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Detection of single copy sequences using BAC-FISH and C-PRINS techniques in sunflower chromosomes

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ABSTRACT: Bacterial artificial chromosome - fluorescence *in situ* hybridization (BAC-FISH) and cycling-primed *in situ* labeling (C-PRINS) techniques were evaluated for integration of physical and genetic maps of sunflower (*Helianthus annuus* L.). Single-site SSR markers were selected from three linkage groups of a high-density sunflower genetic map. This selection was based on previously identified QTL associated to *S. sclerotiorum*. These markers were used to select BACs contaning single copy sequences for BAC-FISH aplication. Blocking of highly dispersed repetitive sunflower sequences reduced unspecific hybridization, and allowed the detection of specific signals for BACs containing SSR markers HA4222 and HA2600, anchored to LG 16 and LG 10, respectively. Single-site FISH signal detection was optimized by adjusting the relative quantity and quality of unlabelled repetitive sequences present in the blocking DNA. The SSR marker ORS1247 anchored to the LG 17 was detected by C-PRINS, which yielded fluorescence signals that were specific and intense. This progress in localizing single-copy sequences using BAC-FISH and indirect C-PRINS strategies in sunflower will facilitate the integration of genetic and physical maps, allowing the identification of chromosomes containing key genes and/or QTL associated to agronomic important traits in sunflower.

Introduction

Sunflower (*Helianthus annuus* L.) is the third most important source of edible vegetable oil worldwide. It belongs to the *Asteraceae* family which includes over forty economically important crops, such as lettuce, chamomile and chicory among others. It is a diploid species with 2*n*=2*x*=34 with an estimated genome size of about 3,500/1C Mb (Baack *et al*., 2005).

In the last decade, significant progress has been made in sunflower genomics regarding the development of a large number of molecular markers such as SSR or Simple Sequence Repeat (SSR) (Paniego *et al*., 2002; Tang *et al*., 2002, 2003), Expressed Sequence Tags (EST) databanks (http://cgpdb.ucdavis.edu/sitemap.html; www.genoplante.com (INRA Member Group, France); (Gentzbittel *et al*., 1999; Fernández *et al*., 2003; Heesacker *et al*., 2008) and functional markers derived from ESTs as SNPs (Lai *et al*., 2005; Liu and Burke, 2006; Kolkman *et al*., 2007; Fusari *et al*., 2008), SSR-ESTs and Indels (Kumpatla and Mukhopadhyay, 2005; Pashley *et al*., 2006; Heesacker *et al*., 2008). In addition, the construction of several genetic linkage maps

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including both neutral and functional markers (Tang *et al*., 2002, 2003; Yu *et al*., 2003; Al-Chaarani *et al*., 2004; Lai *et al*., 2005; Kiani *et al*., 2007) as well as the development of several BAC libraries (Gentzbittel *et al*., 2002; Özdemir *et al*., 2004; Bouzidi *et al*., 2006; Feng *et al*., 2006) have been reported. In spite of these advances, the association between linkage groups (LGs) and chromosomes has not been established yet.

Reliable cytological techniques as fluorescence *in situ* hybridization (FISH) and primed *in situ* labelling (PRINS) represent key tools for association of physical and genetic maps. FISH has been the method of choice for mapping repetitive sequences and thus, enabling the characterization of the chromosome complement in different species (Minelli *et al*., 2000; Ceccarelli *et al*., 2007). However, the application of these strategies to localize single locus DNA sequences has been hampered by technical difficulties related, at least in part, to the use of rather short sequences as probes (Danilova and Birchler, 2008). The use of probes containing few kilobases of complementary DNA leads to lower sensitivity in conventional FISH assays, particularly in applications involving large genome size plants like sunflower. This technical difficulty can be overcome by using large-insert genomic clones like BACs (Bacterial Artificial Chromosomes) as probes in FISH (Jiang *et al*., 1995; Lapitan *et al*., 1997; Desel *et al*., 2001; Islam-Faridi *et al*., 2002; Koorneef *et al*., 2003; Lee *et al*., 2003) leading to the strategy known as BAC-FISH (Shizuya *et al*., 1992). However, the identification of individual BAC clones suitable for FISH is not trivial because of the frequent presence of repetitive DNA in these clones that may jeopardize the detection of specific unique sequences. The use of overlapping oligonucleotides (overgo) for identification of BACs containing specific-unique-site-sequences, has been successfully reported for different plant species, including sunflower (Chen *et al*., 2002; Gardiner *et al*., 2004; Yüksel and Paterson, 2005; Feng *et al*., 2006).

PRINS technique (Koch *et al*., 1989) represent an alternative to the use of FISH for localization of DNA sequences in plant chromosomes and it has also been reported for different plant species (Menke *et al*., 1998; Kubaláková *et al*., 2001). This strategy was found to be particularly useful for detection of high-copy tandem repeats. However, for the detection of low-copy repeats, a more sensitive variant of PRINS named cycling PRINS (C-PRINS), which involves serial thermal cycles analogous to polymerase chain reaction (Gosden *et al*., 1991; Kubaláková *et al*., 2001), was recommended. Compared to FISH, PRINS has several advantages including the minimal sequence information required for oligonucleotide primer design, the easiest and fastest detection of the technique based on primer hybridization to homologous targets within densely structured chromatin, compared to the rather large probes commonly used for FISH, the avoidance of probe labeling and the possibility to perform the primer extension even into flanking scattered repetitive sequences, thus strengthening the signal for a short unique sequence (Menke *et al*., 1998).

In the present work we report the use of the BAC-FISH and C-PRINS techniques for the detection of single-copy DNA sequences on sunflower chromosomes in order to evaluate the potential of both techniques for further applications in the integration of physical and genetics maps.

Materials and Methods

Selection and screening of BAC clones for FISH

The BAC clones used in this study were selected from an 8.3x genome coverage sunflower BAC library with an average insert size of 125 kbp (BAC: HA_HBa. www.genome.clemson.edu).

Unique genomic sequence flanking 32 different microsatellites selected from a high-density genetic map of sunflower and anchored to LG 9, 10 and LG 16, (Kiani *et al*., 2007) were used to design overlapping oligonucleotide probes (http://genome.wustl.edu/gsc/overgo/overgo.html) (McPherson *et al*., 2001).

Overgos were designed using the Overgo 1.02i program (http://www.mouse-genome.bcm.tmc.edu/webovergo/OvergoDescription.asp) and oligoSpawn software (http://oligospawn.ucr.edu). Overgos were synthesized and then individually labelled with [α-^{32}P]dATP and [α-^{32}P]dCTP, according to the method developed by J. D. McPherson (http://www.tree.caltech.edu). Pairs of overgo primers (10 pmol for each primer) were added to 3.5 µl H_2O and denatured at 80°C for 5 min, followed by 10 min at 37°C and afterwards cooled on ice. Heat-denatured overgo primer pairs were mixed with 0.1 mg/µl BSA, 2 µl OLB (oligo labeling buffer without dATP, dCTP, and random hexamers), 2 U Klenow DNA polymerase fragment (Invitrogen, USA), 0.5 µl [α-^{32}P]dATP (3,000 Ci/mmol) and 0.5 µl [α-^{32}P]dCTP (3,000 Ci/mmol), in a final 10 µl reaction volume. Subsequently, the reaction mixture was incubated for 1 h at 37ºC, followed by a 10 min denaturing step performed at room temperature, with the addition of NaOH at 0.2 N final concentration. Removal of unincorporated ra-

dioactive nucleotides was performed using "QiAquick® Nucleotide Removal Kit" (Qiagen, Germany).

Two BAC-library high-density filters were screened with pooled 10 to 12 overgo probes.

Filters were prehybridized in sodium polyanethol sulfonate hybridization buffer (PAES) at 60°C for 4 h. Labelled probes were denatured and then added to the hybridization tubes and then allowed to hybridize overnight at 60°C. The filters were washed in a series of buffers with increasing stringency: 2X SSC and 0.1% SDS at 60°C for 5 min, 1.5X SSC and 0.1% SDS at 60°C for 15 min, 1X SSC and 0.1% SDS at 60°C for 15 min, and 0.5X SSC and 0.1% SDS at 60°C for 15 min (Sambrook *et al*., 1989). Filters were sealed in plastic sheets, exposed for 72 h and then scanned with a Typhoon trio (Amersham Biosciences, UK) using the program ImagenQuant (Amersham Biosciences, UK).

PCR screening, BAC DNA purification and probe labeling

DNAs from BAC positive clones were purified using NucleoBond® Xtra Midi Plus (Machery-Nagel, Germany). PCR verification of positive clones was carried out using SSR specific primers and 80 ng of DNA from each clone identify in the screening in the presence of 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 0.75 U of *Taq* DNA polymerase (Invitrogen, USA), 1X *Taq* polymerase buffer and 0.25 µM of each primer, in a final volume of 25 µl. Following an initial 5 min denaturalization at 95°C for, 35 PCR cycles of 95°C for 40 s, 62°C for 40 s and 72°C for 2 min and a final extension at 72°C for 10 min were performed. PCR products were separated using 2% standard TAE agarose gel electrophoresis.

DNAs from BAC were labelled with digoxigenin-11-dUTP by random priming labeling reaction (Boehringer Mannheim, Germany) and used as probes for fluorescence *in situ* hybridization (FISH).

Analysis of repetitive/transposon content in BAC clones using repetitive DNA as probes

Three repetitive DNA regions previously described in sunflower were amplified using genomic DNA from the inbred line HA89 and the following specific primers: SSR-Ha785 (GenBank AN: GF100475) with similarity to a highly repetitive sequence family of *Helianthus annuus* (GenBank AN: AJ009965) was amplified using forward (5' GTCTCGGGATCCAAGATTGA 3') and reverse (5' TCACGACGGTTGTAAAACGA 3') primers (Paniego *et al*., 2002); HaRetro3 (GenBank AN: DQ229838.1) with similarity to a *gypsy*-like retrotransposon family, was amplified using forward (5' AGGGCATTCAAATGGCTATG 3') and reverse (5' GTCTCATCCGGAAGATCCAA 3') primers (Tang *et al*., 2006) and HaRep1 (GenBank AN: AJ009967) with similarity to a *copia*-like retrotransponson family, was amplified using forward (5' TCTCAGAACCTCGGCAATCT 3') and reverse (5' GGCAGCAAAAGAGAAAATG 3') primers (Santini *et al*., 2002).

PCR reaction containing 100 ng of HA89 DNA and the following reagents: 10 mM PCR buffer, 0.25 µM of each specific primer, 0.2 mM of dNTPs, 0.75 U of *Taq* polymerase (Roche, France), was conducted in an Eppendorf thermocycler, with an initial denaturing step of 3 min at 94ºC followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC, 30 s at 72ºC, and a final extension step of 10 min at 72ºC. PCR amplification products were run at 80 V on 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. The corresponding bands were excised and purified with Qiaex II gel extraction kit (QIAgen, Germany). Probes were labelled with [α-^{32}P]-dCTP by random priming according to the manufacturer's recommendations (Promega Biotech, USA). These highly repetitive DNA fragments were used as probes in Southern blot hybridization assays to characterize the single-locus microsatellite containing BAC clones and to block unspecific hybridization in BAC-FISH experiments (see bellow).

Southern-blot hybridization

Positive clones were isolated and purified using a QIAgen Plasmid Midi Kit (QIAgen, Germany) and digested with *Eco*RI restriction enzyme, electrophoresed in 0.8% agarose gels and blotted to nylon membranes Hybond N^+ (GE Healthcare, UK) as described by Sambrook *et al*. (1989). The filters were prehybridized using a buffer containing PAES at 65ºC for 16 h. Denatured [α-P^{32}]-labelled probes (either Ha785, HaRep1 or HaRetro3 amplified sequences) were added and allowed to hybridize overnight at 65ºC, during the course of separate assays. Filters were washed in a series of buffers with increasing stringency: 2X SSC and 0.1% SDS at room temperature for 20 min, 1.5X SSC and 0.1% SDS at room temperature for 15 min, and 0.5X SSC and 0.1% SDS at 65ºC for 15 min (Sambrook *et al*., 1989). Filters were sealed in plastic bags, exposed for three days to X-rays sensitive screens, and then scanned with a Typhoon trio scanner (Amersham Biosciences, UK).

Chromosome preparations

Inbred line HA89 was selected for chromosome preparations. Root tips, obtained from seeds germinating over moisturized paper, were treated with 0.05% colchicine for 2 h at room temperature, fixed in absolute ethanol/acetic 3:1 (v/v) and stored at 4°C for several weeks. For cell wall digestion, root tips were treated with in an enzymatic solution (2% cellulose [w/v] plus 20% pectinase [v/v]) for 3 h at 37°C and stored in 0.01 M citric acid-sodium citrate buffer at 4°C for 24 h. Digested material was transferred to a slide containing a single drop of 45% acetic acid and flamed before squashing.

Fluorescence in situ hybridization (FISH)

FISH was conducted according to the methods described by Schwarzacher and Heslop-Harrison (2000). Chromosome slides were incubated 10 min in 3:1 (v/v) ethanol: acetic acid and then washed twice in absolute ethanol for 10 min.

Chromosome slide preparations were pre-treated with RNase for 1 h at 37°C and fixed with 4% paraformaldehyde. Hybridization mixture, 30 µl per slide, was prepared by adding 50% deionized formamide, 10% dextran sulphate, 2X SSC (0.3 M NaCl, 0.03 M Na_3-citrate), 0.1% SDS (sodium dodecyl sulphate), 0.3 mg/ml of sheared salmon sperm DNA (Sigma, USA), 100 ng of probe and 10 to 80 times of blocking DNA. Taking into account the outcome of BAC-Southern blot assays, DNA fragment from SSR-Ha785 and / or HaRep1 were used as blocking DNA. Hybridization mixture was denatured at 70ºC for 15 min. After chilling on ice for 5 min, 30 µl of the denatured mixture was applied to each slide and hybridization was performed using a thermocycler (Eppendorf Mastercycler, Germany) at 75°C for 7 min, 55-60°C 30 s, 45°C 5 min, 38°C 5 min and 37°C 10 min and then incubated overnight at 37°C in a humid chamber. Stringent washes were made in 2X SSC at 42°C for 10 min, 20% formamide/0.1X SSC at 42°C for 10 min, followed by two washes in 0.1X SSC at 42°C for 5 min, 2X SSC at 42°C for 5 min, 4X SSC/0.2% Tween at 42°C for 5 min and 4X SSC/0.2% Tween at room temperature for 5 min. Slides were blocked for 5 min at room temperature with 5% bovine serum albumin (BSA) (w/v) in 4X SSC/0.2% Tween.

Detection of digoxigenin hybridization sites was carried out by incubating the slides in a solution of 4 µg/ml of anti-digoxigenin-FITC fluorescein isothiocyanate (Roche, France) in 5% BSA (w/v) in 4X SSC/0.2% Tween for 1 h at 37°C. Afterwards, slides were washed in 4X SSC/0.2% Tween three times for 10 min each. Immediately, DNA was stained with 4 µg/ml DAPI (4', 6'-diamidino-2-phenylindole) and washed briefly with 4X SSC/Tween 0.2%. After rinsing, Vectashield antifade solution (Vector Laboratories, USA) was applied.

Cycling-Primed in situ labeling (C-PRINS)

PCR oligonucleotide primers from single locus microsatellite ORS1247 (Accession number: BV006602), anchored in the LG 17, of 339 pb in lenght was selected to carry out C-PRINS. This SSR was selected based on a previously identified QTL associated to *S. sclerotiorum* (Maringolo, personal comunication).

C-PRINS technique was conducted according to methods described by Kubaláková *et al*. (2001) with few modifications. Extension mix was performed in a

TABLE 1.

Name, type and repetition motif of SSRs contained in the BAC clones used for BAC-FISH in sunflower chromosomes.

BAC name	Clone ID	SSR marker	Repeat type	Repeat motif	LG number	Accession
BAC1	131-J09	HA928	(Di –Trinucleotide)	GT-ATT	10	BV728013
BAC2	132-N05	HA928	(Di –Trinucleotide)	GT-ATT	10	BV728013
BAC3	50-A15	HA4222	Trinucleotide	ATT	16	BV728199
BAC4	20-J02	HA2063	(Di-Dinucleotide)	GA-GT	9	BV728113
BAC5	49-K06	ORS805	Dinucleotide	GA	9	BV006216
BAC6	236-E09	HA2600	Dinucleotide	GA	10	BV728055

volume of 50 µl containing 1X DNA *Taq* polymerase buffer (Invitrogen, USA), 2.5 mM $MgCl_2$, 2 µM of each primer, 100 µM of dATP, dCTP and dGTP, 34 µM dTTP, 8 µM digoxigenin-11-dUTP (Roche Applied Science, France), 2 U *Taq* DNA polymerase and deionized H_2O.

Glass slides were sealed within a frame chamber (Bio-Rad, USA). Subsequently, 30 µl of reaction mix was added over the delimited area and a polyester coverslip was placed over it. Slides were then placed on a thermocycler (Eppendorf, Mastercycler, Germany) and subjected to the following amplification program: 91°C 4 min followed by 35 cycles of 91°C for 45 s, 55-60°C for 45 s and 72°C for 45 s and, at last, 72°C for 10 min.

Detection of the reaction was carried out by adding 100 µl of stop buffer (2.923 g of NaCl, 1.861 g of Na_2EDTA in 100 µl of deionized H_2O, pH 8) and incubated 2 min at 70°C. One hundred µl of BSA were added to each preparation, which was later incubated with 50 µl of anti-Dig fluorescein at 37°C for 30 min.

Slides were washed with 4XSSC/0.2% Tween for 5 min at room temperature for three times and later counterstained with 75 µl of DAPI (2 µg/ml) for 15 min. Chromosome preparations were mounted in Vectashield antifade solution (Vector Laboratories, USA).

Image analysis

Slides were examined with an epifluorescence microscope (Leica DMLB, Germany). Individual images obtained from each filter were captured using a Leica DFC 350 FX camera. These images were optimized for best contrast and brightness with Adobe Photoshop CS2 program.

Results

In order to evaluate BAC-FISH and C-PRINS methods, SSR markers targeting three LGs of a high-density sunflower genetic map (Kiani *et al*., 2007) were chosen. LGs 10, 16 and 17 were selected based on previous identification of QTL for resistance to *S. sclerotiorum* (Maringolo, 2007).

Different groups of probes, each containing 10-12

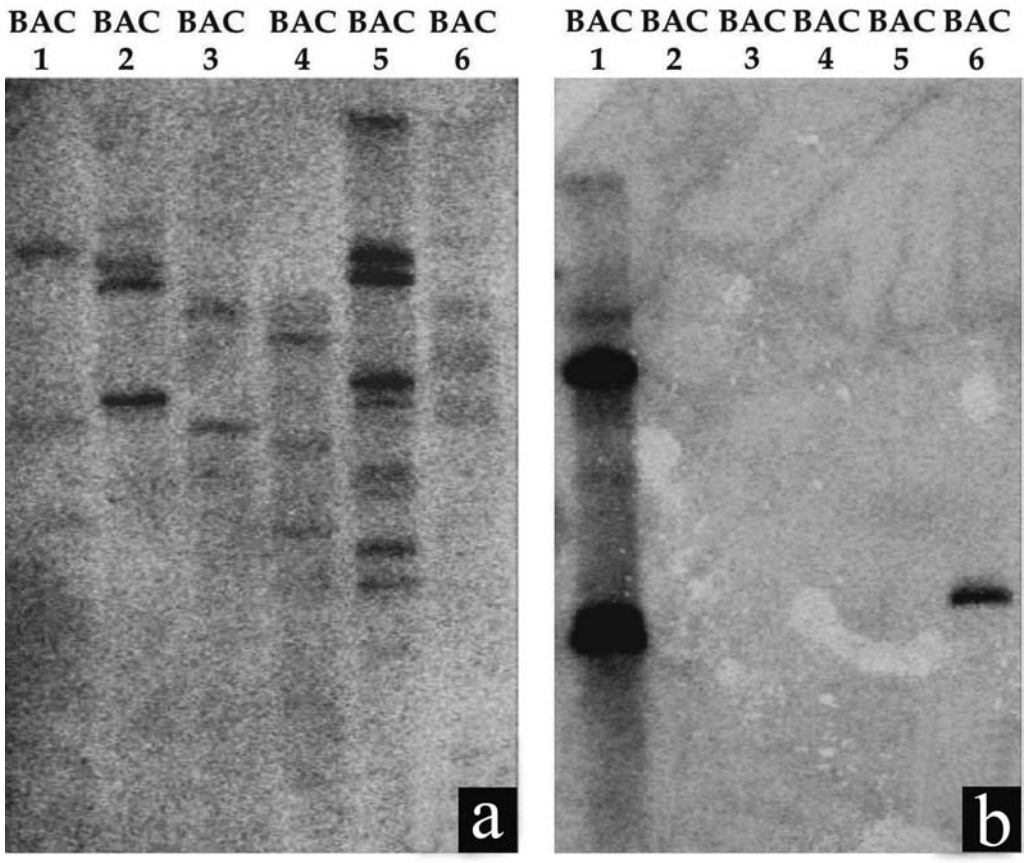

Figure 1. BAC-Southern blot analysis. Positive BAC clones were isolated, purified, *Eco*RI digested, electrophoresed, blotted to nylon membranes and hybridized to [α-P^{32}]-labelled probes. **(a)** Hybridization signals observed in the six BACs clones using SSR-Ha785 as probe. **(b)** Hybridization signals observed in BAC 1 and BAC 6 clones using HaRep1 as probe.

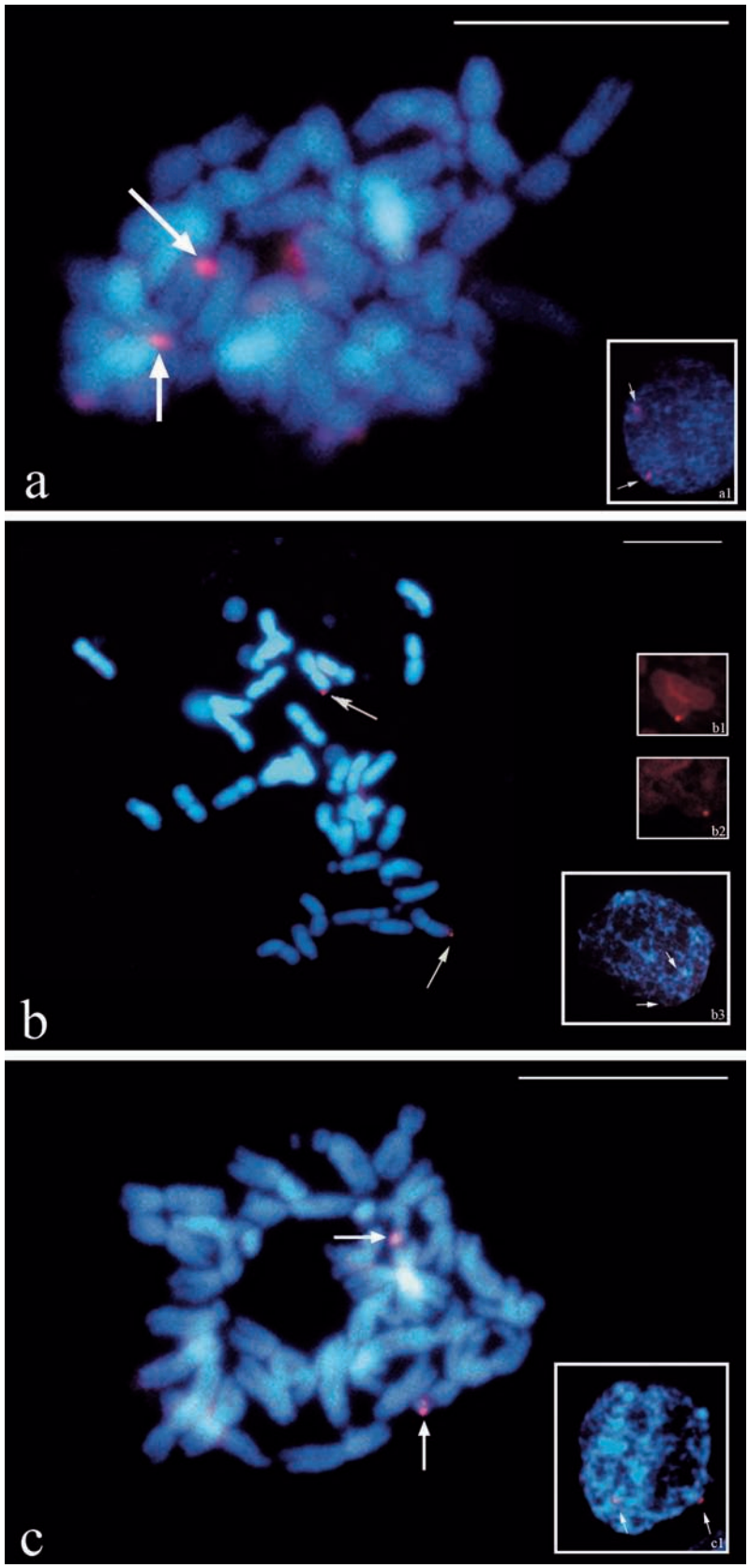

Figure 2 BAC-FISH: **(a)** mitotic metaphase, the arrows show hybridization signals in homologous chromosomes using BAC3 containing the SSR HA4222 (LG 16) as probe. Relation probe: DNA blocking = 1: 20. **(a1)** interphase nucleus showing two signals. **(b)** mitotic metaphase, the arrows show hybridization signals in homologous chromosomes using BAC6 containing the SSR HA2600 (LG 10) as probe. Relation probe: DNA blocking = 1: 20. **(b1-b2)** chromosomes staining with digoxigenin showing hybridization signals on the chromosomes **(b3)** interphase nucleus showing two signals. **C-PRINS**: **(c**) mitotic metaphase, the arrows show bright fluorescent signal in two chromosomes using primers of SSR ORS1247 as probe. **(c1)** interphase nucleus showing two signals. The counterstaining was made with DAPI. Bar =10 μm.

overgoes were used for hybridization to BAC filters as described in Materials and Methods. More than 70 positive clones were obtained, out of which, 35 were selected for further analysis. Six BAC clones representing three linkage groups were confirmed by PCR directed to the SSR tags and reconfirmed by means of Southern blot hybridizations (Table 1).

When each one of the six BAC clones were used as probe in FISH experiments, hybridization signals were found scattered over all the chromosomes (data not shown). This result strongly suggests that these BACs contain samples of the repetitive DNA sequences scattered within the sunflower chromosomes.

These six clones were characterized using Southern blot by hybridization with different repetitive sequences (Table 1). This evaluation confirmed that the hybridization patterns observed in FISH experiments were due to transposable elements present in each BAC clone. Among these 6 clones, BAC2 and BAC5 showed the highest repetitive element copies particularly when hybridized with SSR-Ha785 probe (Fig. 1a). When the HaRep1 sequence, which showed similarity to *copia*-like retrotransposons (Santini *et al*., 2002), was used as probe, strong hybridization signals were detected in BAC1 and BAC6 clones (Fig. 1b). On the other hand, no signals were observed when a probe with similarity to *gypsy*-like retrotransposon was used (Tang *et al*., 2006) (data not shown).

Based on these results, two BAC clones (BAC3 and BAC6) were selected to perform BAC-FISH using the repetitive sequences SSR-HA785 and / or the HARep1 as blocking DNA.

Specific signal were obtained in both cases and confirmed after thorough observation of 30 different cells at metaphase stage. The relative concentrations of probe DNA and blocking DNA were adjusted for each BAC clone used as probe for FISH. When BAC3 containing SSR HA4222, anchored to LG 16 was used as probe, one pair of specific signals were detected (Fig. 2a). In this case a DNA concentration ratio of 1 to 20 between BAC3 and SSR-Ha785 was used. Figure 2b shows two terminal signals in two chromosomes with similar size and morphology, when BAC6 (containing SSR HA2600) was used as probe. Here, the DNA was blocked with both SSR-Ha785 (DNA concentration ratio = 1/15) and HaRep1 (DNA concentration ratio = 1/10). Regarding C-PRINS strategy, indirect C-PRINS was applied for the localization of one single SSR site in the sunflower genetic map. To this aim, oligonucleotides corresponding to the flanking regions of ORS1247 anchored to LG 17 were used as primers. Consequently, two strong signals were observed in a pair of SAT-chromosomes corresponding to ORS1247 (Fig. 2c).

Discussion

In the present work, BAC-FISH and C-PRINS techniques were evaluated for the detection of single-copy DNA sequences on sunflower chromosomes, corresponding to already mapped single-site SSR markers (Kiani *et al*., 2007).

The detection of unique targeted sequences in plants with large genomes by FISH using probes with molecular sizes lower than 10 kbp turned out to be very difficult even after using amplification methods such as tyramide amplification (Khrustaleva and Kik, 2001; Qian and Lloyd, 2003). Thus, the use of large insert size of BAC clones turned out as an attractive strategy for physical mapping of single copy sequences on different plant species (Lapitan *et al*., 1997; Wang and Chen, 2005), although it requires the identification of suitable BAC specific clones.

In this work, the use of pools of overgo probes to identify BAC clones containing target single-site SSRs was more efficient than a traditional screening using specific individual probes. Through this technique, six BAC clones containing five markers of unique location, previously anchored to three linkage groups of interest: LG 10 (HA928, HA2600), LG 16 (HA4222) and LG 9 (HA2063, ORS805) were identified. These LGs encompassed previously reported QTL for resistance to *S. sclerotiorum* (Maringolo, 2007). The BAC-FISH technique using these BAC clones as probes in *in situ* hybridization assays delivered a scattered pattern of hybridization which indicates the presence of repetitive elements in these clones, as confirmed by the BAC-Southern blot performed using specific repetitive DNA probes. We observed different hybridization patterns for each BAC clone as well as differences in signal intensity for the different evaluated repetitive sequences (repetitive sequences previously reported in sunflower as well as sequence with similarity to *copia*-like and *gypsy*-like retrotransposon families). These results confirm previous reports indicating the disperse distribution of sequences with similarity to retrotransposons in the sunflower genome (Natali *et al*., 2006; Tang *et al*., 2006). Due to this reason, it was necessary to block these repetitive DNA sequences to detect single copy sequences. Thus, blocking strategies were assayed for the identification of unique target sequences by BAC-FISH.

Our study showed that the BAC-FISH technique allowed the identification of specific signals in metaphasic chromosomes for SSR HA4222 marker anchored to LG 16 and for SSR HA2600 anchored in LG 10. These results confirm that BAC-FISH is a useful tool for the detection of single copy sequences in plant species with large genomes such as sunflower. However, since the presence of large amounts of repetitive elements present in BAC clones impairs the localization of unique sequences in different species (Kim *et al*., 2002), the blocking conditions for these sequences for each BAC-FISH assay was optimized. We determined that the sunflower repetitive sequences, SSR-Ha785 and the HaRep1, turned out to be efficient blocking DNAs suitable for BAC-FISH applications.

In parallel, C-PRINS was tested as a simpler and faster alternative to BAC-FISH for localization of single copy sequences like SSRs anchored to know LGs. Intense fluorescent signals were detected by primer extension using oligonucleotides directed to the SSR marker ORS1247 anchored to the LG 17. As expected, when primers for ORS1247 were tested, signals were observed in two homologous chromosomes.

In this work we demonstrate that indirect C-PRINS is a useful method to detect single copy sequences in sunflower. These results agree with those reported by Kubaláková *et al*. (2001), who found that the C-PRINS technique is a more sensitive variant of PRINS technique and more useful for the detection of low copy number sequences. C-PRINS technique appears as a valuable alternative or complementary strategy to the BAC-FISH technique for localization of single copy sequences in the sunflower genome. In comparison, C-PRINS technique has the advantage of being easy to implement, faster, cheaper and more specific avoiding the detection of repetitive DNA.

In conclusion, we found that both techniques, BAC-FISH with the use of blocking repetitive sequences and indirect C-PRINS, are suitable strategies to detect single copy sequences in sunflower. The application of these techniques to the integration of genetic and physical maps will contribute to the chromosomal location of key genes and/or QTL associated to agronomic important traits.

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