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Construction of a potato YAC library and identification of clones linked to the disease resistance loci *R1* and *Gro1*

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Abstract A yeast artificial chromosome (YAC) library was constructed from high-molecular-weight DNA of potato (*Solanum tuberosum*). Potato DNA fragments obtained after complete digestion with four different rare-cutter restriction enzymes were cloned using the pYAC-RC vector. The library consists of 21 408 YAC clones with an average insertion size of 140 kb. The frequency of YAC clones having insertions of chloroplast or mitochondrial DNA was estimated to be 0.5% and 0.3%, respectively. The YAC library was screened by PCR with 11 DNA markers detecting single genes or small gene families in the potato genome. YACs for 8 of the 11 markers were detected in the library. Using 2 markers that are linked to the resistance genes *R1* and *Gro1* of potato, we isolated two individual YAC clones. One of these YAC clones was found to harbour one member of a small family of candidate genes for the nematode resistance gene *Gro1*.

Key words Potato · YAC library · Disease resistance · *R1* · *Gro1*

Introduction

Yeast artificial chromosome (YAC) libraries are instrumental in the physical mapping of complex eukaryotic genomes and for cloning genes based on map position (Burke et al. 1987). When used in combination with high-density restriction fragment length polymorphism (RFLP) maps, methods for efficiently saturating specific regions of a genome with polymerase chain reaction (PCR)-based DNA markers (Michelmore et al. 1991; Williams et al. 1990; Vos et al. 1995) and molecular maps of high genetic resolution, YAC technology provides access to novel genes by chromosome walking or landing strategies even in large plant genomes (Tanksley et al. 1995). Only a few YAC libraries have been constructed from higher plant DNA: from *Arabidopsis thaliana* (Ward and Jen 1990; Grill and Somerville 1991), maize (Edwards et al. 1992), tomato (Martin et al. 1992), barley (Kleine et al. 1993), sugarbeet (Del Favero et al. 1994) and rice (Umehara et al. 1995). The construction of plant YAC libraries is technically demanding due to unique features of plant cells such as rigid cell walls, large cell size and high cellular concentrations of polysaccharides and DNA hydrolyzing enzymes. The average insert size of YAC libraries of higher plants is usually smaller, therefore, than those obtained, for example, from the human genome. The CEPH human YAC library has an average insert size of 900 kbp (Cohen et al. 1993), whereas average insert size of plant YAC libraries ranges from 100 kbp to 350 kbp (Umehara et al. 1995).

RFLP linkage maps have been constructed for the 12 chromosomes of potato (Gebhardt et al. 1991, 1994). These maps have been used to locate in the potato genome major genes for resistance to the late blight fungus *Phytophthora infestans* (*R1*, Leonards-Schippers et al. 1992) and the root cyst nematode *Globodera rostochiensis* (*Gro1*, Barone et al. 1990). High-resolution genetic maps have been constructed for the chromosome segments harbouring the *R1* and *Gro1* resistance loci (Meksem et al. 1995; Ballvora et al. 1995), and family of genes tightly linked with the *Gro1* locus has been identified. The members of this gene family are candidates for the *Gro1* resistance allele (Leister et al. 1996). However, for positional cloning of

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the *R1* gene and for further characterization of the physical organization of the *Gro1* candidate gene family large insert genomic libraries are required.

Here we report on the construction and analysis of a potato YAC library derived from DNA of a genotype that carries both resistance alleles *R1* and *Gro1* and show that the library can be used to isolate genomic sequences in the region of the *R1* and *Gro1* locus.

Materials and methods

Plant material, yeast strain, YAC vectors and media

The dihaploid potato lines P6/210 and 1/52 were used as sources of high-molecular-weight (HMW) DNA. Line P6/210 is heterozygous for both *Gro1*, a dominant allele conferring resistance to the root cyst nematode *Globodera rostochiensis*, and *R1*, a dominant allele conferring race-specific resistance to *Phytophthora infestans*. Line 1/52 is heterozygous for the *Gro1* allele. *Saccharomyces cerevisiae* strain AB 1380 (MATa, *ura3-52*, *trp1*, *lys2-1*, *his5*, *ade2-1*, *can1-100*; Burke et al. 1987) was obtained from Biozym (Hameln, Germany), and the plasmid vectors pYAC-RC (Marchuk and Collins 1988) and pYAC-4 (Burke et al. 1987) were obtained from the American type culture collection (ATCC). Isolation of plasmid DNA, restriction digests and other standard techniques were performed as described in Sambrook et al. (1989). The yeast media YPD, AHC and SD were prepared according to Sherman et al. (1986) except that arginine was excluded from the SD medium and 60 mg/l canavanine (Sigma, Deisenhofen, Germany) was added.

Fragmentation of HMW potato DNA and preparation of the vector

High-molecular-weight DNA embedded in agarose plugs was prepared from leaf protoplasts and digested with restriction enzymes according to Ballvora et al. (1995). Partial digestion of HMW-DNA was performed as described by Edwards et al. (1992). Mechanically sheared DNA fragments were obtained according to Ward and Jen (1990).

YAC vectors pYAC-RC and pYAC-4 were linearized and dephosphorylated according to Martin et al. (1992) except that pYAC-RC was digested with the rare-cutter enzymes *Cla*I, *Mlu*I, *Bss*HII or *Sac*II instead of *Eco*RI and pYAC-4 was digested with *Sma*I instead of *Eco*RI for blunt end ligation.

Construction of the YAC library

In agarose ligation

After restriction digestion, DNA-containing agarose plugs were incubated in ESP buffer (0.5 *M* EDTA, pH 9.0, 1% *N*-Laurylsarcosine, 1 mg/ml proteinase K) for 4 h at 50°C, washed three times in TE + phenylmethylsulfonylfluoride (PMSF) (10 m*M* TRIS HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* PMSF) and three times in TE. After the addition of a 30- to 50-fold excess of restricted and dephosphorylated pYAC-RC vector, the mixture was melted at 65°C for 10 min. After cooling to 37°C, 1/10 volume of the 10 × T4-ligase buffer (Boehringer, Mannheim) was added, and T4-ligase was added to a final concentration of 0.1 U/µl. Ligation was performed overnight at room temperature. For the construction of some of the *Mlu*I and *Sac*II clones, size fractionation was performed after ligation using the CHEF-system (CHEF-DR II, BioRad: 120°, 30 s, 16 h, 12°C, 160 V).

Transformation

A 1/50 volume of 50 × gelase buffer (Biozym, Hameln, Germany) was added to the ligation products. The mixture was then melted at 65°C for 10 min. After cooling to 45°C Gelase (Biozym) was added to a final concentration of 1 U/150 mg agarose plug weight. Incubation was for 2 h at 45°C. A 40-µl aliquot of the mixture was used to transform 200 µl yeast spheroplasts as described by Burgers and Percival (1987). Transformants were first selected on solidified SD medium lacking uracil and supplemented with canavanine (SD + Can, − Ura). Transformed colonies were grown at 30°C for 3–4 days before they were transferred into 96-well microtitre plates containing 100 µl double-selection liquid medium (AHC, − Ura, − Trp). After 2 days of growth at 30°C, one volume 50% glycerol was added and the plates were stored at −70°C.

Preparation of DNA from YAC clones

Yeast clones were grown in 2 ml AHC medium overnight, and yeast HMW-DNA was prepared as described (Del-Favero et al. 1994). For the isolation of DNA from pooled yeast clones, cells were transferred from microtitre plates to AHC agar plates using a 96-prong handling device. The yeast colonies grown were pooled, and DNA was extracted according to Sherman et al. (1986). Yeast DNA for the identification of individual positive clones by PCR was prepared according to Kwiatkowski et al. (1990).

Determination of sizes of YACs

HMW yeast DNA was prepared according to Kleine et al. (1993) and separated by PFGE (Rotaphor, Type IV, Biometra, Göttingen, Germany) using the supplier's instructions for the separation of DNA molecules between 100 and 2000 kb. The DNA was nicked in the gel by UV light (302 nm) for 5 min and then transferred to Hybond N filters (Amersham, Braunschweig, Germany) by standard capillary transfer (Sambrook et al. 1989). Hybridization was performed using the ECL detection system (Amersham) according to the supplier's instructions. Plasmid pBR322 linearized by *Eco*RI digestion was used as probe.

Screening of the library for chloroplast, mitochondrial, low-copy and single-copy sequences

All library screens were performed using PCR (Green and Olsen 1990). DNA was extracted from 96 clones grown on AHC (platepools). Superpools were constructed by mixing the DNA isolated from four platepools (384 clones), resulting in 54 superpools for the entire library. Screening was first carried out on superpools. One hundred nanograms of superpool DNA was amplified using marker-specific primers that were designed to anneal at 55°C. Cycling conditions were: denaturation at 93°C for 2 min 30 s, then 35 cycles of 30 s at 93°C, 45 s at 55°C, 1 min 30 s at 72°C and finally 1 round of 10 min at 72°C.

For positive superpools, the four platepools were subsequently analysed and, finally, for identification of individual positive clones, pools of lanes and rows (one-dimensional pools) were screened. Marker genes for chloroplast and mitochondrial sequences were the large subunit of ribulose-bisphosphate carboxylase (*rbcL*) and the mitochondrial complex I subunit of NADH-dehydrogenase (*nad 9*), respectively. Sequences of *rbcL* (accession number: M76402) and *nad9* (accession number: X79774) of *Solanum tuberosum* were used for designing PCR primers (Table 1). PCR primers for RFLP marker loci *GP21* and *GP179* are described in Meksem et al. (1995). Primers for RFLP marker loci *CP56* (CP56-5′,1 and CP56-3′1) and *CP113*

Table 1 PCR primer sequences

Primer	Sequences (5′ → 3′)	Expected product size (bp)
nad9 sense	CCAAGCAATGCCCAAAAGTC	402
nad9 antisense	TCGTCTGCACTGGTTTGTAC	
rbcL sense	CCAGTCTTGATCGTTACAAAG	581
rbcL antisense	AGTCATGCATTACGATCGGAA	
332s	CACATAGATCAGTTAAATGCATT	102
332as	AAACTTTCATAATTGTTTAAAGTTTT	
334s	CATAGAACAGTTAGACGCATTA	100
334as	GCTTTCATATTTGTATAAAGTTCC	
11s	CAATGATGAGAGGGTGAAGAA	562
11as	ATGGATAATCTTTAGGAAATATTG	
121s	AGAAATTGGTTTAACGGTTGAC	277
121as	CCAACCTCTTGAAATTCTGAAT	
123s	TGAAACCTCTCCGGCATATC	450
123as	TGGGAACATGATCCACCATG	
GP516s	CCTTTCTGCCCGATGTATCCTG	270
GP516as	GTCATTCATGGCGCTCAGATTGG	

(CP113-5′1 and CP113-3′1) are described in Niewoehner et al. (1995). The SPUD237 primers are described in De Jong et al. (1997), and primer sequences for markers *GP516*, 1.1, 1.2.1, 1.2.3, 3.3.2 and 3.3.4 are also listed in Table 1.

The frequency p of repetitive sequences in the YAC library was estimated as follows: The probability, *P*, that no positive clone is present in 1 superpool of 384 clones is

$$P = (1 - p)^{384} \quad \text{or} \quad P = Z/n$$

where Z is the number of superpools with no positive signal and not containing, therefore, a positive clone and where *n* is the number of superpools tested ($n = 54$). Solving the equation $Z/n = (1 - p)^{384}$ for p gives $p = 1 - \log_{384}(Z/54)$.

Southern gel blot analysis

Total DNA of potato (4–5 μg) and 0.15 μg yeast DNA were restricted with *Taq*I, electrophoresed on 4% denaturing polyacrylamide gels, blotted and hybridized according to Gebhardt et al. (1989).

Results

Construction and characterization of the YAC library

Preliminary pilot experiments considered different strategies for the construction of potato YAC clones. The cloning of randomly sheared HMW-DNA according to Ward et al. (1990) resulted in YACs with small insertions of around 50 kb. The cloning of fragments obtained after partial digestion of HMW-DNA with *Eco*RI and subsequent size selection by pulsed-field-gel electrophoresis gave rise to YACs with an average insert size of 350 kb. The transformation efficiency was, however, very low with only about 10 clones per microgram DNA. When HMW-DNA was digested to completion with *Mlu*I or *Sac*II and size selected after ligation, YACs with relatively large insertions were obtained (Table 2). The transformation efficiency was highly variable (50–500 clones per microgram DNA). Moreover, genome representativity may be a problem when selecting against a size fraction after complete digestion with restriction enzymes. One thousand and eight hundred YAC clones were obtained after size selections from HMW-DNA of potato line 1/52, and these were included in the YAC library. The major part of the YAC library was constructed by cloning HMW-DNA of line P6/210 without size selection and after complete digestion with the four rare-cutter restriction enzymes *Sac*II, *Mlu*I, *Cla*I and *Bss*HII. Between 4000 and 6000 YAC clones were collected for each of the four enzymes. Average insert size was determined by the analysis of subsets of randomly picked clones (Table 2). A total of 21 408 YAC clones were collected, which represents approximately 3000 megabases or three potato genome equivalents (Table 2, Fig. 1).

Screening the library for chloroplast and mitochondrial sequences

DNA of the 54 superpools, each superpool corresponding to 384 individual YAC clones, was used as template for PCR analysis with chloroplast and mitochondrial gene-specific primers. The PCR assay identified chloroplastic *rbcL* sequences in 46 superpools and mitochondrial *nad9* sequences in 38 superpools. The frequency of YACs with insertions derived from chloroplasts or mitochondria was estimated using the formula given in the Materials and methods. According to this estimate 0.5% of YAC clones carried chloroplast DNA and 0.3% mitochondrial DNA.

Table 2 Composition of the potato YAC library

Cloning enzyme	Number of clones analysed	Average insertion size (kb)	Total number of clones	Cloned genome (Mb)
*Bss*HI	25	90	5 184	467
*Cla*I	25	160	5 952	952
*Mlu*I	25	130	3 936	512
*Sac*II	25	110	4 320	475
*Mlu*I[a]	90	380	864	328
*Sac*II[a]	27	240	960	230
Total	217	140	21 408	2964

[a] Clones were obtained after size selection

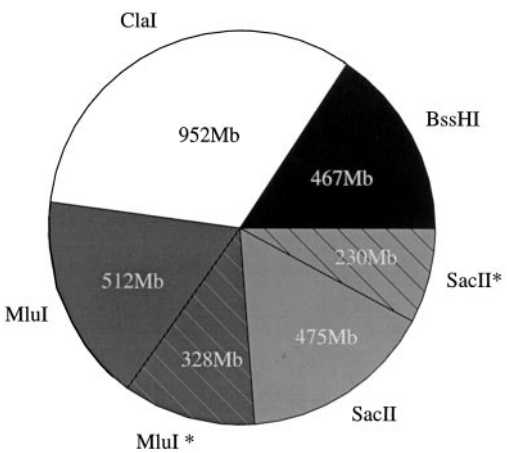

Fig. 1 Megabases of the potato genome covered by the six subsets of YAC clones. Subsets *Mlu*I* and *Sac*II* originated from HMW-DNA of line 1/52 that was size-selected after ligation. The remaining subsets originated from HMW-DNA of line P6/210 that was cloned without size selection

Screening the library with markers detecting single genes or small gene families

The 54 superpools were submitted to PCR analysis with 11 primer pairs specific for markers that detect single- or low-copy number sequences (Table 3). Markers GP21, GP179 and SPUD237 are linked to the *R1* resistance locus (Meksem et al. 1995). GP21 and GP179 primers were derived from single-copy RFLP markers, whereas SPUD237 primers were derived from an amplified fragment length polymorphism (AFLP) marker (De Jong et al. 1997). Markers GP516, CP56, 3.3.4 and 3.3.2 are tightly linked to the cyst nematode resistance gene *Gro1* (Ballvora et al. 1995; Leister et al. 1996). CP56 corresponds to a single-copy gene, whereas GP516 detects, besides the locus on chromosome, at least two additional loci on different chromosomes. The 3.3.2 and 3.3.4 primers amplify several resistance gene homologous sequences belonging to a gene family likely to include the *Gro1* resistance allele (Leister et al. 1996). CP113 is linked to the cyst nematode resistance locus *H1* (Barone et al. 1991; Gebhardt et al. 1993) and detects copies of a small, clustered gene family. The 1.1, 1.2.1 and 1.2.3 primers amplify, similarly as 3.3.2 and 3.3.4, resistance gene homologous sequences located in different regions of the potato genome. Altogether, the 11 markers tested detect at least 25 physical loci and identified 24 superpools containing at least 1 positive YAC clone each. No positives were found with 3 of the markers (Table 3).

Table 3 Superpools positive for single or low-copy number markers

Marker	Loci on chromosome no.	Number of copies	Number of positive superpools	Cloning enzyme
GP21	V	1	1	*Cla*I
GP179	V	1–2	1	*Cla*I
SPUD237	V	1	0	–
CP113	V	5–10	5	*Bss*HI (3), *Sac*II[a], *Mlu*I
GP516	VII, X, XII	$\geqslant 3$	6	*Bss*HI (2), *Sac*II, *Mlu*I (2), *Cla*I
CP56	VII	1	0	–
3.3.4	VII	3–8	1	*Mlu*I
3.3.2	VII	5–8	0	–
1.1	III, XI	$\geqslant 2$	5	*Cla*I (3), *Sac*II (2)
1.2.1	II, XI	$\geqslant 2$	4	*Cla*I (2), *Mlu*I[a], *Sac*II
1.2.3	II	1	1	*Cla*I

[a] Superpool of YACs obtained after size selection

Isolation and analysis of single YAC clones containing markers linked to *R1* and *Gro1*

YAC clones positive for markers GP21 and 3.3.4, respectively, were isolated from the YAC library by the PCR screening of superpools, single-plate pools and pools of rows and columns of individual plates. Candidate yeast clones of rows or columns were analysed individually by PCR. One positive YAC clone was isolated for each of markers GP21 and 3.3.4. The GP21-YAC had an insert size of around 100 kb, while the 3.3.4-YAC had an insert size of 270 kb. When this YAC clone was analysed by Southern gel blot analysis and together with line P6/210 used to construct the library, it was shown that it contained one copy of the gene family detected by the 3.3.4 probe. This copy is characterized by a 1.7-kb *Taq*I restriction fragment, which is present in the nematode-resistant lines P6/210 and 40 and also in the susceptible lines 18 and 41 (Fig. 2).

Discussion

Approximately 3000 megabases of potato DNA have been cloned in yeast artificial chromosomes. To the best of our knowledge, the 21 000 YAC clones obtained represent the first YAC library of potato. Provided that the generation of YAC clones was unbiased, the potato YAC library covers three haploid genome equivalents with a 95% probability of containing any sequence in the genome at least once.

A simplified strategy was adopted for constructing the major part of the library. Instead of cloning size-selected DNA fragments resulting from partial digestion with a single restriction enzyme, we cloned fragments obtained after complete digestion with four different restriction enzymes without size selection. A large number of YAC clones can be generated this way within a relatively short time, with good cloning efficiency and requiring lower quantities of HMW-DNA. For achieving genome representativity, we used four different restriction enzymes. The choice of the enzymes was based on their ability to produce a high proportion of long restriction fragments. Depending on the enzyme, average insert sizes of between 90 kb and 160 kb were obtained, which are comparable to the ones reported for YAC libraries of *Arabidopsis*, maize, tomato, sugarbeet and barley (Grill and Somerville 1991; Edwards et al. 1992; Martin et al. 1992; Kleine et al. 1993; Del Favero et al. 1994) but are smaller than the 350 kb of a recently generated YAC library of rice (Umehara et al. 1995). Large insert YAC libraries suffer, however, from a high percentage of chimeric YACs (Umehara et al. 1995; Green et al. 1991). Although the frequency of chimeric YACs in our potato YAC library has not yet been determined, it is expected to be a low due to the moderate insert size.

As basis for chromosome walking and landing strategies in plants, libraries with moderately sized inserts of between 100 kb and 200 kb may also be sufficient, as very effective strategies are now available which make possible the saturation of a genomic target region with DNA markers that are physically linked to the target gene within less than 100 kb (Michelmore et al. 1991; Williams et al. 1990; Vos et al. 1995). In the cases of the *R1* and *Gro1* resistance loci of potato, the genomic regions harbouring these loci have been enriched with RAPD and AFLP markers in addition to already available RFLP markers (Ballvora et al. 1995; Meksem et al. 1995).

The screening of the potato YAC library with a number of single- or low-copy number markers revealed the presence of positive clones in all four of the sublibraries constructed with four restriction enzymes. Not all sequences tested were represented, however. Two of 5 single-copy markers and 1 of 6 markers detecting small gene families were not detected in the library. This indicates that the library is not as representative as expected from the theoretical genome coverage. The four restriction enzymes used for library construction are methylation-sensitive, and all contain CpG in the recognition sequence, which is the preferred site for methylation in plant DNA. Restricted access of the enzymes to methylated recognition sites may have resulted in a fraction of very large DNA fragments that was inefficiently cloned.

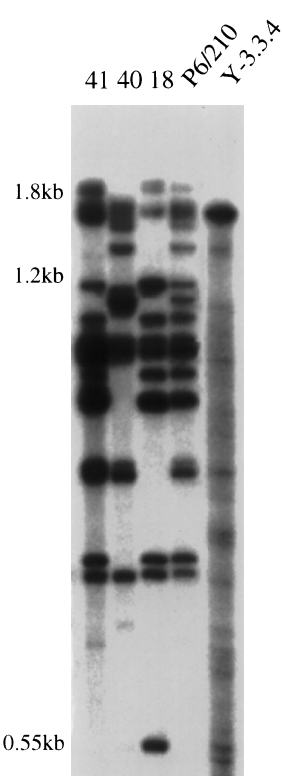

Fig. 2 Southern gel blot analysis of YAC clone 3.3.4 and dihaploid potato clones P6/210, 41, 40 and 18. Clone P6/210, from which YAC 3.3.4 was derived, is an F_1 hybrid of the cross 41 by 40. P6/210 and 40 are resistant to the nematode, whereas 41 and 18 are susceptible. DNAs were restricted with *Taq*I. The filter was hybridized to the resistance gene homologous probe 3.3.4. The fragment present in YAC 3.3.4 is present in all four potato clones. The size range of the fragments detected by the probe is indicated on the *left*

Complete coverage of the potato genome may be approached by constructing additional sets of YAC clones using methylation insensitive rare-cutter restriction enzymes or by complementing the YAC library with BAC libraries (BAC = bacterial artificial chromosomes, Shizuya et al. 1992; Wang et al. 1995; Choi et al. 1995; Marek and Shoemaker 1996).

One of the markers used to screen the YAC library detects a family of three to eight physically linked genes including the nematode resistance allele *Gro1* (Leister et al. 1996). One 270-kb YAC clone was isolated which harbours one member of this gene family. The fact that this gene copy is the only one present on the YAC indicates that it is either physically well separated from the other members of the same gene family or that the YAC clone represents one distal end of the gene cluster.

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