ml in water) for 1 h at 37°C, and washed in 2× SSC for 2–5 min. To remove cytoplasm, the slides were treated with pepsin (Sigma-Aldrich; 0.1 mg/ml) in 0.01 M HCl at 38°C for 10 min, followed by a wash in 2× SSC for 2–5 min. Subsequently, the slides were post-fixed in 4% formaldehyde in 2× SSC for 10 min, washed in 2× SSC (2–5 min), and dehydrated in an ethanol series (70, 80, and 96%).

Labeled BAC DNAs were pooled and precipitated to reduce the probe volume. For a single slide, the probe was dissolved in 20 μl of hybridization mix (50% formamide, 10% dextran sulfate in 2× SSC) at 37°C overnight. Probe and chromosomes were denatured together on a hot plate at 80°C for 2 min and incubated in a moist chamber at 37°C for 48 h. Post-hybridization washing was performed in 50% or 20% (CCP in *N. caerulescens*) formamide in 2× SSC for 3–5 min at 42°C. DNA labeled by biotin-dUTP was detected using avidin~Texas Red (Vector Laboratories) and amplified by goat anti-avidin~biotin (Vector Laboratories) and avidin~Texas Red. Probes labeled by digoxigenin-dUTP were visualized by mouse anti-digoxigenin (Jackson ImmunoResearch) and goat anti-mouse~Alexa Fluor 488 (Molecular Probes). Chromosomes were counterstained with DAPI (2 μg/ml) in Vectashield (Vector Laboratories).

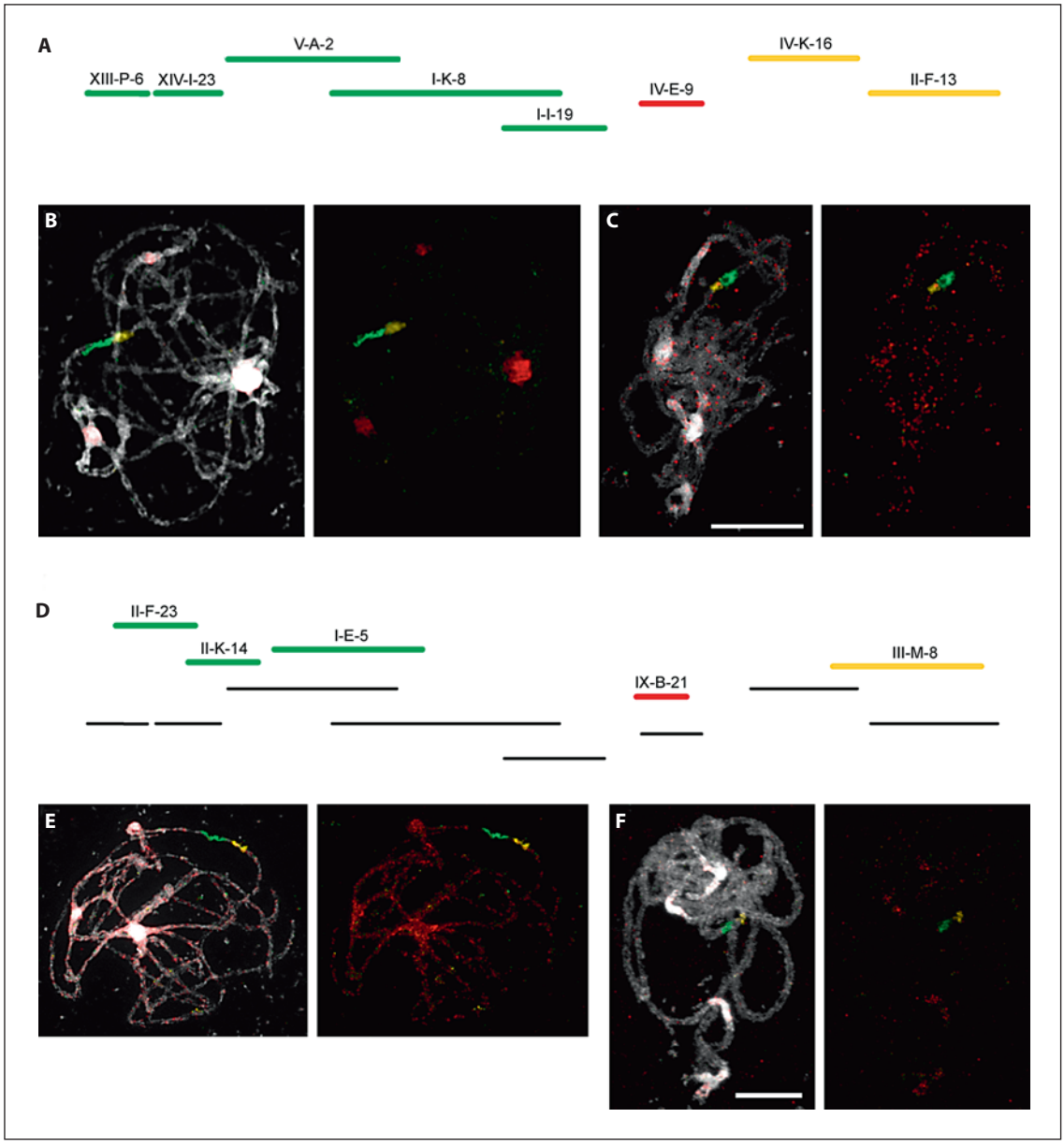

Fig. 1. BAC painting in *Arabidopsis halleri* and *A. thaliana*. **A** Labelling scheme of *A. halleri* BAC contigs A and B, and BAC clone IV-E-9. **B**, **C** In situ hybridization of the labeled probes (**A**) to pachytene chromosomes of *A. halleri* (**B**) and *A. thaliana* (**C**), respectively. **D** Labelling scheme of *A. halleri* BAC contigs A and B, and BAC clone IX-B-21. Black lines correspond to BAC clones labeled in (**A**). **E**, **F** In situ hybridization of the labeled probes (**D**) to pachytene chromosomes of *A. halleri* (**E**) and *A. thaliana* (**F**), respectively. Bar (10 μm) has the same magnification in (**B**) and (**C**), and in (**E**) and (**F**), respectively.

Preparations were observed using an Olympus BX-61 epifluorescence microscope, and images were acquired separately for all fluorochromes using appropriate excitation and emission filters (AHF Analysentechnik) using an AxioCam CCD camera (Zeiss). The 4 monochromatic images were pseudocolored and merged using the Adobe Photoshop CS2 software (Adobe Systems).

Results and Discussion

We have hybridized 2 *A. halleri* BAC contigs to pachytene chromosomes of *A. halleri* and *A. thaliana*. The 8 and 3 BAC clones of the A (c. 0.55 Mb in *A. thaliana*) and

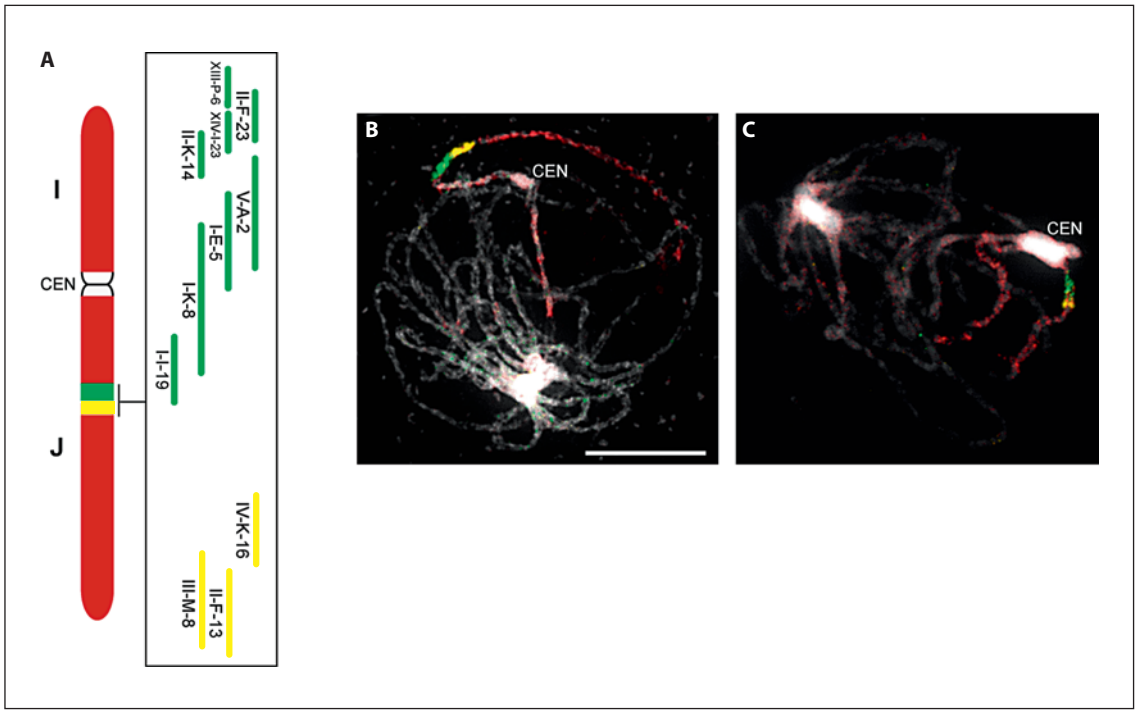

Fig. 2. BAC chromosome painting in *Arabidopsis halleri* and multi-species chromosome painting in *Noccaea caerulescens*. **A** Labeling scheme of *A. halleri* BAC contigs A and B (green, yellow), and *A. thaliana* supercontigs corresponding to genomic blocks I and J (red) of the Ancestral Crucifer Karyotype [Schranz et al., 2006]. Simultaneous in situ hybridization of *A. halleri* and *A. thaliana* BAC contigs to pachytene chromosome of *A. halleri* (**B**) and *N. caerulescens* (**C**). Bar (10 μm) has the same magnification in (**B**) and (**C**).

B (c. 0.27 Mb) contigs, respectively, have been differentially labeled (fig. 1A, D), and tested in both *Arabidopsis* species in the absence of blocking DNA. In *A. halleri*, the digoxigenin- (green) and Cy3-dUTP (yellow) labeled contigs A and B identified the expected chromosome region on the bottom arm of chromosome Ah4 (fig. 1B, E). The same contigs cross-hybridized to *A. thaliana* pachytene chromosomes provided a comparably strong signal on the bottom arm of chromosome At2 (fig. 1C, F), corresponding to the collinear region of Ah4 [Kuittinen et al., 2004; Schranz et al., 2006].

We have also tested 2 BAC clones positioned between the contigs A and B and presumably containing repetitive elements [Lacombe et al., 2008]. In *A. halleri*, BAC clones IV-E-9 and IX-B-21 hybridized to all centromeres and pericentromeres, respectively (fig. 1B, E). In *A. thaliana*, red signals of the IV-E-9 were irregularly scattered along all chromosomes with some preferential hybridization at pericentromeric regions (fig. 1C), whereas BAC IX-B-21 (red) hybridized to pericentromeric regions, though the hybridization signal intensity was weaker than in *A. halleri* (fig. 1F). The hybridization of BAC IV-E-9 to centromeric heterochromatin is due to *gypsy*- and CACTA-like mobile elements, whereas the clone IX-B-21 contains presumably a *copia*-like retrotransposon(s) [Lacombe et al., 2008].

Furthermore, we demonstrate how BAC contigs of both *Arabidopsis* species can be combined and simultaneously used to paint specific chromosome regions in a third species (fig. 2A–C). The 2 *A. halleri* contigs along with 3 *A. thaliana* BAC supercontigs homeologous to the genomic blocks I and J on ancestral chromosome AK4 [Schranz et al., 2006], except the part covered by *A. halleri* BACs, have been hybridized to pachytene chromosomes of *A. halleri* and *Noccaea (Thlaspi) caerulescens*. The latter species is phylogenetically closely associated with the crucifer Lineage II [Mandáková and Lysak, 2008], which is relatively distantly related to the genus *Arabidopsis* of the Lineage I. We assumed that the *A. halleri* karyotype resembles that of *A. lyrata* (n = 8) [Kuittinen et al., 2004],

and hence the Ah4 chromosome will have a structure similar to Al4 of *A. lyrata*. The overall conserved collinearity between 2 other *A. halleri* chromosomes (Ah6 and Ah7) and chromosomes Al6 and Al7 of *A. lyrata* has been confirmed previously [Lysak et al., 2003]. In *N. caerulescens* (n = 7), chromosome Nc4 also resembles the ancestral structure of AK4 [Mandáková and Lysak, 2008]. In both analyzed species, a strong green/yellow signal of the *A. halleri* contigs was observed. Somewhat weaker red fluorescence of the *A. thaliana* supercontigs labeled the remaining parts of chromosome Ah4 and Nc4, respectively (fig. 2B, C). The weaker hybridization signal intensity of *A. thaliana* contigs reflected the selective use of approximately each third clone of the BAC tiling path. Multi-species painting of chromosomes Ah4 and Nc4 along the entire length confirmed their conserved AK4-like structure.

We have shown that BAC clones of *A. halleri* can be successfully used as reliable chromosome painting probes in this species, congeneric *A. thaliana* as well as in the more distantly related species *N. caerulescens*. Comparative cytogenetics in the Brassicaceae has been until now limited to the use of chromosome-specific BAC supercontigs of *A. thaliana*. As the genome of *A. halleri* (0.24 pg C^{-1}) [Lysak et al., 2009] is 1.5-fold larger than the *A. thaliana* genome (0.16 pg C^{-1}) [Bennett et al., 2003], it can be expected to find a higher percentage of repetitive elements in the *A. halleri* genome. Indeed, the sequence data analysis of Lacombe et al. [2008] corroborated in the present study identified 2 BAC clones containing transposon and retrotransposon elements. These data suggest that the selection of BAC clones suitable for chromosome painting in *A. halleri* and other crucifer species with a similar or larger genome must be carried out with even more caution than in *A. thaliana* [see Lysak et al., 2003]. In species with larger genomes, undesirable cross-hybridization of repetitive sequences could be reduced through the application of a highly repetitive Cot-100 genomic fraction or sheared genomic DNA as shown for Solanaceae species [Iovene et al., 2008; Tang et al., 2008]. Repetitive elements present in 2 *A. halleri* BAC clones (IV-E-9 and IX-B-21) are shared by the 2 *Arabidopsis* species, though the weaker and more scattered hybridization signals in *A. thaliana* suggest that the repeats are less abundant and/or more divergent in *A. thaliana* as compared to *A. halleri*.

As the karyotype structure of *A. thaliana* is relatively complex due to chromosome rearrangements accompanying the chromosome number reduction (from n = 8 to n = 5) in this species [Lysak et al., 2006; Schranz et al., 2006], chromosome-specific BAC (super)contigs of closely related n = 8 species would be more suitable as CCP probes. The present data suggest that *A. halleri* BAC contigs can be successfully employed as CCP probes, and even more extensive resources should become available for *A. lyrata* and *C. rubella* (both n = 8) within the DOE genome sequencing program (http://www.jgi.doe.gov). Moreover, by hybridizing *A. halleri* and *A. thaliana* BAC contigs to chromosomes of *N. caerulescens*, we showed for the first time that multi-species comparative chromosome painting in a plant species is feasible. Current experiments prove that (i) painting BAC probes derived from a pair of species can be used reciprocally in the respective species (*A. halleri* ↔ *A. thaliana*), and (ii) BAC contigs of one species can be substituted or supplemented by painting probes of another closely related taxon (*A. halleri* + *A. thaliana* → *N. caerulescens*). We anticipate that the latter approach, namely multi-species CCP, will significantly facilitate and expedite comparative reconstructions of karyotype evolution in Brassicaceae and other plant families.

As previously reported [Lacombe et al., 2008], physical mapping based on the conserved synteny among *Arabidopsis* species can be used to assemble BAC contigs. However, the presence of repetitive elements such as transposons or retrotransposons hampers the use of this strategy for building supercontigs and constructing complete physical maps. In the present study we showed that cytogenetic mapping can anchor individual BAC clones and contigs on chromosomes, build supercontigs and identify gaps between them. Therefore, BAC painting is an efficient alternative to chromosome walking in constructing genome-wide physical maps.

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