Theor Appl Genet (2000) 101:1001–1007

F. Dong · J. Song · S.K. Naess · J.P. Helgeson
C. Gebhardt · J. Jiang

Development and applications of a set of chromosome-specific cytogenetic DNA markers in potato

Received: 19 January 2000 / Accepted: 27 March 2000

Abstract Reliable and easy to use techniques for chromosome identification are critical for many aspects of cytogenetic research. Unfortunately, such techniques are not available in many plant species, especially those with a large number of small chromosomes. Here we demonstrate that fluorescence in situ hybridization (FISH) signals derived from bacterial artificial chromosomes (BACs) can be used as chromosome-specific cytogenetic DNA markers for chromosome identification in potato. We screened a potato BAC library using genetically mapped restriction fragment length polymorphism markers as probes. The identified BAC clones were then labeled as probes for FISH analysis. A set of 12 chromosome-specific BAC clones were isolated and the FISH signals derived from these BAC clones serve as convenient and reliable cytological markers for potato chromosome identification. We mapped the 5S rRNA genes, the 45S rRNA genes, and a potato late blight resistance gene to three specific potato chromosomes using the chromosome-specific BAC clones.

Key words Chromosome identification · Physical mapping · FISH · BAC · Molecular cytogenetics · Potato

Communicated by B.S. Gill

F. Dong · J. Song · J. Jiang (✉)
Department of Horticulture, University of Wisconsin-Madison, Madison, WI 53706, USA
e-mail: jjiang1@facstaff.wisc.edu
Fax: +1 608–262–4743

S.K. Naess · J.P. Helgeson
USDA/ARS Plant Disease Resistance Research Unit, Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706, USA

C. Gebhardt
Max-Planck-Institut für Züchtungforschung, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

Introduction

The development of reliable and easy to use techniques for chromosome identification is critical for future advances in cytogenetics. Unfortunately, chromosome identification is a major challenge in many plant species with small chromosomes. Pachytene chromosome identification was successfully applied in cytogenetic studies in a number of plant species, most notably in maize (McClintock 1929), tomato (Rick and Barton 1954) and rice (Khush et al. 1984). However, pachytene chromosome preparation is often an elaborate process, the material is not available year-round, often all chromosomes cannot be identified in a single cell, and the distribution patterns of heterochromatin and euchromatin are not always sufficient for unambiguous identification of every chromosome. Cytogenetic identification of individual chromosomes using banding techniques (Gill and Kimber 1974a, b) has revolutionized cytogenetics research for many plant species. In general, plant species with large chromosomes have benefited from banding techniques more than those with small chromosomes. Small chromosomes usually show fewer characteristic bands that are critical for chromosomal identification. With the advent of non-isotopic in situ hybridization techniques in plants (Rayburn and Gill 1985), a single repetitive DNA probe could be used for molecular cytogenetic karyotyping and chromosome identification (see review by Jiang and Gill 1994). However, such repetitive DNA probes are available in only a few plant species.

It is relatively easy to generate a linkage map of an experimental organism using DNA marker technologies, especially with restriction fragment length polymorphism (RFLP) markers. For cytogenetic studies, genetic linkage maps must be correlated to physical chromosomes. In the absence of aneuploid stocks, in situ hybridization is the method of choice for physical mapping of RFLP genetic markers. However, most RFLP probes (0.5–4.0 kb) are too small and do not generate consistent and reliable in situ hybridization signals. Instead, Jiang and Gill (1994) suggested that RFLP marker-specific

large insert DNA clones should be used and the utility of this method has been demonstrated in several plant species (Woo et al. 1994; Hanson et al. 1995; Jiang et al. 1995; Fuchs et al. 1996; Zhu et al. 1996; Lapitan et al. 1997). The in situ hybridization signals derived from RFLP marker-tagged large insert DNA clones can also be used as cytological markers to identify individual chromosomes. In this report we demonstrate the feasibility of the ''chromosome-specific cytogenetic DNA marker'' strategy in chromosome identification and cytogenetic mapping in potato. A set of BAC clones specific to each of the 12 potato chromosomes was isolated. We used these BACs to map the 5S rRNA genes, the 45S rRNA genes, and a potato late blight resistance gene, to three specific potato chromosomes

Materials and methods

Materials

The BAC library used in this study was constructed from a diploid potato species *Solanum bulbocastanum* (2n = 2*x* = 24) (Song et al. 2000). This library consists of 23808 clones, with an average insert size of 155 kb, representing approximately 3.7 equivalents of the potato genome. Most of the potato restriction fragment length polymorphism (RFLP) markers with known chromosomal locations (Gebhardt et al. 1991, 1994; Tanksley et al. 1992) were developed at the Max-Planck-Institut für Züchtungforschung, Germany, with a few provided by Dr. S. D. Tanksley at Cornell University, USA. Plasmids pTa71 and pTa794 (Gerlach and Bedbrook 1979; Gerlach and Dyer 1980) were used to detect the potato 45S and 5S rRNA genes, respectively. A RAPD (randomly amplified polymorphic DNA) marker, G02–625, was closely linked to a late blight resistance gene on potato linkage group 8 in four different mapping populations (Naess et al. 2000). A BAC clone, 32A07, was isolated by screening the BAC library using a PCR (polymerase chain reaction) -based approach using sequence-specific primers designed from the RAPD fragment (S. K. Naess and J. P. Helgeson, unpublished data). BAC 32A07 was used for cytogenetic mapping in the present study.

Filter preparation and library screening

Hybond filters (11 × 7.5 cm) (Amersham, U.K.) were placed on Whatman paper saturated with sterilized water and inoculated with BAC clones using a Multi-Blot 384-pin replicator (V and P Scientific, USA). Each filter was inoculated with 1536 clones. The filters were then placed on LB agar and incubated at 37°C for 12–18 h until the colonies were 1–1.5-mm in diameter. Colonies were lysed according to Nizetic et al. (1990) and the BAC DNA was bound to filters by baking at 80°C for 2 h.

The inserts of the RFLP probes were released from the plasmids with appropriate enzymes, recovered using a Qiaex II gel extraction kit (Qiagen, USA) and labeled with ^{32}P. Pre-hybridization and hybridization were performed at 65°C in 7% SDS, 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA and 100 µg/ml of denatured salmon sperm DNA. Post-hybridization washes were performed in 1 × SSC and 0.1% SDS at 65°C for 30 min. BAC clones with positive hybridizations to the RFLP probes were picked for FISH analysis.

Somatic metaphase chromosome preparations

A haploid potato (*S. tuberosum*, 2n = 2*x* = 24) clone US-W 1, provided by Dr. R.E. Hanneman at the University of Wisconsin-Madison, USA, was used for chromosome preparations. Potato root tips about 1-cm long were harvested from young plants grown in greenhouses, pre-treated in 2 mM of 8-hydroxyquinoline (Sigma, USA) at room temperature for 3 h, and fixed in a 3:1 solution of methanol : glacial acetic acid for 1–2 days. After washing twice in distilled water (10 min each), the meristematic portions of the roots were removed and incubated in a solution of 2% cellulase (Sigma, USA) and 1% pectolyase (Sigma, USA) at 37°C for 45 min. The root tips were then washed carefully with distilled water twice, for 10-min each, and re-fixed in the 3:1 solution for 20 min. A single root tip was transferred to a pre-chilled glass slide and macerated with a drop of 3:1 fixation solution using a fine-pointed forceps. The slide then was warmed over an alcohol flame. After air-drying, slides with good metaphase chromosome spreads were stored in a –80°C freezer.

Fluorescence in situ hybridization

BAC DNA was isolated by using an alkaline-lysis method (Sambrook et al. 1989), purified by CsCl ultracentrifugation, and labeled with either biotin-16-dUTP or digoxygenin-11-dUTP (Roche Diagnostics, USA) by standard nick translation reactions. For the majority of the potato BAC clones it was necessary to include potato C_ot-1 DNA in the hybridization mixture to block the hybridization of repetitive DNA in the BAC probes. Potato genomic DNA was isolated from the haploid clone US-W1 and the C_ot-1 fraction of the genomic DNA was prepared according to Jiang et al. (1996).

FISH was conducted as previously described (Jiang et al. 1996). Slides were dehydrated in an ethanol series (70%, 90% and 100%, 5-min each). The chromosomal DNA on the slides was then denatured in 70% formamide for 1.5 min at 80°C and followed by a –20°C ethanol series (70%, 90% and 100%, 5-min each). The hybridization mixture (10 µ l for each slide) contained 10 ng of labeled probe DNA, 50% formamide, 10% dextran sulfate, 2 × SSC, 10 µg of sheared salmon sperm DNA, and an appropriate amount of potato C_ot-1 DNA (see Table 1). The mixture was denatured at 100°C for 5 min and incubated at 37°C for 5 h before adding to the slides. After overnight incubation at 37°C and washing at 42°C in 2 × SSC, biotin-labeled probes were detected with 1% fluorescein isothiocyanate (FITC)-conjugated anti-biotin antibody (Vector, USA), and digoxygenin-labeled probes with 1% rhodamine-conjugated anti-digoxygenin antibody (Roche Diagnostics, USA). Propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) (Sigma, USA) in an antifade solution (Vector, USA) was used to counterstain the chromosomes. Slides were examined under an Olympus BX60 fluorescence microscope. Chromosome and FISH signal images were captured using a SenSys CCD (charge-coupled device) camera (Photometrics, USA) and merged using IPLab Spectrum software (Signal Analytics, USA).

Results

Isolation of chromosome-specific BAC clones of potato

To isolate BAC clones that hybridize to each of the 12 potato chromosomes, we screened the BAC library with 21 genetically mapped potato RFLP probes. Only half of the library (12288 clones) was screened with all the RFLP probes, except for probe CP44, for which the entire library was used. Three probes failed to hybridize to any BAC clones in the portion of the library used. The rest of the probes identified at least one positive clone. Table 1 summarizes the screening results from 12 RFLP probes and the BAC clones selected as chromosome-specific cytogenetic DNA markers. On average each

Table 1 BAC library screening results, genetic and physical locations of the 12 chromosome-specific BAC clones selected and their blocking requirements in FISH

Chrom. no.	RFLP markers	Genetic locations[a]	No. of positive clones[c]	Selected BACs	Physical locations[d]	Blocking DNA
1	GP264	North arm	1	06M21	Short arm	50×
2	TG14	South arm	1	36H11	Long arm	50×
3	GP295	South arm	2	57C05	Long arm	100×
4	GP180	North arm	3	23K12	Short arm	50×
5	GP22	South arm	1	37L16	Long arm	100×
6	GP79	North arm	2	39P07	Short arm	100×
7	CP43	South arm	5	38O02	Short arm	50×
8	GP170	South arm	1	46A08	Short arm	50×
9	CP44	North arm	2	37J14	Short arm	Not required
10	GP247	–[b]	5	37L15	Long arm	100×
11	GP125	North arm	1	25O15	Long arm	25×
12	GP229	North arm	2	54J18	Short arm	25×

[a] The positions of the RFLP markers on potato/tomato genetic linkage maps (Gebhardt et al. 1991, 1994; Tanksley et al. 1992)
[b] This marker is located in the middle of potato genetic linkage group 10 (Gebhardt et al. 1994). Its position in terms of north arm or south arm could not be determined
[c] No. of positive clones from 12288 clones for all RFLP markers, except CP44 for which 23808 clones were used for screening
[d] Physical locations of the FISH signals from the BAC clones in terms of the short arm or long arm of specific potato chromosomes

RFLP probe hybridized to 2.3 BAC clones from the 12288 clones screened. Had the complete library (23808 clones) been used, there should have had about 4.5 hits by each RFLP probe. This result is in accordance with the estimation that the library is approximately 3.7 equivalents of the potato genome (Song et al. 2000).

In situ hybridization of the BAC clones to potato chromosomes

The positive BAC clones identified by the chromosome-specific RFLP probes were hybridized to potato somatic metaphase chromosomes using FISH. If multiple BAC clones were identified by a single RFLP marker, the clone with the strongest hybridization to the RFLP probe was selected for FISH analysis.

The percentage of repetitive DNA sequences within the BAC clones tested for FISH varied substantially, resulting in FISH signals with different contrast and intensity. The BAC probes can be classified into three groups based on the specificity and intensity of their FISH signals. The first group, represented by BAC 37J14 for chromosome 9, generated distinctive FISH signals without a C_ot-1 pre-annealing procedure (Fig. 1I), suggesting little, if any, dispersed repetitive DNA within this BAC clone. The second group of BAC clones contains large amounts of repetitive DNA. Distinctive FISH signals can not be generated from these clones after pre-annealing with more than 200-fold C_ot-1 DNA. Thus, these BAC clones cannot be used as chromosome-specific cytogenetic DNA markers. About 10% of the tested BAC clones belonged to this group. Most of the BAC clones fall into a third group. These clones contain a limited amount of repetitive DNA sequences that can be effectively blocked with a C_ot-1 pre-annealing procedure. BACs in this group generated distinctive FISH signals which are specific to a single pair of metaphase chromosomes, although the intensity of the signals varied among the BACs. Minor FISH signals were often observed on other chromosomes but they can be readily distinguished from the chromosome-specific signals (Fig. 1).

We selected a set of 12 BACs as potato chromosome-specific cytogenetic DNA markers (Fig. 2A). The suggested amount of C_ot-1 DNA for each BAC clone in FISH experiments is listed in Table 1. These 12 BACs consistently produced distinct chromosome-specific signals in our experiments and therefore are reliable for potato chromosome identification.

Application of the chromosome-specific markers

FISH has been widely used to physically map DNA sequences on plant chromosomes (Jiang and Gill 1994). However, the cytological identification of the individual chromosome(s) with the hybridization signal(s) was not reported in the majority of the FISH mapping experiments. To test the utility of the potato chromosome-specific DNA markers, we chose the potato 5S rRNA genes, 45S rRNA genes, and a BAC clone linked to a late blight resistance gene, for cytogenetic mapping.

The 5S rRNA genes were mapped proximal to the centromere on the short arms of a single pair of potato chromosomes (Fig. 2B). The major cluster of the 45S rRNA gene was mapped to the distal ends on the short arms of a different pair of chromosomes (Fig. 2C). Previous genetic mapping information in tomato (Vallejos et al. 1986; Lapitan et al. 1991) suggested that the 5S and 45S rRNA genes are located on potato/tomato genetic linkage groups 1 and 2, respectively. By co-

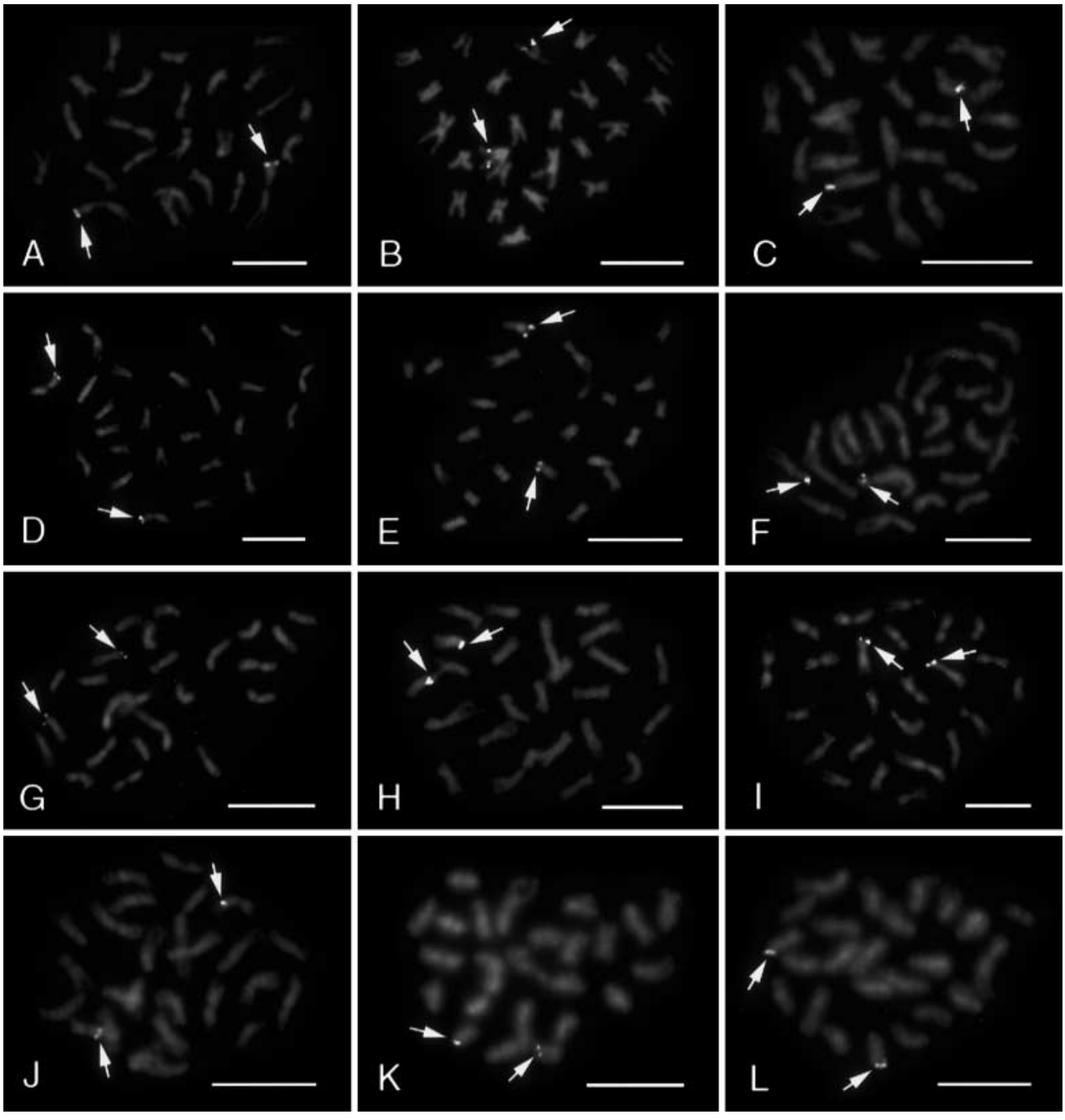

Fig. 1A–L Potato chromosome spreads hybridized with chromosome-specific BAC clones. **A** to **L** show hybridization of BAC probes (Table 1) to potato chromosomes 1 to 12, respectively. Arrows point to the chromosome-specific FISH signals. All bars are 10 μm

hybridizing the rDNA probes with chromosome-specific BAC clones, we demonstrated that the potato 5S and 45S rRNA genes are located on chromosomes 1 and 2, respectively (Fig. 2B, C).

S. bulbocastanum, a wild diploid potato species, is highly resistant to *Phytophthora infestans*, the fungus that causes late blight of potato (Helgeson et al. 1998). A RAPD marker, G02–625, is closely linked to a late blight resistance gene derived from *S. bulbocastanum* (Naess et al. 2000). Sequence-specific primers were designed from this RAPD marker and a BAC clone, 32A07, was identified using a PCR-based library screening approach (S.K. Naess and J.P. Helgeson, unpublished results). FISH analysis showed that BAC 32A07 is located on the long arm of chromosome 8. The distance from the FISH signals of 32A07 to the telomere is approximately 25% of the long arm (Fig. 2D).

Discussion

The somatic metaphase chromosomes of potato are about 1–3.5 μm in size. It is not possible to distinguish

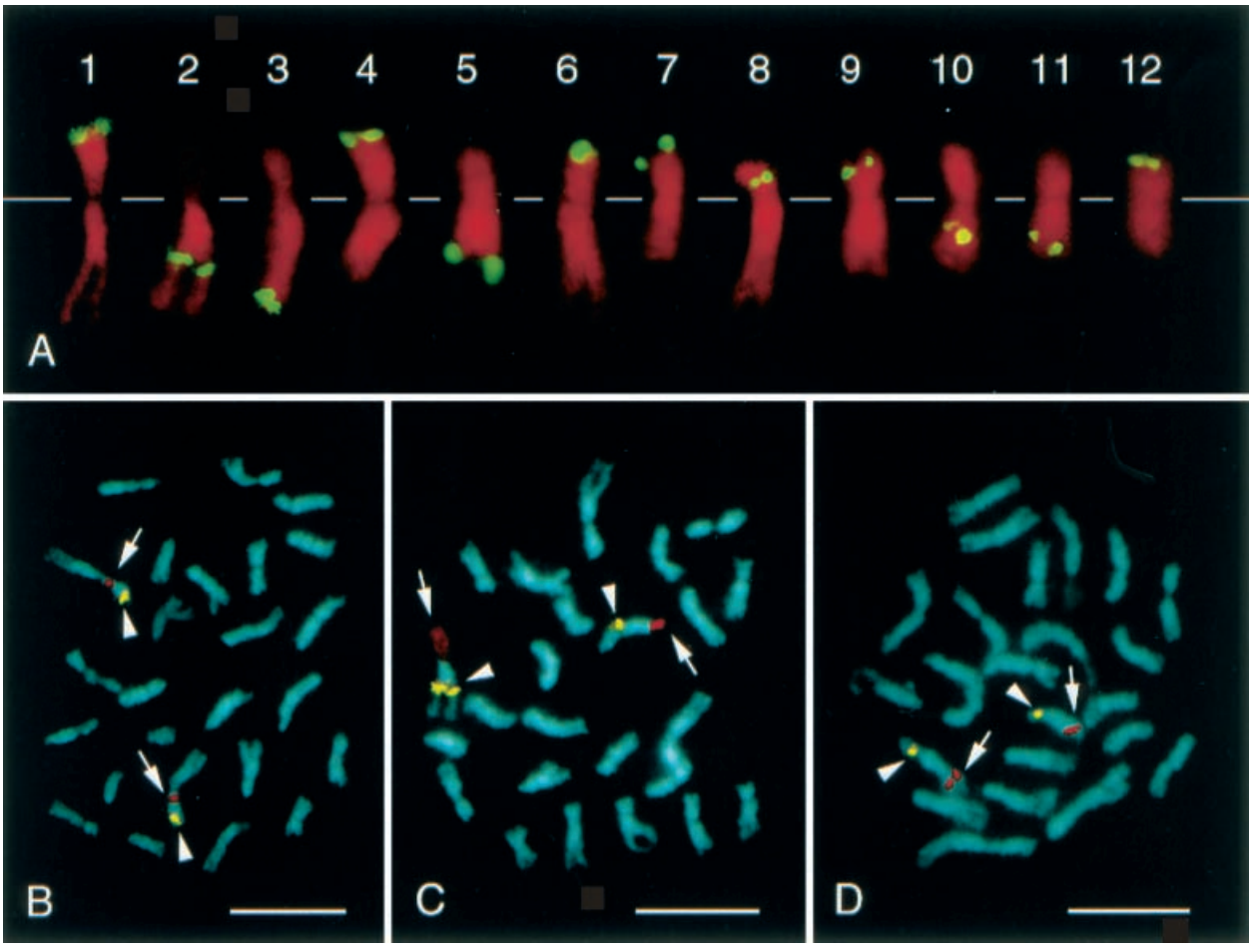

Fig. 2 A Twelve individual potato chromosomes with FISH signals derived from the chromosome-specific BAC clones. **B** FISH mapping of potato 5S rRNA genes. The 5S rRNA genes (*red color, arrows*) are located near the centromeres at the same chromosome as chromosome 1-specific BAC 06M21 (*yellow color, arrowheads*). **C** FISH mapping of potato 45S rRNA genes. The 45S rRNA genes (*red color, arrows*) were mapped to the distal region on the short arm of the same chromosome where chromosome 2-specific BAC 36H11 (*yellow color, arrowheads*) was located. **D** FISH mapping of a BAC clone, 32A07, which is linked to a potato late blight resistance gene. BAC 32A07 (*red color, arrows*) was mapped to the long arm of the same chromosome where the chromosome 8-specific marker 46A08 (*yellow color, arrowheads*) was mapped. The physical distance from 32A07 to the telomere is approximately 25% of the long arm. All *bars* are 10 μm

the 12 potato chromosomes based on their morphology. Giemsa-banding analyses of potato chromosomes have been reported by several laboratories (Mok et al. 1974; Lee and Hanneman 1976; Pijnacker and Ferwerda 1984; Wagenvoort et al. 1994). However, the banding techniques do not produce a sufficient number of characteristic bands for reliable and routine chromosome identification. Mid-pachytene chromosomes of potato average 40 μm (Yeh and Peloquin 1965) and the unique distribution patterns of euchromatin and heterochromatin allow the identification of every potato chromosome (Yeh and Peloquin 1965; Ramanna and Wagenvoort 1976; Wagenvoort 1988). However, pachytene analysis is elaborate in potato. Several potato pachytene chromosomes, which have a similar distribution pattern of heterochromatin in the proximal regions, are difficult to distinguish from each other. In addition, pachytene analysis can be applied only on diploid clones, while cultivated potato and many wild potato species are autopolyploids which are not accessible with this technique.

We demonstrated that the 12 potato chromosomes can be identified, in a straight-forward fashion, by the presence of FISH signals with chromosome-specific cytogenetic DNA markers. The major advantages of this method as compared to the traditional pachytene and banding analysis include the following. (1) It gives each potato chromosome a distinctive feature to be differentiated from the rest of the chromosomes, whereas it is difficult to distinguish chromosomes with a similar morphology or banding pattern by pachytene or banding analysis. Therefore, this system will be particularly valuable for analyzing the mitotic and meiotic behavior of a particular chromosome. (2) The quality of the chromosome preparations is not important for chromosome identification using this system but it is critical for banding and pachytene analysis. (3) It can be used for both diploid and polyploid species, whereas pachytene analysis can be used only for diploid species/clones. (4) This system can be applied to any plant species, especially those with large numbers of small chromosomes. Many

plant species with small chromosomes receive little benefit from the traditional techniques for chromosome identification. BAC libraries have been constructed in numerous plant species (www.genome.clemson.edu/lib_frame.html; hbz.tamu.edu/cgi-bin/htmlassembly?bacs). Chromosome-specific cytogenetic DNA markers can be easily established in these species by isolating chromosome-specific BAC clones.

As in other plant species, potato chromosomes were previously arranged based on their lengths in conventional karyotyping, with the longest one being chromosome 1 and the shortest one chromosome 12 (Swaminathan 1954). When the potato chromosomes were distinguished based on Giemsa-banding patterns, they were named either alphabetically, from chromosome A to chromosome L (Mok et al. 1974; Wagenvoort et al. 1994), or numerically, from chromosome 1 to chromosome 12 (Pijnacker and Ferwerda 1984). Pachytene chromosomes were named using a Roman number system (Yeh and Peloquin 1965). These chromosome numbering systems do not correlate with one another and none of them are associated with the numbering system for potato genetic linkage groups. In the current chromosome-specific cytogenetic DNA marker system, the potato chromosomes are identified and numbered in accordance with the genetic linkage groups (Gebhardt et al. 1991, 1994). Chromosome identification based on chromosome-specific DNA markers will also be consistent among different genotypes and possibly different species within the genus *Solanum*, assuming that there are no rearrangements involving different chromosomes in these species. Since the genetic linkage maps of potato were aligned with that of its relative, tomato (Bonierbale et al. 1988; Gebhardt et al. 1991, 1994; Tanksley et al. 1992), the potato chromosomes can be aligned cytologically to tomato chromosomes and, through the tomato chromosomes, to chromosomes in other Solanaceae species.

Since the physical locations of the potato chromosome-specific BAC clones also represent the arm locations of the potato RFLP markers which were used for BAC isolation, we were able to find the orientation of 11 potato genetic linkage maps corresponding to the potato chromosome arms (Table 1). The north/south orientation of the current potato genetic linkage groups 1, 2, 3, 4, 5, 6, 9 and 12 (Gebhardt et al. 1991, 1994) match with the short arm/long arm orientation; but linkage groups 7, 8 and 11 have an opposite orientation (Table 1).

A different FISH-based chromosome identification system involves the application of repetitive DNA probes which generate unique hybridization patterns on each chromosome. This system is very much similar to the conventional chromosome banding technique and has been primarily used in plant species with large chromosomes, such as *Vicia faba* (Fuchs et al. 1994), wheat (Mukai et al. 1993; Pedersen and Langridge 1997), and barley (Busch et al. 1995). The distribution of repetitive DNA elements can be highly variable in eukaryotic genomes. The hybridization patterns of a repetitive DNA probe may vary in different species within the same genus and even in different ecotypes within the same species. Thus, the hybridization patterns derived from repetitive DNA probes may not always be diagnostic for every chromosome of different accessions within a single species.

Technical improvements will make the detection of small DNA probes on plant chromosomes more routine in the future. Thus it is possible to map genetically mapped RFLP probes directly on plant chromosomes, rather than mapping RFLP marker-tagged large-insert genomic DNA clones. Nevertheless, the quality of the FISH signals, especially their intensity and detection frequency, derived from large-insert clones, such as BACs, is much better than that derived from small RFLP probes. Thus, large-insert DNA clones will serve as better chromosome-specific DNA markers than small DNA probes.

Acknowledgements This work was supported by a Plant Genome grant 96–35300–3720 from USDA, and Hatch funds 142-D395 and 142—3935, to J.J.

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