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Construction of a bacterial artificial chromosome library, determination of genome size, and characterization of an *Hsp70* gene family in *Phytophthora nicotianae*

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Abstract

The oomycete plant pathogen *Phytophthora nicotianae* causes diseases on a wide range of plant species. To facilitate isolation and functional characterization of pathogenicity genes, we have constructed a large-insert bacterial artificial chromosome (BAC) library using nuclear DNA from *P. nicotianae* H1111. The library contains 10,752 clones with an average insert size of 90 kb and is free of mitochondrial DNA. The quality of the library was verified by hybridization with 37 genes, all of which resulted in the identification of multiple positive clones. The library is estimated to be 10.6 haploid genome equivalents based on hybridization of 23 single-copy genes and the genome size of *P. nicotianae* was estimated to be 95.5 Mb. Hybridization with a nuclear repetitive DNA probe revealed that 4.4% of clones in the library contained 28S rDNA. Hybridization of total genomic DNA to the library indicated that at least 39% of the BAC library contains repetitive DNA sequences. A BAC pooling strategy was developed for efficient library screening. The library was used to identify and characterize BAC clones containing an *Hsp70* gene family whose four members were identified to be clustered within ~18 kb in the *P. nicotianae* genome based on the physical mapping of eight BACs spanning a genomic region of ~186 kb. The BAC library created provides an invaluable resource for the isolation of *P. nicotianae* genes and for comparative genomics studies.

Index descriptors: Phytophthora nicotianae; Oomycete; BAC library; Genome size; Heat shock protein 70; Tobacco black shank

1. Introduction

The availability of large-insert genomic DNA libraries is essential for physical analysis of large chromosomal regions, map-based gene isolation, and gene structure and function analysis. Two commonly used vector systems to clone large DNA fragments (>100 kb) are yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs). The latter vector, in particularly, has been preferred for library construction because of its capacity to maintain large DNA fragments (up to 300 kb), easy handling and, most importantly, its stability (Shizuya et al., 1992). BAC libraries are particularly useful for cloning of genes of unknown sequences such as avirulence genes by chromosome walking and have been successfully established for several oomycete species including *Phytophthora infestans* (Randall and Judelson, 1999; Whisson et al., 2001), *Phytophthora sojae* (F. Arrendondo and B.M. Tyler, personal communication), and *Peronospora parasitica* (Rehmany et al., 2003). BAC libraries are also useful resources for genome analysis.

Phytophthora species represent a large group of important oomycete plant pathogens that are phylogenetically distinct from true fungi. Species in this genus are very diverse in terms of their epidemiology, genomics, and manner of interacting with host plants (Cooke et al., 2000). Unlike the model species *P. infestans* and *P. sojae* that have few host species (Judelson, 1997; Kamoun, 2003; Tyler, 2002), *Phytophthora nicotianae* has a wide host range, capable of infecting over 72 plant genera (Hickman, 1958), yet it interacts with tobacco in a gene-for-gene manner (Perrone et al., 2000).

We are interested in the developmental regulation of asexual reproduction and the molecular basis of host

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recognition and infection of *P. nicotianae*. To facilitate genome analysis, physical mapping, and gene cloning in *P. nicotianae*, we present here the creation of a large-insert BAC library using nuclear DNA from our model strain H1111, and the development of an efficient procedure for library screening. The high quality of the library was confirmed by hybridization of numerous expressed sequence tag (EST) clones, all of which resulted in the identification of multiple positive clones. Characterization of the library also allowed the genome size of *P. nicotianae* to be estimated. We used the library to isolate and characterize a small gene family encoding Hsp70 proteins, a class of proteins playing important roles in protein folding, transport, and complex formation, and many other cellular processes essential for normal cell function. We demonstrate the usefulness of the BAC library and the library screening method in the isolation of allele-specific genomic clones by analyzing in detail the genomic organization of the *P. nicotianae Hsp70* gene family.

2. Materials and methods

2.1. Phytophthora nicotianae isolate and culture conditions

Phytophthora nicotianae isolate H1111 (ATCC MYA-141) was originally isolated from tobacco plants in Queensland, Australia. Under appropriate conditions, it produces large numbers of zoospores and has been used as a model strain in our laboratory.

The *P. nicotianae* culture was routinely maintained on 10% (v/v) cleared V8 juice agar (1.5%, w/v) supplemented with 0.002% (w/v) β-sitosterol and 0.01% (w/v) $CaCO_3$. For preparation of mycelia for DNA isolation, half of a V8 agar plate fully covered with *P. nicotianae* was cut into five pieces and placed in a 2 L flask containing 300 ml of 10% (v/v) cleared V8 juice broth and incubated with shaking at 150 rpm for 5 days before blending the whole liquid culture with a blender followed by addition of 500 ml of 10% cleared V8 broth and continued incubation for 2 more days with shaking at 22.5 °C. The mycelia were collected by filtering through one layer of miracloth, frozen in liquid nitrogen and stored at −80 °C for later use.

2.2. DNA isolation and BAC library construction

For the isolation of total genomic DNA for Southern analysis, *P. nicotianae* H1111 mycelia were ground into a fine powder in liquid nitrogen in a mortar before dispersing into DNA extraction buffer (Dudler, 1990). For Southern analysis, 2 μg of total DNA was digested with appropriate restriction enzyme(s), resolved in 0.8% agarose, and transferred to Hybond-N^+ nylon membranes (Amersham–Pharmacia) using 0.4 N NaOH as the transfer agent following standard procedures (Sambrook et al., 1989).

For isolation of megabases of chromosomal DNA for BAC library construction, *P. nicotianae* nuclei were isolated from mycelia following a procedure essentially the same as that of Wang et al. (1995). The purified nuclei (2.2 ml) from 15.8 g of mycelia were mixed with 3 ml of low melting point agarose in H_2O and 0.5 ml of proteinase K (10 mg/ml). The plugs were soaked in ESP solution (0.5 M EDTA, 1% *N*-lauroylsarcosine, 5 mg/ml proteinase K, pH 9.5) and incubated at 50 °C for 15 h. The plugs were placed in fresh ESP solution and incubated at 50 °C for a further 2 days before being stored at 4 °C in 50 mM EDTA (pH 9.5). Before partial digestion, each of the resulting agarose plugs (200 μl, contain approximately 10 μg of DNA) was cut into two pieces and treated twice with 900 μl TE (10 mM Tris–HCl, 1 mM EDTA, pH 9) for 1 h each and one time with 900 μl weak TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.6) supplemented with 2 mM PMSF (phenylmethyl sulphonyl fluoride) for 1 h, followed by soaking for 1 h in 1.1× *Hin*dIII buffer (Buffer 2, New England Biolabs) supplemented with 2 mM spermidine (Sigma) and 1× BSA, all at 50 °C. The soaking solution was replaced with 200 μl of 1× *Hin*dIII buffer supplemented with 2 mM spermidine and 1× BSA (100 μg/ml), bringing the total volume to 300 μl, and placed on ice for 5 min. Serial *Hin*dIII partial digestions were performed to optimize the condition for enriching fragments in the size range of 100–300 kb. Under our experimental conditions, the best partial digestion was achieved by addition of 12 U of *Hin*dIII and equilibration on ice for 2 h before performing digestion in a water bath at 37 °C for 1 h. The digestion was terminated by adding 35 μl of 0.5 M EDTA (pH 9). The partially digested nuclear DNA was separated by running a two-step PFGE (pulsed-field gel electrophoresis) gel in 0.5× TAE buffer using the Pulsaphor system (Pharmacia) following the method of Zhu et al. (1997) and the agarose gel containing fragments in the size range of 100–300 kb was cut and placed in a dialysis bag before electroeluting the DNA in modified 0.5× TAE buffer using standard methods (Zhu et al., 1997). The eluted DNA was directly used for ligation.

The BAC vector pBeloBACII was propagated in *Escherichia coli* strain Electromax DH10B (Invitrogen, San Diego, USA) and isolated by the standard method of alkaline lysis followed by purification by CsCl gradient centrifugation. The vector was linearized by *Hin*dIII and dephosphorylated to reduce background self-ligations with HK phosphatase (Epicentre, Madison, WI, USA) following manufacturer's recommended procedure.

To perform the ligation, 150 μl of electroeluted DNA was mixed with 50 ng of linearized vector DNA in 200 μl volumes with 0.9× T_4 ligase buffer and 25 U of T_4 ligase

(Promega, Madison, WI, USA) and incubated at 4 °C overnight. Ten microliters of ligation mixture were mixed with 150 μl of Electromax *E. coli* DH10B cells and electroporation was done in a 0.2 cm gap cuvette at 2.5 kV with resistance of 200 Ω and capacitance of 25 μF using a Gene Pulser (Bio-Rad, CA, USA). The transformation mix was transferred into 6 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7) and incubated at 37 °C for 1 h with shaking at 120 rpm before plating onto five large LB plates supplemented with 12.5 μg/ml chloramphenicol, 80 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and 0.005% (w/v) X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). The plates were incubated for 24 h at 37 °C and kept overnight at 4 °C before picking recombinant white colonies into 384-well microtitre plates in which each well was filled with 90 μl freezing medium (LB broth with 35 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 5.8 mM $(NH4)_2SO_4$, and 4.4% (v/v) glycerol) supplemented with 12.5 μg/ml chloramphenicol. The microtitre plates were incubated at 37 °C for 24 h and the entire library was duplicated into microtitre plates using a 384-pin replicator. Following incubation, the plates were sealed and stored at −80 °C.

2.3. Creation of BAC colony filters

To make BAC colony filters, the whole BAC library arrayed in twenty-eight 384-well microtitre plates was replicated onto seven microtitre plate-sized Hybond-N^+ nylon membranes each containing 1536 clones, by offsetting replication from four microtitre plates. Clones from four 384-well microtitre plates (Nunc, USA) were replicated by hand onto single Hybond-N^+ nylon membranes (Amersham–Pharmacia) using a 384-pin replicator (VP scientific, San Diego, USA) following manufacturer's instructions. Prior to colony transfer, the nylon membranes were pre-wetted on LB agar (1% bacto-tryptone, 0.5% bacto-yeast extract, 10 g NaCl per liter, 1.5% agar, pH 7) plates supplemented with 12.5 μg/ml chloramphenicol. The membranes were incubated colony side up on the LB plates at 37 °C for about 20 h or until the colonies reached the appropriate sizes. The membranes were treated sequentially with solutions to lyse the cells, then release and fix the plasmid DNA on the membrane surface, following the procedure described by Wang et al. (1995).

2.4. EST clones for library hybridization

All DNA probes used in this study were part of EST clones identified by a *P. nicotianae* EST project (W. Shan and A.R. Hardham, unpublished data). DNA probes used for *P. nicotianae* genome size determination were verified by genomic Southern blot analysis. Hybridization condition was the same as BAC library screening as described in Section 2.5.

2.5. BAC library screening

To make BAC pool DNA, BAC clones from each microtitre plate were replicated into individual 75 ml LB broth cultures before adding LB broth to 375 ml supplemented with 12.5 μg/ml chloramphenicol. The culture was shaken at 250 rpm at 37 °C for 16 h. The plasmid DNA from each 375 ml culture forms BAC sub-pool DNA and was isolated using Qiagen Midiprep plasmid isolation kit (Qiagen GmbH, Germany) following manufacturer's protocol with minor modifications in the amount of extraction buffers used in which 20 ml P1, 20 ml P2, and 20 ml P3 solution was used instead of 4 ml each as recommended for midi plasmid preparations. This BAC sub-pool DNA was finally dissolved in 150 μl H_2O. Each BAC pool DNA was made by combining BAC DNA from four sub-pools, which correspond to BAC clones from four microtitre plates.

The 375 ml cultures typically yielded 40–50 μg of BAC DNA, which is sufficient for making 150 blots. About 1.2 μg of BAC pool DNA were digested to completion with 10 U of *Hin*dIII (New England Biolabs, USA), separated in 1% agarose gels in 1× TAE buffer at 2.0 V/cm for 3–4 h and transferred to Hybond-N^+ nylon membranes using 0.4 N NaOH as transfer agent as described (Sambrook et al., 1989). Gel-purified DNA fragments were used to make radiolabel probes using the Megaprime kit (Amersham–Pharmacia). Hybridizations were done at 65 °C for 16 h using either 6× SSPE or 6× SSC with 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll 400, 0.02% PVP, and 0.02% BSA) and 0.1% SDS as hybridization buffer as described (Sambrook et al., 1989). Following hybridization, the membranes were washed at 65 °C twice in 2× SSC and 0.1% SDS each 15 min, and twice in 0.2× SSC and 0.1% SDS each 15 min.

2.6. Characterization of Pn-Hsp70-hybridizing BACs by PCR

To analyze genomic organization of the *Pn-Hsp70* gene family members, eight out of 10 BAC clones hybridizing to the *Pn-Hsp70* EST clone WS187 were subjected to detailed analysis. T7 end sequencing was done for BACs 1D19, 2M8, 2O3, 22I13, and 26B22, and SP6 end sequencing was done for the BAC 7K23. Direct BAC end sequencing was done at the Australian Genome Research Facility (Brisbane, Queensland), using 1 μg BAC DNA purified by Qiagen Midi Plasmid Kit as described above. These BACs appeared to overlap across the *Pn-Hsp70* locus, based on restriction and Southern blot analysis. Gene specific primers, 38F (CCTGTACGTTCAGAAGTATGTG, derived from

the 1D19 end sequence), 41F (CGTTGCAAAC CAAGGATTCC, derived from the 7K23 end sequence), 41R (CGGTTGTGCTAGATAGTTTACG, derived from the 7K23 end sequence), 44F (CAAGGTCTT GGAGTAGCAAG, derived from the 22I13 end sequence), and 44R (ACGGCAGTTCAAGACAATGC, derived from the 22I13 end sequence), were designed from these BAC end sequences and the *Pn-Hsp70a* coding region (P1, CCCTTGAACTGCACCGTGAT, and P4a, CGTCTATGGTGCTGATCAAG). Various primer combinations were used to analyze the eight WS187-hybridizing BACs by PCR. For PCR amplification, standard (VentR DNA polymerase, New England Biolabs, USA) or hotstart (JumpStart *Taq* DNA polymerase, Sigma–Aldrich, Germany) conditions were used following manufacturer's recommendation with minor modifications in which 5 ng BAC DNA was used, $MgCl_2$ was supplemented to 2 mM final concentration, and dNTP mix was used at 200 μM final concentration. PCR amplification was done in a PTC-200 Peltier Thermal Cycler (MJ Research, Finland) using the following conditions: initial denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 56–60 °C for 40 s, and 72 °C for 1–3.5 min, and a final 5 min extension at 72 °C.

2.7. BAC subcloning

BAC DNA isolated from 1.5 ml overnight cultures was digested to completion with appropriate restriction enzymes, separated in 0.8% agarose, gel-purified for the target fragments using an Ultraclean 15 DNA purification kit (Mobio, CA, USA). The purified fragment was ligated with the corresponding enzyme-linearized pBluescript KS following standard procedures (Sambrook et al., 1989) and transformed into *E. coli* DH10B cells by electroporation.

3. Results

3.1. Library construction

To facilitate characterization of selected candidate genes, we used our model *P. nicotianae* isolate H1111 to construct a large-insert genomic bacterial artificial chromosome (BAC) library. Chromosomal DNA from *P. nicotianae* H1111 (distinct bands in excess of 1 Mb, data not shown) was isolated from nuclei embedded in agarose and partially digested by *Hin*dIII. The resulting fragments in the range of 100–300 kb were ligated with pBeloBACII (Kim et al., 1996) and transformed into *E. coli* DH10B cells. Based on the ethidium bromide-stained PFGE gels, 12 U of *Hin*dIII per 200 mg agarose plug produced the highest amount of DNA in the 100–300 kb size range when the partial digestion was done at 37 °C for 1 h in 300 μl volume. The library was constructed with double size selection of partially digested DNA according to Zhu et al. (1997). Pilot analysis of over 20 randomly selected clones was performed for each ligation reaction before large-scale selection of recombinant BAC clones. A total of 10,752 clones was finally picked and arrayed in twenty-eight 384-well microtitre plates, duplicated and stored at −80 °C.

3.2. Library characterization

To determine the size distribution of BAC clones, a random sample of five to eight clones per plate (200 clones in total) was analyzed by restriction digestion with *Not*I and PFGE analysis (Fig. 1) and digestion with *Hin*dIII followed by standard gel electrophoresis. This analysis indicated that the average insert size of the BAC library was 90 kb (Fig. 2). Eight percent of BAC clones carried small inserts (<20 kb) or no insert at all. Only 1.5% of clones carried 20–40 kb inserts. The remaining BAC clones contained inserts in the range of 40 kb to over 120 kb. The majority of the clones (65.5%) carried inserts of 60–120 kb. Contamination of size selected large fragments with small ones was observed in many reported BAC libraries and has been a common problem for BAC library construction (Zhu et al., 1997; Zhang and Wu, 2001). The relatively small portion of small clones (8%) in the BAC library constructed in the present study further confirmed the efficient double size selection procedure developed by Zhu et al. (1997).

To determine possible contamination by organellar DNA, the whole library was hybridized with WS359 and WS364 probes, both containing cloned *P. nicotianae* DNA showing homology to *P. infestans* mitochondrial DNA (Paquin et al., 1997). The mitochondrial DNA probes were identified in an EST project (W. Shan and A.R. Hardham, unpublished results) and consisted of fragments separated by 5 kb on the ~38 kb mitochondrial genome (Paquin et al., 1997). Using these two

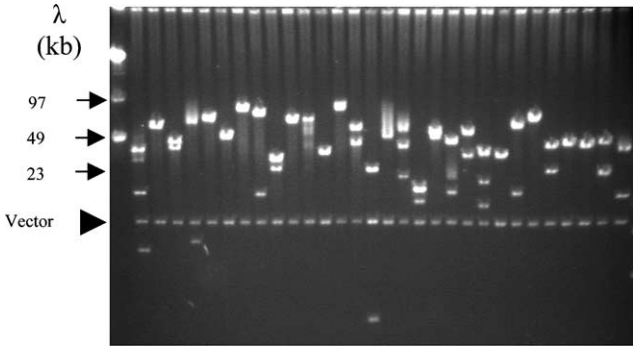

Fig. 1. Analysis of 30 randomly selected *P. nicotianae* BAC clones. BAC DNA was digested to completion with *Not*I and separated by pulsed-field gel electrophoresis (PFGE). The λ size marker and vector band are indicated by arrows.

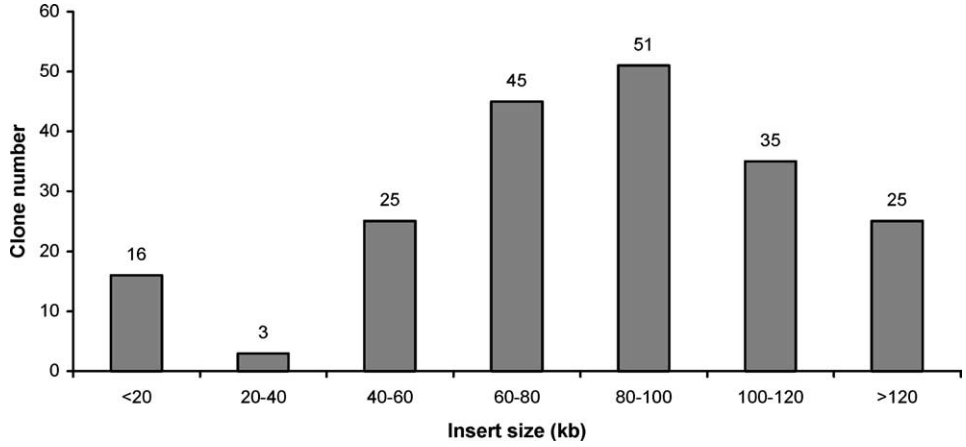

Fig. 2. Insert size distribution of randomly selected BAC clones in the *P. nicotianae* BAC library. The sizes were determined by insert sizes released from 200 randomly selected BAC clones by *Not*I (PFGE analysis) and *Hin*dIII.

probes, only one probe gave a positive result and it reacted with only one clone, representing <0.01% of the library. Since these two probes span about 32% of the *Phytophthora* mitochondrial genome, as determined by Southern blot analysis and their physical distance, it is likely that less than 4 of the 10,752 BAC clones carry mitochondrial DNA. This demonstrated that the nucleus isolation and subsequent size selection of partially digested DNA were successful in excluding the small mitochondrial DNA molecules.

The BAC library was screened with an EST sequence, WS210, carrying putative *P. nicotianae* 28S rDNA identified from our EST project and matching the 28S rRNA gene of *Phytophthora megasperma* (Van der Auwera et al., 1994). The WS210 probe hybridized with 134 of the 3072 BAC clones tested, indicating that the ribosomal DNA was present in 4.4% of the clones in the library. As the 28S ribosomal DNA is dispersed on many chromosomes in higher organisms, its high-frequency representation in the library (4.4% versus the estimated 0.1% frequency for a single copy DNA element) suggests that it is likely to be present on multiple chromosomes in *P. nicotianae* as well.

To estimate the content of repetitive sequences, the same 3072 BAC clones screened with the 28S rDNA probe were hybridized under stringent conditions with a total genomic DNA probe from *P. nicotianae*. At least 39% of clones gave detectable signals after a 40-min exposure and are thus likely to contain repetitive sequences (Fig. 3). While at least 12% of these clones contained highly repetitive sequences as predicted from their intense signals, at least 26% of the clones with intermediate signal intensities apparently contain moderately repetitive DNA elements. At least 36% of the clones identified as containing highly repetitive sequence cross hybridized with the 28S rDNA probe. The majority of the BAC library clones contain single or low copy number sequences.

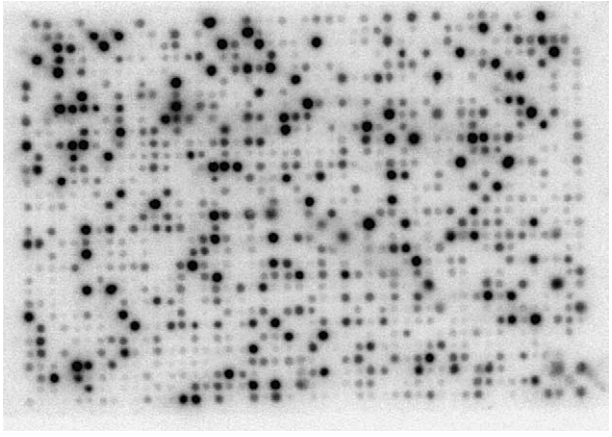

Fig. 3. Colony hybridization of BAC clones to total genomic DNA probe of *P. nicotianae*. Total genomic DNA of *P. nicotianae* H1111 was radiolabeled and hybridized with a BAC colony filter containing 1536 clones.

3.3. Library screening

We developed an efficient BAC pooling procedure for BAC library screening and initial characterization of selected genes (Fig. 4). In brief, the whole library was divided into seven pools each consisting of BAC clones from four microtitre plates. BAC pool DNA and corresponding BAC colony filters were prepared as described in Section 2. The DNA from the seven BAC pools was digested to completion with *Hin*dIII to release all inserts and loaded in parallel to *P. nicotianae* H1111 genomic DNA cleaved to completion with restriction enzymes including *Hin*dIII. This allows identification of BAC pools containing fragments corresponding to those of total *P. nicotianae* genomic DNA. The whole library can thus be loaded onto a 5×8 cm nylon membrane. In our method, multiple blots containing the whole BAC library can be easily prepared and used simultaneously

for hybridization of multiple probes. The second round hybridization is directed to the identification of individual BAC clones from positive BAC pools. In this way, we identified positive BAC clones corresponding to all 37 EST clones screened. *P. nicotianae* is a heterothallic oomycete. This suggests that a substantial amount of heterozygosity may be present across its chromosomes. Indeed, different BAC clones carrying both gene alleles were identified for a number of genes (examples included in Fig. 5). By restriction analysis of large-insert BAC clones, allelic relationships of target genes can easily be verified for organisms such as *P. nicotianae* that have relatively small genome sizes. The efficiency of the library screening procedure for the isolation of allele-specific genomic DNA was confirmed for a number of genes derived from our EST project (data not shown). Most genes analyzed so far were single or low copy number genes, based on Southern blot analysis of *Eco*RI, *Hin*dIII, and *Bam*HI restricted genomic DNA and positive BAC clones.

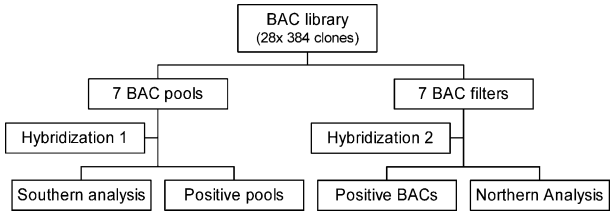

Fig. 4. A strategy for BAC library screening and gene characterization. The whole library is divided into seven pools each of which contains 1536 BAC clones from four microtitre plates, and which are also replicated onto a single microtitre plate-sized nylon membrane to form corresponding BAC colony filters. The BAC pool DNA, as well as *P. nicotianae* genomic DNA, was digested to completion with *Hin*dIII, separated in standard agarose gel electrophoresis, and blotted onto nylon membranes for Southern analysis. This enables identification of BAC pools carrying targeted genomic DNA corresponding to fragments identified in the genomic Southern blots. Genomic DNA cut with other restriction enzymes can be included to assess copy number of the target gene. Second-round hybridization identifies individual positive BAC clones. The relatively low level of template DNA on BAC filters allows Northern blots to be included in the same bottles used for second-round hybridization to analyze the transcriptional levels of the target gene.

3.4. Analysis of genome representation

The estimation of nuclear genome representation was carried out by hybridization of 23 single-copy EST probes identified from our *P. nicotianae* EST project. The single-copy nature of these EST clones was verified by Southern blot analysis of *P. nicotianae* genomic DNA digested by *Eco*RI, *Bam*HI, and *Hin*dIII. Restriction fragment length polymorphisms were observed for a number of gene loci in *P. nicotianae* (as exemplified in Fig. 5), based on the detailed analysis of individual

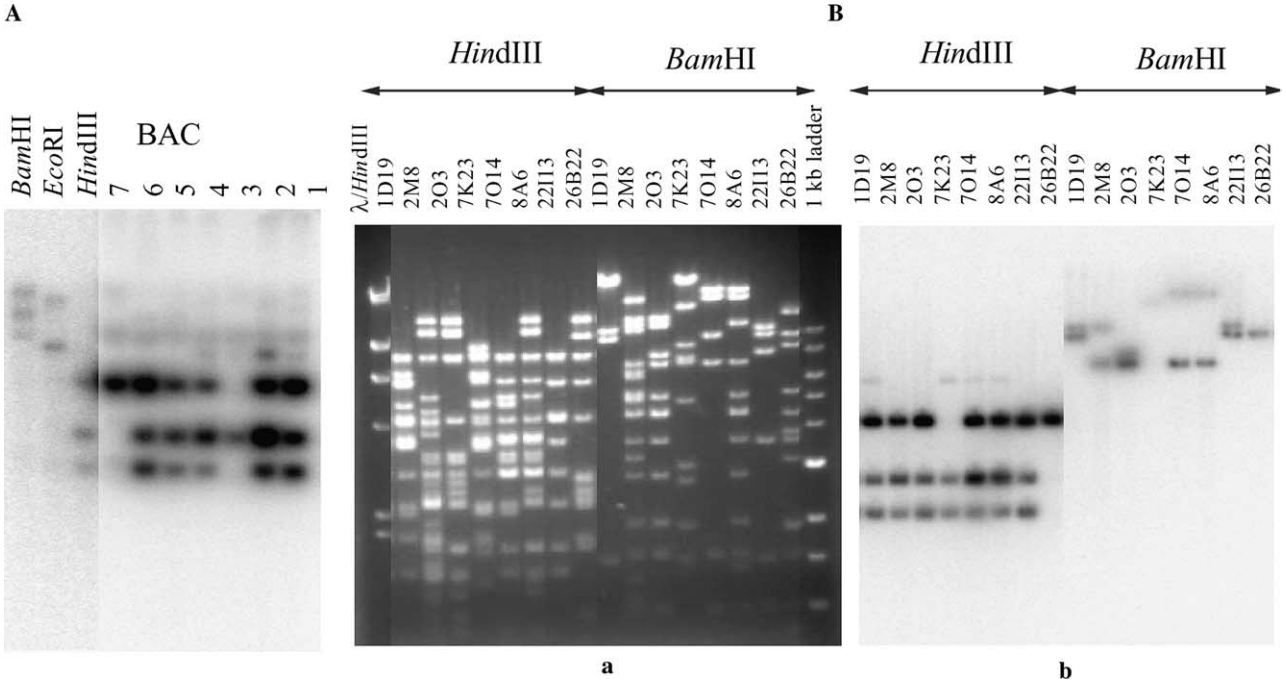

Fig. 5. Isolation and characterization of a *P. nicotianae Hsp70* gene family. (A) A BAC pooling strategy was used to identify BAC clones hybridizing to WS187, an EST homologous to *Hsp70* genes in the databases. *P. nicotianae* genomic DNA has been cut with *Hin*dIII, *Bam*HI, and *Eco*RI; lanes 1–7 are BAC pool DNA from seven samples each representing clones from four microtitre plates and digested with *Hin*dIII. (B) Eight individual positive BAC clones digested with *Hin*dIII and *Bam*HI (a) and hybridized with the WS187 probe (b). Note that BACs 1D19, 7O14, and 8A6 contain all the *Bam*HI hybridizing bands in the genomic Southern blot (A) whereas the other BACs only partially span the *Pn-Hsp70* locus, based on the *Bam*HI digestion pattern (b), BAC end-sequencing and PCR analysis (not shown).

positive BAC clones for a number of EST clones (unpublished data). This is consistent with the fact that *P. nicotianae* is a heterothallic oomycete and suggests the possible presence of a substantial amount of genetic polymorphism across its chromosomes. Thus all EST clones giving one or two hybridizing bands were regarded as single-copy genes. The results of hybridization are shown in Table 1. The 23 EST clones used to hybridize to the BAC pool DNA all identified 2–6 positive pools. On average, each single-copy probe hybridized with DNA in four BAC pools. Two colony BAC filters corresponding to positive BAC pools were hybridized with the 23 single-copy genes, and 2–10 positive BAC clones were detected for each probe. At least two BAC clones were identified in all cases, with an average number of 2.3 clones per positive BAC pool for each of the 23 probes characterized (Table 1). In our identification of individual clones for a number of genes from positive BAC pools, we observed that for a given probe the number of positive clones from each positive pool does not vary significantly. We therefore estimated the total number of positive clones based on the colony hybridization data from two membranes (pools). In this way, we estimated a 10.13-fold coverage of the haploid genome of *P. nicotianae* by the BAC library.

Based on the average insert size of 90 kb of the library and subtraction of the clones carrying mitochondrial DNA, the library is calculated to cover a total of 968 Mb of *P. nicotianae* nuclear DNA sequences. Dividing this value by the 10.13-fold genome coverage estimated from the hybridization data of the 23 single-copy genes, the haploid genome size of *P. nicotianae* was estimated to be 95.5 Mb.

3.5. Isolation and characterization of an Hsp70 gene family

WS187, an EST homologous to the *Hsp70* gene of *Bremia lactucae* (Judelson and Michelmore, 1989), an obligate oomycete plant pathogen, was identified in our EST project and was used to screen the BAC library using the developed BAC pooling procedure. This 2236 bp EST was sequenced by primer walking and putatively encodes a polypeptide with 657 residues (GenBank Accession No. CF891694). All seven BAC pools hybridized with the 2.3 kb EST clone and five contained BAC clones carrying *Hin*dIII fragments of sizes corresponding to all three *Hin*dIII genomic DNA fragments. BAC filters corresponding to BAC pools 1, 2, 6, and 7 (Fig. 5) were subjected to a second round of hybridization to locate individual positive BAC clones. This led to the identification of 10 BAC clones. Restriction and Southern blot analysis of eight randomly selected positive clones revealed that these contained 1–3

Table 1
Hybridization of *P. nicotianae* BAC library with 23 single-copy EST clones

EST clone	Gene homology	GenBank Accession Nos.	BAC pools[a]	BAC clones[b]	Total[c]
WS009	Zinc finger protein	CF891671	4	2	4
WS015	Zinc finger protein	CF891672	6	10	30
WS016	50 kDa glycoprotein elicitor	CF891673	3	3	4.5
WS017	Unknown	CF891674	3	4	6
WS021	S-phase-specific protein	CF891675	5	5	12.5
WS024	G-protein β-subunit	CF891676	6	4	12
WS041	Unknown	CF891677	5	5	12.5
WS043	Unknown	CF891678	5	6	15
WS056	Translation elongation factor 2	CF891679	4	4	8
WS057	Unknown	CF891680	2	2	8
WS064	Cyclophilin	CF891681	4	8	16
WS066	Plasma membrane H^+-ATPase	CF891682	4	7	14
WS084	Unknown	CF891683	3	6	9
WS100	Unknown	CF891684	5	4	10
WS105	Unknown	CF891685	5	4	10
WS151	Annexin	CF891686	5	3	7.5
WS192	Translation elongation factor 3	CF891687	5	5	12.5
WS196	Unknown	CF891688	2	5	5
WS220	Cyclophilin	CF891689	4	4	8
WS225	Phosphate transporter protein G7	CF891690	2	2	2
WS227	Phosphoglyceromutase 1	CF891691	2	4	4
WS235	ADP/ATP carrier protein T1	CF891692	5	5	12.5
JM310	Unknown	CF891693	5	4	10
Average			4.1	—	10.13

[a] Number of positive BAC pools/filters.
[b] Number of positive BAC clones from two randomly selected positive BAC pools/filters.
[c] Total number of positive BAC clones hybridizing to each single-copy probe as estimated by (a) and (b).

hybridizing *Hin*dIII fragments (4.2, 2.8, and 2.3 kb), indicating that the hybridizing fragments were physically linked (Fig. 5). Since there was no *Hin*dIII site in the WS187 clone, it is likely that the cloned *Hsp70* gene family consists of at least three members in the haploid genome of *P. nicotianae*.

Detailed physical mapping indicated that all BAC clones analyzed overlap with each other as revealed by restriction analysis with *Eco*RI (not shown), *Hin*dIII (Fig. 5), *Bam*HI (Fig. 5), PCR analysis (not shown), and DNA sequencing. The eight positive BACs cover a genomic region of ~186 kb and contain four physically linked *Pn-Hsp70* genes (Fig. 6). Based on restriction analysis and hybridization signal intensities, two different alleles in the *Hsp70* locus can be further differentiated among the overlapping BACs (Fig. 6). This was based on restriction patterns generated by *Bam*HI. BACs 1D19 and 22I13 contain two *Bam*HI fragments (9 and 10 kb, respectively) that correspond to the doublet *Bam*HI bands in the genomic Southern blot (Fig. 5A) and were designated to represent allele 1. Detailed analysis by BAC end-sequencing and PCR indicated that BAC 22I13 partially spans the *Pn-Hsp70* locus. BAC end sequencing and PCR analysis were also used to confirm that BACs 7K23 and 26B22 contain allele 1 sequences but they do not overlap and both only partially cover the *Pn-Hsp70* locus. BACs 7O14 and 8A6 contain ~6 and ~23 kb *Bam*HI fragments which correspond to the largest and smallest *Bam*HI bands in the genomic Southern blot (Fig. 5A) and were designated to represent allele 2. Similarly, BACs 2O3 and 2M8 contain allele 2 sequences and partially span the *Pn-Hsp70* locus. The physical mapping result was further confirmed by *Eco*RI restriction analysis, DNA sequencing, and PCR.

A 2.2 kb *Eco*RI or ~6 kb *Hin*dIII fragment that hybridized weakly with WS187 was present in genomic Southern blots and was identified in BACs 1D19, 7O14, and 8A6. Repeated experiments excluded possible partial digestion and indicated that it was physically located ~15 kb apart from the main four-member cluster.

All three hybridizing *Hin*dIII fragments from BAC 22I13 (representing allele 1) and one *Bam*HI fragment from BAC 7O14 (representing allele 2) were cloned and the recombinant clones were characterized by PCR analysis and partially sequenced. Partial alignment of N-terminal sequences indicated that the largest hybridizing 4.2 kb *Hin*dIII fragment (*Pn-Hsp70a*, GenBank Accession No. AY456093) shared ~90% nucleotide sequence identity with the other *Hin*dIII hybridizing fragments, *Pn-Hsp70b* (2.8 kb, GenBank Accession No. AY456094) and *Pn-Hsp70c* (2.4 kb, GenBank Accession No. AY456095). *Pn-Hsp70b* and *Pn-Hsp70c* share 99% sequence identity over the coding region. The fourth member *Pn-Hsp70d* co-migrates with *Pn-Hsp70b* when digested with *Hin*dIII. All four members in this gene family are clustered in an ~18 kb genomic region (Fig. 6). The 4.2 *Hin*dIII fragment contained the functional *Hsp70* gene that transcribed WS187. It was

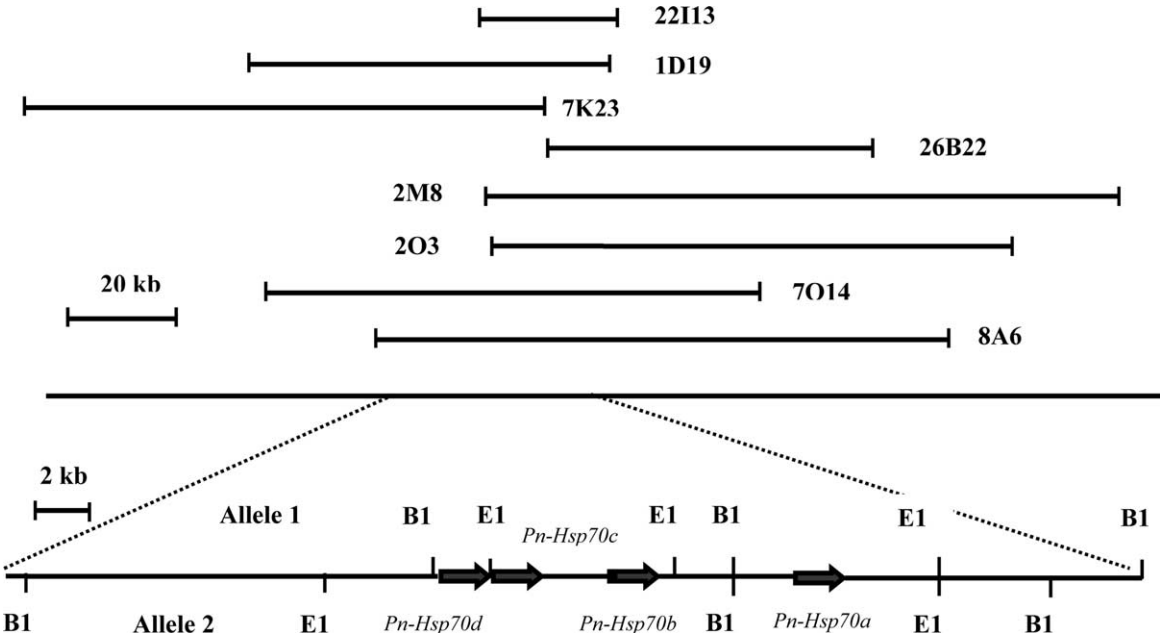

Fig. 6. Physical organization of the *Pn-Hsp70* gene locus determined by DNA sequencing and PCR, restriction and Southern blot analysis. Based on the restriction mapping and hybridization signal intensities, the eight positive BAC clones clearly carry two different alleles of the *Hsp70* locus, with BACs 1D19, 7K23, 22I13, and 26B22 carrying allele1 and BACs 2M8, 2O3, 7O14, and 8A6 carrying allele 2 sequences. Transcription directions for *Pn-Hsp70* genes (*a–d*) are shown by arrows. *Eco*RI (E1) and *Bam*HI (B1) restriction sites in allele 1 and allele 2 are shown above and below the *Pn-Hsp70* cluster diagram, respectively.

sequenced by primer walking and contains a continuous open reading frame of 1971 nucleotides encoding a polypeptide of 657 residues. Both of the predicted Pn-Hsp70b and Pn-Hsp70c proteins have 655 residues and differ in only three residues. The predicted Pn-Hsp70a and Pn-Hsp70b proteins share 92.7% sequence identity. Partial sequence alignment indicated that the *Bam*HI fragment cloned from BAC 7O14, which represents allele 2, contains sequences allelic to *Pn-Hsp70a* (not shown). The *Bam*HI site is also present in the *Hin*dIII fragment containing *Pn-Hsp70a* (Fig. 6).

Blast searches against the PGC (https://xgi.ncgr.org/pgc/) and JGI (http://genome.jgi-psf.org/physo00) databases identified homologues in both *P. sojae* and *P. infestans*. Partial sequence alignment indicated that the *Pn-Hsp70a* gene shares at least 91% sequence identity at the nucleotide sequence level and at least 95% sequence identity at the predicted protein sequence level to both *P. sojae* and *P. infestans* homologues.

Probing of RNA blot with WS187 (representing *Pn-Hsp70a*) showed that the *Pn-Hsp70* genes were expressed in three examined asexual developmental stages such as vegetative hyphae, motile zoospores, and germinating cysts, but significantly downregulated during asexual sporulation (Fig. 7). However, no attempt was directed to differentiate mRNA of *Pn-Hsp70a* from that of *Pn-Hsp70b*, *Pn-Hsp70c*, and *Pn-Hsp70d*. *Pn-Hsp70b* and *Pn-Hsp70c* share ~90% nucleotide sequence identities with *Pn-Hsp70a* and both contain complete Hsp70 open reading frames. They also share the same translation consensus sequence as *Pn-Hsp70a* but are very dissimilar in their promoter regions to *Pn-Hsp70a*, suggesting that they may respond and function in different developmental or environmental conditions.

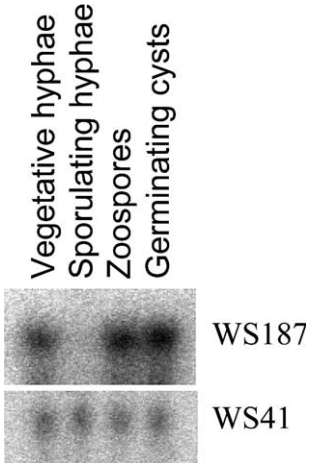

Fig. 7. Northern blot analysis of *Pn-Hsp70* genes. Each lane contains 10 μg total RNA and the blot was sequentially probed with WS187 (upper panel) and WS41 (lower panel), an EST constitutively expressed and encoding a protein with unknown function, which is used to show equal loading of RNA samples.

Subclones containing the *Pn-Hsp70d* sequence were not identified.

The deduced amino acid sequence of *Pn-Hsp70a* has a higher level of sequence identity with Hsp70 proteins of the oomycete species *B. lactucae* (91%) (Judelson and Michelmore, 1989) and *Achlya klebsiana* (91%) (LeJohn et al., 1994) than it does to those of any other organism (<77% sequence identities) including insects, eukaryotic microbial parasites, mammals, plants, and nematodes. In the C terminus, the deduced Pn-Hsp70a protein from *P. nicotianae* is missing a domain of ~21 residues compared with that of *B. lactucae*, and this is a major contributor to the smaller size of *P. nicotianae* Hsp70 protein compared to that from *B. lactucae*. This domain contains tandem repeats and is also missing in the *A. klebsiana* Hsp70 protein.

4. Discussion

Large-insert genomic libraries made with a single-copy, *F* factor-based plasmid (Shizuya et al., 1992) are a very valuable resource for positional cloning of genes of interest and for genomic studies, and have been constructed for a number of plant species (Zhang and Wu, 2001). By contrast, only a few BAC libraries have been constructed for fungal species: three BAC libraries have been reported for the rice blast fungus *Magnaporthe grisea* (Diaz-Perez et al., 1996; Nishimura et al., 1998; Zhu et al., 1997) and one for the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Pedersen et al., 2002). To facilitate positional cloning of avirulence genes and genome analysis, four BAC libraries have been established for three oomycete species including *P. infestans* (Randall and Judelson, 1999; Whisson et al., 2001), *P. sojae* (F. Arrendondo. and B.M. Tyler, personal communication), and *P. parasitica* (Rehmany et al., 2003).

To aid functional characterization of candidate genes, we constructed in this study a large-insert genomic BAC library using chromosomal DNA from our model *P. nicotianae* isolate H1111. The library contains 10,752 clones arrayed in 28 microtitre plates with an average insert of 90 kb and is essentially free of mitochondrial DNA. The quality of the BAC library was confirmed by hybridization with 37 EST clones, all of which resulted in the identification of multiple positive BAC clones.

Library characterization was done with several probes. Using total genomic DNA, relatively few clones hybridized strongly to the probe. Given the large-insert nature of the library and the reported 50–65% proportion of repeated DNA in the genomes of oomycete pathogens *B. lactucae* (Francis et al., 1990) and *P. sojae* (Mao and Tyler, 1991), this value might be indicative of a lower content of repeated DNA sequences in

P. nicotianae. However, because this analysis was conducted using high stringency hybridization and membrane washing conditions, this lower value for repeated DNA may reflect hybridization only to abundant and highly conserved DNA elements. The BAC library was estimated to be equivalent to 10.13-fold haploid genomes of *P. nicotianae*, based on hybridization results of 23 single-copy EST clones.

The genome size of *P. nicotianae* has not been previously reported. However, based on the size of the BAC library and the genomic representation of 23 selected single-copy genes in the library, the genome size of *P. nicotianae* was estimated to be 95.5 Mb. This is more similar to the genome size of *P. sojae* (Rutherford and Ward, 1985; Voglmayr and Greilhuber, 1998) than to that of *P. infestans* (Tooley and Therrien, 1987). Various methods have been used to estimate the genome size of fungal and oomycete species. These generally include various nuclear DNA staining methods, reassociation kinetics, genomic reconstruction, and direct CHEF (Contour-clamped homogeneous Electric Field) gel analysis of chromosome numbers and sizes. These methods, however, have led to inconsistent estimations of the genome size in some species, particularly those whose genomes are large and contain chromosomes that in CHEF gels are larger than the size markers and difficult to resolve. These include several *Phytophthora* species examined so far such as *P. parasitica* and *Phytophthora palmivora* (Tooley and Carras, 1992). Genome size estimation based on DNA reassociation kinetics data is particularly dependent on how repetitive DNA classes are modeled (Mao and Tyler, 1991).

The genome size of *P. sojae* was estimated to be 46.5 Mb from analysis of CHEF gel electrophoresis (Tooley and Carras, 1992), which was about 50% of the value estimated from nuclear DNA staining methods (Rutherford and Ward, 1985; Voglmayr and Greilhuber, 1998), and 75% of that estimated by using DNA reassociation kinetics and genomic reconstructions (Mao and Tyler, 1991). Very different genome size values (ranging between 50 and 115 Mb for isolates from the same plant species *Lactuca sativa*) were also obtained for *B. lactucae* using nuclear Feulgen staining and image analysis (Voglmayr and Greilhuber, 1998), reassociation kinetics and genomic reconstruction (Francis et al., 1990), and CHEF gel analysis (Francis and Michelmore, 1993). Voglmayr and Greilhuber (1998) obtained less variation in genome size estimations over a wide range of genome sizes using Feulgen staining and image analysis than achieved using a variety of other approaches. Our method for determining the *P. nicotianae* genome size was based on the number of times randomly selected, single-copy genes were represented in the size-defined large-insert genomic library. This provides an alternative approach for genome size determination. According to our results, *P. nicotianae* has a genome more similar in size to the 86–102 Mb of *P. sojae*, as determined by the nuclear DNA staining methods (Rutherford and Ward, 1985; Voglmayr and Greilhuber, 1998) than it has to that of 62 Mb, as determined by the reassociation kinetics method (Mao and Tyler, 1991). This value is much smaller than the genome size of *P. infestans*, which was determined to be 250 Mb by using the DAPI (4,6-diamidino-2-phenylindole) microfluorometry method (Tooley and Therrien, 1987).

The large-insert BAC library that was constructed in the present study is a useful genetic resource and will greatly facilitate the functional characterization of *P. nicotianae* genes identified in EST projects and particularly the cloning of genes with unpredictable sequences such as avirulence genes that govern recognition specificities in the pathogen against host plants. Avirulence genes have been cloned from a number of fungal plant pathogens (Laugé and De Wit, 1998) but, unlike their corresponding resistance genes in plants, they share no common features and cannot be cloned by homology-based approaches. Instead, a map-based gene cloning approach is ideal for such genes and for other genes with unknown sequence information and has led to the physical mapping across several avirulence gene loci in *P. infestans* (Whisson et al., 2001), *P. sojae* (MacGregor et al., 2002), and *Pe. parasitica* (Rehmany et al., 2003), and the cloning of the *Avr1b-1* of *P. sojae*, the first avirulence gene to be cloned from an oomycete (Shan et al., 2004). The BAC library will also aid the understanding of genome structure in *P. nicotianae* by comparative genomics with other oomycetes, notably *P. sojae* and *P. infestans* in which genetic maps have been created (May et al., 2002; Van der Lee et al., 1997; Whisson et al., 1995) and in which EST clones are being mapped onto BAC clones to create high density EST physical maps (Tyler, 2001). The usefulness of BAC libraries will be enhanced by the increasing genomics resources that are available for *Phytophthora* species including the impending completion of the *P. sojae* and *P. ramorum* draft sequences in the JGI (http://genome.jgi-psf.org/physo00) and the EST sequences in PGC (https://xgi.ncgr.org/pgc/).

We also developed an efficient procedure for rapid identification of genomic clones containing genes of interest and initial characterization of these genes in terms of their copy number and genomic organization. The BAC pool strategy described here was sensitive enough to identify single positive clones among BAC pools each containing 1536 BAC clones representing ~1.4× haploid genome equivalent and 14.3% of the library. In the hybridization of 23 EST clones with 46 BAC filters, in 12 cases we were able to identify single positive clones in individual BAC pools. In our method, we prepared BAC pool DNA from individual plates of 384 BAC clones for all 28 plates and combined four to form a BAC pool for library screening. This might contribute

to higher levels of efficiency detecting positive clones from BAC pools of four microtitre plates, and the practical limit to such detection could be 6–8 or even more 384-well plates. In the screening of a sorghum BAC library, Lin et al. (2000) routinely achieved identification of a single positive band from BAC pools made from a single plate of 384 BACs, and suggested the practical limit to reliably detect positives is about 3–4 384-well plates.

The large-insert nature of BAC libraries enables the initial analysis of candidate genes in terms of their copy number in the genome, genomic organization, and the allelic relationship of hybridizing fragments revealed in the Southern blot analysis of genomic DNA. The two-step approach, first screening the whole library divided into seven lanes to identify BAC pools containing positive clones, followed by re-screening of colony blots corresponding to positive pools to identify individual positive BAC clones, provides an efficient procedure for identification of allele-specific genomic sequences. Other approaches developed include a two-dimensional PCR screening strategy in which BAC DNA was pooled from rows and columns (Crooijmans et al., 2000), or hybridization-based screening of colony BAC filters of whole libraries. However, both these library-screening strategies are not efficient for identification of clones carrying locus-specific fragments. The procedure described in the present study will be particularly useful for physical mapping and isolation of individual members of multigene families in complicated genomes arising from gene and chromosome duplications.

The Hsp70 protein is a class of heat shock proteins serving a variety of functions and the encoding genes have been identified from diverse organisms, including two oomycetes, *B. lactucae* (Judelson and Michelmore, 1989) and *A. klebsiana* (LeJohn et al., 1994). In most organisms, Hsp70 proteins are encoded by multigene families (Guy and Li, 1998), as revealed by genomic Southern blot and PCR analysis. However, in most cases the genomic organization of these gene family members is not clear except for the *hsp70* genes in *Leishmania infantum* and *Leishmania major*. In *L. infantum*, at least six *hsp70* genes have been identified including five clustered in tandem and one in a different locus (Quijada et al., 1997). In *L. major*, five *hsp70* genes have been identified, including four clustered and one in a separate locus (Lee et al., 1988). We present here for the first time a detailed analysis of the genomic organization of the *Hsp70* gene family in the oomycete, *P. nicotianae*. In *P. nicotianae*, the *Pn-Hsp70* gene locus contains four highly homologous members clustered in a genomic region of ~18 kb. Sequence analysis indicated that the predicted Pn-Hsp70 proteins are more similar to those in other oomycete species than to yeast, higher fungi, and plant species, consisting with the notion that oomycetes are dissimilar to true fungi.

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