Funct Integr Genomics (2005) 5: 97–103
DOI 10.1007/s10142-004-0127-9

ORIGINAL PAPER

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Direct targeting and rapid isolation of BAC clones spanning a defined chromosome region

Received: 3 August 2004 / Revised: 22 October 2004 / Accepted: 26 October 2004 / Published online: 22 January 2005

Abstract To isolate genes of interest in plants, it is essential to construct bacterial artificial chromosome (BAC) libraries from specific genotypes. Construction and organisation of BAC libraries is laborious and costly, especially from organisms with large and complex genomes. In the present study, we developed the pooled BAC library strategy that allows rapid and low cost generation and screening of genomic libraries from any genotype of interest. The BAC library is constructed, directly organised into a few pools and screened for BAC clones of interest using PCR and hybridisation steps, without requiring organization into individual clones. As a proof of concept, a pooled BAC library of approximately 177,000 recombinant clones has been constructed from the barley cultivar Cebada Capa that carries the *Rph7* leaf rust resistance gene. The library has an average insert size of 140 kb, a coverage of six barley genome equivalents and is organised in 138 pools of about 1,300 clones each. We rapidly established a single contig of six BAC clones spanning 230 kb at the *Rph7* locus on chromosome 3HS. The described low-cost cloning strategy is fast and will greatly facilitate direct targeting of genes and large-scale intra- and inter-species comparative genome analysis.

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Keywords Barley · Pooled BAC library · Screening · Targeting genes of interest

Introduction

The characterisation of large and complex genomes requires large insert DNA libraries. In past years, bacterial artificial chromosome (BAC) libraries (Shizuya et al. 1992) have been constructed from reference genotypes of many plant species and have proven their advantages over the yeast artificial chromosome (YAC) cloning system (Burke et al. 1987). Although BAC libraries allow the generation of many stable clones with a very low level of chimerism (Woo et al. 1994), the established technology is not ideal for large and complex genome organisms that require the generation of an extremely large number of clones. For example, species with large genomes such as barley (5,000 Mb) and hexaploid wheat (17,000 Mb) require 250,000 and 850,000 clones respectively for a fivefold genome coverage library with an average insert size of 100 kb, compared to 7,000 clones for a similar coverage in *Arabidopsis thaliana*. Therefore, the construction of BAC libraries from large and complex genomes is very costly and laborious and cannot be performed from many genotypes of interest. However, in the final steps of map-based cloning, it is desirable to use a genomic library of the cultivar or line that contains the gene of interest, as BAC libraries of related species or lines from the same species might not contain the gene. This problem was revealed by recent comparative studies where disruption of colinearity as well as nonconserved gene content was found between related species or even within the same species. In maize, the *bz* genomic regions of two lines were shown to differ dramatically not only in their repeated elements but more importantly in their gene density and content (Fu and Dooner 2002). In wheat, at the *Lr10* leaf rust resistance gene locus, two haplotypes which were defined by the presence (H1) or the absence (H2) of two resistance gene analogs were found in a collection of 113 wild and cultivated wheat

lines (Scherrer et al. 2002). The isolation of *Lr10* was only possible because the BAC library used in the sub-genome map-based cloning approach was constructed from a genotype belonging to the H1 haplotype (Feuillet et al. 2003).

These recent findings make it clear that it is essential to develop tools that allow the rapid and low cost generation and screening of genomic libraries from any species or lines of interest. Several strategies to directly target orthologous regions, genes or alleles of interest have been previously developed. One of the methods consisted of constructing nongridded BAC libraries, which are pooled and subsequently screened for genes or sequences of interest. Such methods have already been used in mouse (Pierce et al. 1992), soybean (Salimath and Bhattacharyya 1999) and hexaploid wheat (Liu et al. 2000; Ma et al. 2000). Another strategy, successfully used in maize, consisted of the construction of a sublibrary of gene-rich regions in an adapted BAC vector by using the rare-cutting methylation-sensitive enzyme *Not*I and direct screening for favourable alleles (Fu and Dooner 2000).

In barley, so far only one widely available BAC library has been constructed from the North American cultivar Morex (Yu et al. 2000). In the present study, we report the construction of a pooled BAC library of the barley line Cebada Capa and the development of a screening method that allowed the efficient and rapid establishment of a 230-kb contig at the *Rph7*leaf rust resistance locus on chromosome 3HS. This second BAC library will allow the cloning of genes not present in Morex as well as performing intraspecific microcolinearity studies at any locus of interest in barley.

Materials and methods

BAC library construction

The Cebada Capa barley BAC library was constructed as described in Chalhoub et al. (2004) with minor modifications. Three- to 4-week-old plants from the barley line Cebada Capa were kept for 1 week in the dark and leaves were harvested and frozen in liquid nitrogen. Nuclei isolation and high molecular weight DNA preparation were essentially performed as described by Allouis et al. (2003). The plugs were recovered after pre-electrophoresis. Twenty plugs were used for partial restriction digest with *Hin*dIII (GibcoBRL) and the partially digested DNA was subjected to one size selection and electroeluted. Fifty to 100 ng DNA was ligated into the pIndigo BAC vector (Caltech) prepared for efficient cloning according to the "one single tube vector preparation method" (Chalhoub et al. 2004) and the ligated DNA was transformed into DH10B *Escherichia coli* electrocompetent cells (Invitrogen). Transformed cells were plated on selective LB media (12.5 μg chloramphenicol, 0.55 mM IPTG, 80 μg/ml X-Gal) after test plating of each transformation, in order to allow an average of 1,300 colonies per 14-cm-diameter plate. Eight different ligation reactions experiments were conducted and 177,000 barley BAC clones were obtained.

Pooling of the BAC library and conservation of the pools

Between 700 and 3,600 BAC clones (an average of 1,300) were collected from each plate in 3 ml storage buffer (LB supplemented with 10 mM KCl, 20 mM $MgSO_4/MgCl_2$, 40 mM glucose, 25% glycerol) and homogenised for 30 min at 37°C under gentle agitation (220 rpm). Each of the resulting 3 ml cultures represents a pool. The pools were then aliquoted into three tubes, each corresponding to one copy of each pool of the library. The tubes were flash frozen in liquid nitrogen and the three copies stored in separate −80°C freezers. Storage of the pools in a high glycerol (25%) and glucose-enriched medium allows multiple use of the BAC pools with several thawing and freezing cycles without loss of viability.

BAC DNA analysis

DNA was isolated from the pools using a standard alkaline lysis minipreparation method (Sambrook et al. 1989). Recombinant BAC DNA from individual clones was isolated using the same procedure and analysed by *Not*I or *Hin*dIII restriction digestion followed by pulsed field gel electrophoresis (PFGE) according to Chalhoub et al. (2004).

Table 1 Characterisation of the barley Cebada Capa BAC library with simple sequence repeat (SSR) markers (Ramsay et al. 2000) analysed on 96 of the 138 pools (representing approximately 4.3 barley genome equivalents)

SSR[a]	Linkage group	Observed size (bp)[b]	Number of positive pools
WMC1E8	1H	180	7
Bmac0032	1H	230	11
EBmac0415	2H	240	8
HVM36	2H	110	13
Bmag0225	3H	150	7
Bmac0209	3H	220	7
Bmag0353	4H	100	9
HvMLO3	4H	230	10
Bmac0113	5H	200	3
Bmag0223	5H	150	7
Bmac0018	6H	140	7
Bmac0156	7H	140	8

[a]Ramsay et al. 2000
[b]Approximate size observed in barley cultivar Cebada Capa genomic DNA as estimated on agarose gel

Table 2 Screening of the barley Cebada Capa BAC library with primers and probes derived from gene sequences of the *Rph7* orthologous locus in Morex (Brunner et al. 2003)

Gene	Primer sequences	Positive pools and number of verified BAC clones[a]
hvpg1	PG1-7: GTCAAGTCCGTTCAAG′	–
	PG1-8: CCGCCATCACCGATAAGTG′	14 (2)[b], 56 (3), 58 (3), 104 (0), 113 (0), 125 (1)
Hvpg4	PG4-1: CGCCTCGTGATGAACTTC′	–
	PG4-2: GCCTCTTGGTGCGAGTTC′	(14 (2)[b], 68 (1), 127 (3)

[a]Confirmed as redundant copies of unique clones by *Hin*dIII digest
[b]The PG1 and PG4 primers and probes detected one positive pool in common representing the same individual BAC clone

Screening of the pooled library by PCR and DNA hybridisation

The barley BAC library was characterised for genome representation by PCR-identification of positive pools with one to two simple sequence repeat (SSR) markers from each of the seven linkage groups (Table 1) as described (Ramsay et al. 2000). Identification of BAC clones from the *Rph7* locus was done using two single copy genes (*Hvpg1* and *Hvpg4*) located at the *Rph7* locus on barley chromosome 3HS (Table 2; Brunner et al. 2003).

Identification of positive pools was done by PCR. PCR reactions were performed in a 20 µl reaction containing 0.5 units Taq DNA polymerase (Sigma), 1× PCR buffer (Sigma), 100 µM dNTPs and 0.5 µM primers (Tables 1, 2) on 5 µl pool DNA diluted 50 times. Amplifications were performed in a Perkin-Elmer thermocycler 7600 with annealing temperatures and extension times depending on the primers and expected PCR fragment sizes.

To detect the positive clone from each positive pool identified in the first step, aliquots of the glycerol stock were diluted 500,000-fold and plated on 22×22 cm Q-trays (Genetix) with LB-agar media and grown overnight in order to obtain about 3,000–5,000 individual clones on each plate. Further identification of the positive clones in each of the pools was done according to one of following methods.

Organising individual clones from positive pools and PCR screening

BAC clones were picked from each plated Q-tray of positive pools using the Q-Bot (Genetix, Dorset, UK) and organized in 384-well plates. Individual clones from each 384-well plate were then pooled in the row (16 minipools) direction and PCR amplification was directly performed on the resulting row minipool cultures. Individual positive clones were then identified by directly performing 24 PCR reactions on each clone of the positive row.

DNA hybridisation

BAC clones from each pool were plated on LB-agar containing Q-tray plates, and transferred on two nylon membranes (GeneScreen Membrane, NEN). The membranes were then removed and placed on two new LB-agar plates overnight at 37°C. The membranes were successively treated in solution 1 (0.5 M NaOH, 1.5 M NaCl), solution 2 (1.5 M NaCl, 0.5 M Tris-HCl pH 8) and solution 3 (4× SSPE) for 7 min in each solution at room temperature. After drying at room temperature and UV cross-linking, the filters were hybridised with the probes (Table 2). The labelling of the probes with α^{32}P-dCTP was performed as described in Graner et al (1990); hybridisations and washes were performed at 65°C. Positive BAC clones were confirmed by direct PCR when clearly separated from neighbouring colonies. When it was not possible to get a single isolated colony, clones corresponding

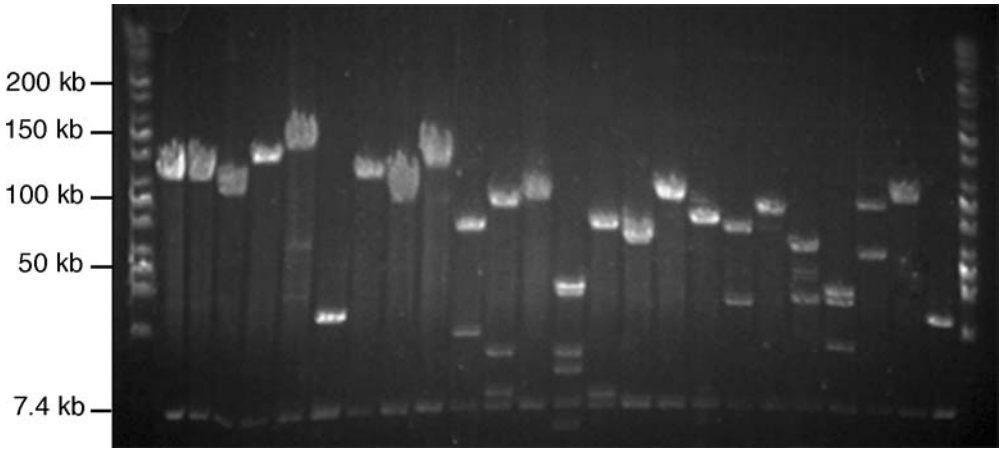

Fig. 1 Pulsed-field gel electrophoresis of 24 randomly selected BAC clones. BAC DNA was digested with *Not*I. The *arrowhead* indicates the 7.4-kb pIndigo vector (Caltech). The molecular weight marker (*L*) is Midrange1 (NEB)

to the positive hybridisation area (positive and neighbouring clones) were collected, grown overnight at 37°C in 4 ml LB-chloramphenicol (12.5 μg/ml) and plated as individual colonies. Ninety-six individual clones were picked and PCR was performed on the individual clones.

Results and discussion

Construction pooling and characterisation of the BAC library

We have constructed a BAC library from the barley cultivar Cebada Capa that carries the *Rph7* leaf rust resistance gene. This library was constructed by *Hin*dIII partial digestion and derived from eight different ligation reactions. It consists of approximately 177,000 recombinant clones. The insert size of the library was estimated from 112 different recombinant clones that were randomly selected from overnight cultures of aliquots from 24 independent pools. Clone insert sizes ranged from 35 to 270 kb with an average of 140 kb. No empty clones (vector carrying no insert) were observed. An example of PFGE analysis of *Not*I digestion from 24 clones is shown in Fig. 1. Based on the average insert size and a haploid barley genome size of 5,000 Mb (Arumuganathan and Earle 1991) the coverage of the library was estimated to be six genome equivalents which gives a probability greater than 99% of recovering any single-copy sequence of interest (Clarke and Carbon 1976).

The Cebada Capa BAC pooled BAC library developed in this study is a highly useful resource for barley genome analysis and gene cloning as it represents the second publicly available BAC library from barley, in addition to the currently available Morex barley BAC library (Yu et al. 2000). The observed larger insert size (140 kb) compared to that of the Morex BAC library (106 kb; Yu et al. 2000) is due to the improved protocol for BAC library construction (Chalhoub et al. 2004). Thus, a reduced number of clones were required for a sixfold coverage library (177,000 clones). This is particularly important for chromosome walking, as larger inserts result in fewer walking steps to span the targeted locus.

Different plating dilutions of transformed cells were tested for each ligation and the BAC library was organised in 138 pools. The number of clones per pool ranged from 700 to 3,600 clones with an average of 1,300 clones in each pool, which represent 0.036 barley genome equivalents. The percentage of recombinant clones was estimated to be 82% based on blue/white selection counts. This high proportion of recombinant clones reduces the problem of a possible bias in the library caused by faster growth of nonrecombinant clones compared to recombinants during the short amplification step. In order to allow use and reuse of the BAC pools by thawing and freezing without loosing clones, the pools were aliquoted into several copies and stored in a high glycerol (25%) and glucose-enriched medium. We tested first whether PCR could directly be made on lysed bacterial cells from each pool but nonreproducible results were obtained (data not shown). In contrast, preparing DNA from pools using the alkaline lysis method (Sambrook et al. 1989) allowed reproducible detection of positive pools by PCR. The amount of DNA prepared from each of the three pool aliquots was shown to allow more that 7,000 screening reactions.

Genome representation of the BAC library

To check the genome representation of the library, 96 pools corresponding to 4.3 barley genome equivalents were screened with 12 SSR markers (Ramsay et al. 2000) from the seven barley linkage groups. An average of eight positive pools per SSR marker was obtained (Table 1) indicating that the overall barley genome is well represented in this BAC library. The discrepancy between barley genome coverage estimated by BAC clone insert sizes for the 96

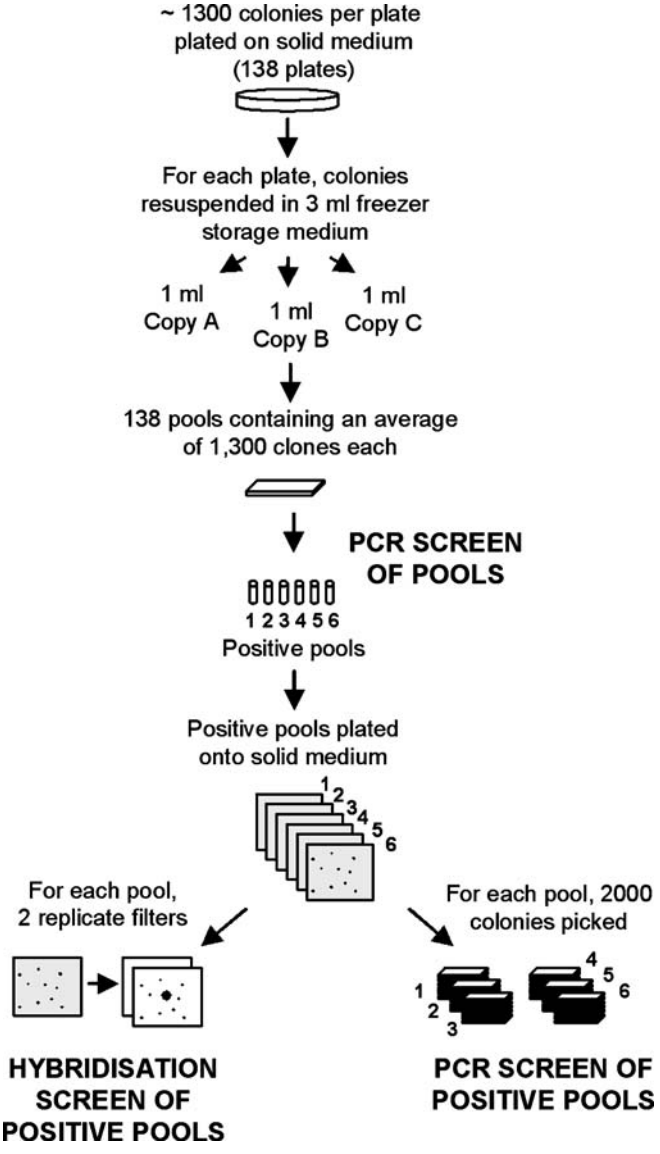

Fig. 2 Schematic representation of the organisation of the BAC library into pools and the different steps of screening

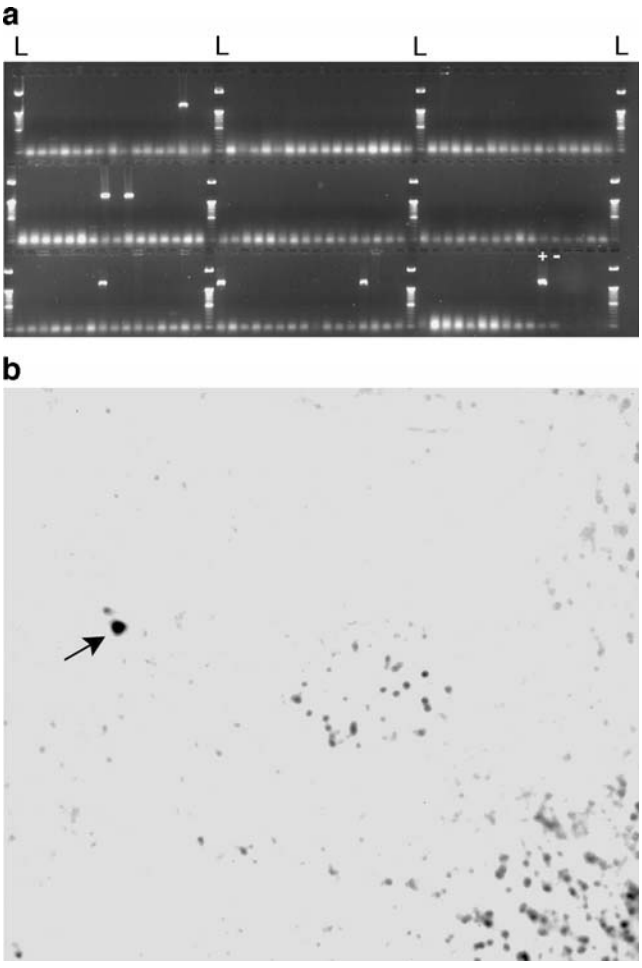

Fig. 3 Screening of the nongridded barley BAC library with the PG1 set of primers and probe. **a** PCR screening of the 138 pools with PG1 primers [*L* 50-pb molecular weight ladder (Invitrogen); + positive control, Cebada Capa genomic DNA; − negative control, H_2O]. **b** Hybridisation screening of the PCR-positive pool number 58 with the PG1 probe. The *black arrow* indicates the positive clone on the hybridisation filter

analysed pools (4.3×) and the SSR markers (8×) may be due to underestimation of the average size of the BAC clones, as several bands were obtained after *Not*I restriction, making calculations relatively difficult.

Establishment of a 230-kb orthologous contig at the *Rph7* locus

As a poof of concept for our strategy, we established a BAC contig at the *Rph7* locus in cv Cebada Capa that is orthologous to the contig recently published from barley cultivar Morex (Brunner et al. 2003). Two single copy genes (*hvpg1* and *hvpg4*) located at the *Rph7* locus on barley chromosome 3HS (Brunner et al. 2003) and separated by 20 kb in the cultivar Morex were used for screening. An efficient and fast strategy based on two steps (Fig. 2) was developed for screening the pooled Cebada Capa BAC library.

The first screening step, consisting of identifying positive pools, was performed by direct PCR on pools using primers corresponding to the two genes (Table 2). Using the primer pair PG1-7/PG1-8, developed from *Hvpg1* sequence, six positive pools (pools number 14, 56, 58, 104, 113, 125) were detected (Table 2, Fig. 3a) whereas with the primers PG4-3/4, developed from *Hvpg4* sequence, only three pools (numbers 14, 68, 127) were identified (Table 2). One pool (number 14) was common to both primer pairs. The eight positive pools were confirmed individually and in the three copies of the library demonstrating that the three copies of each pool are strictly identical (data not shown).

In a second screening step, the positive clone(s) were identified from each of the positive pools identified in the first step. Serial dilutions of aliquots of the glycerol stocks from each positive pool were performed and the optimal dilution (500,000-fold) that allowed the growth of 3,000–5,000 individual BAC clones was plated and grown overnight at 37°C. At this stage, two methods were compared: PCR and hybridisation screen (Fig. 2).

PCR screening

Individual BAC clones were picked from the positive pools (five 384-wells plates) and pooled in row and column direction for PCR screening as described in Materials and methods. PCR reactions were made directly on the bacterial cells, which gave high quality results and reduced potential contamination problems. Using this method we

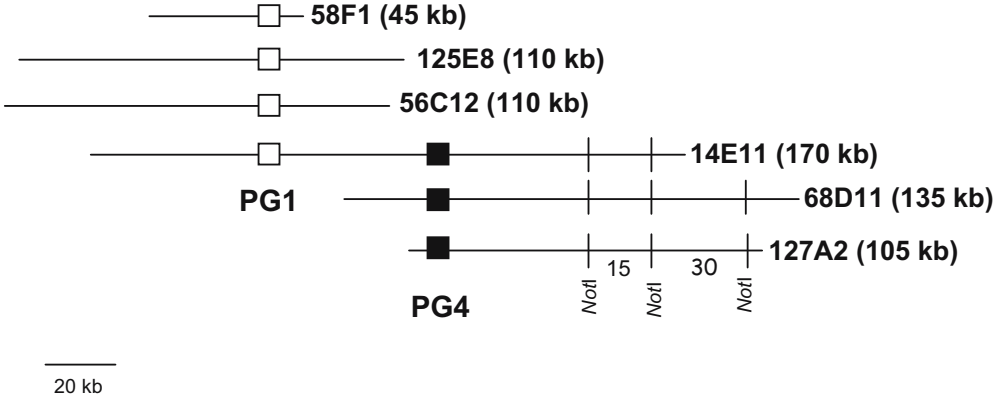

Fig. 4 Physical contig map of 230 kb at the *Rph7* locus on chromosome 3HS in barley cv. Cebada Capa. Six BAC clones are represented by *horizontal lines*, the *black* and *white boxes* represent the PG4 and PG1 genes, respectively, *vertical lines* indicate the presence of *Not*I restriction sites. The size of each clone is shown in *brackets* next to the clone name

rapidly identified one positive BAC clone from pool 14 with the *hvpg1* gene (data not shown).

DNA hybridisation

We also tested screening for positive clones using DNA hybridisation. Our hypothesis was that if we were able to detect one positive clone in 2,000 by PCR then we should also detect at least one positive clone in the 3,000 to 5,000 present on the plated pool by DNA hybridisation. Membrane lifts were taken from each plate and the membranes subsequently hybridised with DNA fragments amplified with the same primers that allowed detection of positive pools by PCR. An example of a hybridisation result of a membrane corresponding to positive pool 58 with the probe derived from the *hvpg1* gene is shown in Fig. 3b. Single isolated positive BAC clones were picked and when it was not possible to clearly separate the positive clone from neighbouring clones, a 1 cm^2 area of the positive regions of each plate was chosen. The resulting mixed cultures were plated and 96 individual colonies were picked for each positive area. Individual positive clones were identified by PCR screening of the bacterial cells and confirmed by hybridisation.

From the eight positives pools, pool numbers 104 and 113 did not show any hybridisation signal with either of the two probes used in this study. We conclude that either these pools were wrongly identified as positive in the first PCR screening step or the positive clone(s) in each pools were not present on the culture plate. For the six other pools, more than one hybridisation signal was observed with either Hvpg1- or Hvpg4-derived probes (Table 2). As each of the pools represents less than 0.036 barley genome equivalents, it is likely that copies of the same original clone were detected in each pool. This hypothesis was confirmed by further characterisation of a maximum of three positive clones (Table 2) from each original pool by fingerprinting using *Hin*dIII (data not shown).

Overall, a total of six different individual BAC clones from the *Rph7* region were identified using a set of two primer pairs and their corresponding probes. The insert sizes ranged from 45 to 170 kb. Clone 14E11 was identified with both*hvpg1* and *hvpg4* genes (Table 2). The BAC clones could be organised into a contig of 230 kb according to hybridisations with the *hvpg1* and *hvpg4*gene-derived probes and *Not*I restriction digests of the clones (Fig. 4).

Similar or different strategies have been used for direct targeting of BAC clones. In avoiding the difficult step of whole genome cloning of the maize genome, Fu and Dooner (2000) constructed a sublibrary of gene-rich regions in an adapted BAC vector by using the rare-cutting methylation-sensitive enzyme *Not*I. On the other hand, production of nongridded libraries and their screening for target clones by hybridization has been very common, since the introduction of the phage and plasmid cloning system about 30 years ago (Sambrook et al. 1989). Such methods have now been used with the BAC cloning system in mouse (Pierce et al. 1992), soybean (Salimath and Bhattacharyya 1999) and hexaploid wheat (Liu et al. 2000; Ma et al. 2000). In comparison, we rapidly cloned into BAC the whole genome of barley using the method of Chalhoub et al. 2004 that insures higher insert size (145 kb). In addition, we developed a complete and accurate strategy that combines PCR screening and hybridization steps to directly target positive BAC clones.

In conclusion, the results obtained in this study demonstrate the power of pooled BAC libraries cloning and screening strategies for direct targeting of interesting genes and alleles from specific genotypes in a short time frame and with low costs. In addition, the easy generation and identification of BAC clones of orthologous loci will be a highly valuable tool for evolutionary studies given recent findings, which show important intraspecies variation in the sequences and the gene content in orthologous regions (Fu and Dooner 2002; Feuillet et al. 2003).

Acknowledgements This work was supported by grant 3100-065114 of the Swiss National Science Foundation and research funds from the French Ministry of Research and Ministry of Agriculture.

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