Bacterial Artificial Chromosome Library
3.1 Introduction

Positional cloning of genes is a method of gene isolation in which a gene for a specific phenotype is identified, with only its known approximate chromosomal location in the candidate region. Initially, the candidate region can be defined using techniques such as linkage analysis, and then positional cloning is used to narrow the candidate region until the gene and its mutations are found. It typically involves isolation of partially overlapping DNA segments from genomic libraries to progress along the chromosome towards a specific gene. For plants and other organisms with large genomes, identification of genes/loci by positional cloning typically requires the construction of a large insert genomic DNA library followed by cloning of the loci using tightly linked molecular markers. The predominant vector system for such work is the yeast artificial chromosome (YAC) and the bacterial artificial chromosome (BAC) system. Despite YACs having the ability to clone sizes up to 1MB of insert many problems have been detected including: chimerism, tedious steps in library construction and low yields of YAC insert DNA. Thus a new E.coli based system has been developed, the BAC system (Yu et al., 2000), which offers many potential advantages over YACs. BAC libraries have been exploited for the development of detailed genetic and physical maps of major crops and positional cloning of genes of interest. Because of their high stability, low chimerism, and easy DNA purification, large-insert BAC libraries, and especially plant-transformation competent binary BAC (BIBAC) libraries have emerged as the large-insert arrayed libraries of choice for plant genome research (Ren et al., 2004).

Large-insert BAC libraries are crucial to physical map construction, genome analysis, clone-based sequencing, and genomic sequence finishing of large, complex genomes (Zhang et al., 1996b; Tao et al., 2001; The International Human Genome Mapping Consortium 2001; The International Human Genome Sequencing Consortium 2001; Venter et al., 2001; Zhang and Wu 2001; Chen et al., 2002; Gregory et al., 2002; Xu et al., 2003; Wu et al., 2004a). As the insert size of the source library increases, the number of clones needed for construction of a genome-wide physical map is reduced proportionally (Zhang et al., 1996b). The physical maps constructed from larger-insert libraries have increased contig sizes and reduced gaps (Zhang et al., 1996b; Gregory et al., 2002).
Since 1994, BAC libraries are available for many crops such as sorghum (woo et al., 1994), Arabidopsis (Choi et al., 1995), apple (Vinatzer et al., 1998) Wheat (Mouillet et al., 1999), Soybean (Meksem et al., 2000), pea (Coyne et al., 2000). Within leguminosae, BAC libraries are available for Phaseolus vulgaris (Vanhouten W. et al., 2000), Vigna radiata (Miagi M. et al., 2004), Trifolium pretense (Sato S et al., 2005) and the model legumes Lotus japonicus (Kawasaki S et al., 2000), and Medicago truncatula (Nam YW et al., 1999). They have been utilized successfully and are widely used in many areas of genomics and genetics research, including high-resolution gene mapping, positional cloning, integrative physical and genetic mapping (e.g., Chang et al., 2001; Tao et al., 2001; Chen et al., 2002; Wu et al., 2004a), region-targeted marker development (Cregan et al., 1999b), and comparative genome analysis.

Clone-based physical maps have been proven to provide essential platforms for advanced genome research (Chang et al., 2001; Tao et al., 2001; The International Human Genome Mapping Consortium 2001; Zhang and Wu 2001; Gregory et al., 2002). Use of multiple complementary BAC libraries constructed with different restriction enzymes can enhance physical map construction. The beneficial effects may derive from the different GC contents of the recognition sites of different restriction enzymes that increase genome representation. The complete genome representation of the source library is a key to development of whole-genome physical maps of high genome coverage. The distribution of recognition sites for a restriction enzyme is uneven among chromosomes and genomes (The Arabidopsis Genome Initiative 2000), so that the clones from some genomic regions cannot be found in a BAC library constructed with a single restriction enzyme. A physical map constructed from such a library would have more and larger gaps. The use of different vector systems for library construction can further enhance the complementarity of libraries because different origins of replication, cloning sites, selectable markers, and insertion sites of different cloning systems can affect cloning ability and insert stability.

3.1.1 BAC (Bacterial Artificial Chromosomes)
Bacterial Artificial Chromosome, a low copy plasmid based upon E.coli fertility plasmid (F-factor), exists in super coiled form (Shizuya et al., 1992). Four essential regions that function in plasmid stability and copy number are parA, parB, and parC, required for parFIA partitioning. parB and parC are also required for incompatibility
with other F-factors. *OriS* is the origin of RepFIA replication and RepFIA protein E encoded by *crepe*, is essential for replication from *oriS*. A chloramphenicol resistance gene was also incorporated.

### 3.1.1.1 The BAC Vector Cloning Site:

Several cloning sites and markers were incorporated in BAC vector and include (1) Two bacteriophage marker, λ cosN and P1 loxP, (2) Three restriction enzyme sites (*BamHI*, *EcoRI*, *HindIII*) for cloning, and (3) a GC rich *NcoI* restriction enzyme site for potential excision of inserts. The cosN site provides a fixed position for bacteriophage lambda enzyme terminase, which provides a convenient way to generate linear form of BAC DNA, besides that it also used to package 50 kb DNA into the bacteriophage lambda head as a particle. The method, known as Fosmid for F-based cosmid system, is extremely efficient and thus very useful for precious and limited amount of DNA. The P1 lox P site allow the retrofitting of the additional component to the BAC vector at a large stage, e.g., the addition of G418 resistance marker for the selection in eukaryotic cell. The lox P can also be used to linearize the BACs through the P1 phage protein Cre, which catalyzes exchange between two DNA strands at the lox P site. (Shizuya *et al.*, 1992).

### 3.1.1.2 Properties of BAC vector

Plasmids are double stranded DNA vectors that are maintained and replicated in bacteria, which are easy to manipulate and maintain. However, the drawback of plasmids is that their non-vector insert size limit is around 10 kilobases (kb) (Hershfield *et al.*, 1974). Cosmids are essentially plasmids with at least one cohesive end site (cos) from a bacteriophage and require viral packaging prior to transfection into bacteria. Cosmids can accommodate non-vector inserts ranging from 5 kb to 23 kb (Koukolikova-Nicola *et al.*, 1988). Inserts up to 35 kb can be achieved, albeit at a sacrifice of packaging and transformation efficiency which are critical for library construction. However, even the increased maximum insert size of a cosmid system is insufficient for the study of a cluster of bacterial genes, large double-stranded DNA viral genomes, genes encoding non-ribosomal polypeptide synthetic proteins, or constructing physical maps of whole genomes.
### Existing BAC Vectors

<table>
<thead>
<tr>
<th>Name</th>
<th>Cloning sites</th>
<th>Recombinant selection</th>
<th>Features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAC108L (6.7 kb)</td>
<td>HindIII, BamHI</td>
<td>No</td>
<td></td>
<td>(Shizuya et al., 1992)</td>
</tr>
<tr>
<td>pBeloBAC11 (7.4 kb)</td>
<td>HindIII, BamHI, SphI</td>
<td>lacZ</td>
<td></td>
<td>(Kim et al., 1996)</td>
</tr>
<tr>
<td>pECSBAC4 (9.3 kb)</td>
<td>EcoRI, HindIII, BamHI</td>
<td>lacZ</td>
<td></td>
<td>(Frijters et al., 1996)</td>
</tr>
<tr>
<td>BIBAC2 (23.5 kb)</td>
<td>BamHI</td>
<td>sacBII</td>
<td>Plant Transformation via Agrobacterium</td>
<td>(Hamilton et al., 1996)</td>
</tr>
<tr>
<td>pBACwich (11 kb)</td>
<td>HindIII, BamHI, SphI</td>
<td>lacZ</td>
<td>Plant Transformation via Site-Specific Recombination</td>
<td>(Choi et al., 2000.)</td>
</tr>
<tr>
<td>pBACe3.6 (11.5 kb)</td>
<td>BamHI, SacI, SacII, MluI, EcoRI, AvaIII</td>
<td>sacBII</td>
<td>High copy number is available</td>
<td>(De Jong et al., 2001)</td>
</tr>
<tr>
<td>pClasper (9.7 kb)</td>
<td>homologous recombination in yeast</td>
<td>LEU2</td>
<td>Yeast and bacteria shuttle vector</td>
<td>(Bradshaw MS, et al., 1995)</td>
</tr>
<tr>
<td>plndigoBAC-5 (7.5 kb)</td>
<td>HindIII, BamHI</td>
<td>lacZ</td>
<td>Low copy number</td>
<td>(Shizuya et al., 1992)</td>
</tr>
</tbody>
</table>

In the present study plndigoBAC-5 vector is used. This vector is derived from pBeloBAC11 and plndigoBAC (Shizuya et al., 1992), it accommodates and stably maintains DNA inserts of more than 100 Kb. plndigoBAC-5 has been linearized at either its unique BamHI or its unique Hind III site. All BAC vectors including pBeloBAC11 and plndigoBAC contain cloning sites that are flanked by T7 and SP6 promoters which can be utilized for DNA sequencing of the insert. plndigoBAC-5 is the first commercially available BAC vector for cloning and preparation of primary BAC libraries.

#### 3.1.1.3 Applications of BACs

BAC libraries are rapidly becoming a central tool in modern genetics research. Such libraries have been made for a host of taxa and employed in a variety of applications. For example:
1. The suitability of BACs as DNA sequencing/PCR templates has led to the development of BAC-end sequencing (Venter et al., 1996; Boysen et al., 1997; Rosenblum et al., 1997), fostered advances in STS-based mapping (Venter et al., 1996, Venter et al. 1998), and provided a means to quickly search well-defined genomic regions for phenotypically significant genes (Bouck et al., 1998).

2. The facility of BACs as a large DNA cloning vector (Shizuya et al., 1992) combined with the development of methods for high-throughput DNA fingerprinting (Marra et al., 1997), contig assembly (Gillett et al., 1996; Soderlund et al., 1997; Ding et al., 1999), BAC-end sequencing, and STS-based mapping have helped investigators bridge gaps between DNA markers in physically-large genomes i.e. physical mapping. Consequently, many interesting and important genes have been isolated (Wang et al., 1996; Nakamura et al., 1997; Yang et al., 1997; Cai et al., 1998; Danesh et al. 1998; Yang et al. 1998; Folkertsma et al., 1999; Moullet et al., 1999; Nam et al., 1999; Patocchi et al., 1999; Salimath and Bhattacharyya 1999; Sanchez et al., 1999). High-throughput physical mapping already has resulted in the construction of BAC contigs encompassing entire chromosomes and/or complete chromosome sets (Mozo et al., 1999).

3. Many of the DNA probes used to make genetic maps can be localized to specific BACs, providing a means of superimposing genetic maps directly onto BAC-based physical maps (Yang et al., 1997; Mozo et al., 1999). This feature also facilitates map-based cloning of genes responsible for specific phenotypes (Danesh et al., 1998; Nam et al., 1999; Patocchi et al., 1999; Sanchez et al., 1999).

4. BAC-based physical mapping enjoys the fundamental advantage of somatic cell genetics in that it does not require DNA polymorphism (Lin et al., 2000). Therefore it provides an alternative to radiation hybrid mapping in which chromosomes are broken by radiation and propagated in cell cultures (Goss and Harris, 1975; Deloukas et al., 1998). Of particular interest to botanists, this feature has also spawned efficient methods to determine the locus specificity of individual BACs that correspond to multi-locus DNA probes in a manner that can efficiently be applied on a large scale (Lin et al., 2000).
5. BAC-based mapping in conjunction with efficient multiplex screening methods (Cai et al., 1998) may open the door to the development of comprehensive “gene maps” (Hudson et al., 1995) for numerous genomes, conferring many of the advantages of complete genome sequencing decades before complete sequences are likely to be available.

6. BACs have successfully been employed as probes in fluorescence in situ hybridization (FISH) (Cai et al., 1995; Hanson et al., 1995; Jiang et al., 1995; Lapitan et al., 1997; Gómez et al., 1997; Morisson et al., 1998; Godard et al., 1999). FISH-based localization of cloned DNA sequences on chromosomes allows molecular and physical maps to be directly superimposed onto the framework of chromosomes, and subsequently provides useful information on the relationship between chromosome structure, DNA sequence, and recombination (Peterson et al., 1999).

7. Full-scale BAC-based genome sequencing efforts are underway (Venter et al., 1998).

In order to clone the loci for *Fusarium* wilt, it was imperative to have a large insert genomic library of Chickpea. In recent years two chickpea BAC libraries and one BIBAC library have been reported (Rajesh et al., 2004 and Lichtensveig et al., 2004), Chickpea BAC libraries having 23,780 and 14,976 clones respectively and BIBAC library having 23,040 clones. Although these BAC and BIBAC libraries provide sufficient clone resources for Chickpea and since *Fusarium* resistance is known to be genotype specific, therefore it was imperative to construct a Chickpea BAC library for a resistant cultivar, WR-315 against foc1.
3.2 Material and Methods

3.2.1 Pre-treatment of plant material
Fusarium wilt resistance cultivar WR 315 was used for the preparation of the BAC library. The seeds after germination were grown for 14 days in the growth chamber. Whole plant tissue was harvested and frozen in liquid nitrogen for the preparation of high molecular weight (HMW) DNA isolation.

3.2.2 HMW DNA isolation

- For the isolation of nuclei, 20g of frozen tissue was ground into a powder in liquid nitrogen and transferred in to an ice cold beaker containing 200ml of homogenization buffer (10X HB = 0.1M Tris, 0.8M KCl, 0.1M EDTA, 10mM of Spermidine, 10mM Spermine, pH 9.4) + 300µl β-Mercaptoethanol + 100ul of Triton X-100.
- After transfer the contents were swirled with a magnetic stir bar for 10 minutes on ice.
- The slurry was filtered using a cheese cloth and centrifuged in a fixed angle rotor (Beckman) at 1800g at 4°C for 20 min.
- The supernatant was discarded and the pellet was mixed with 1ml of ice cold wash buffer (HB + 0.5x Triton X-100 with no β-Mercaptoethanol).
- The nuclei were resuspended with assistance of paint brush soaked in ice cold buffer and were pelleted by centrifugation at 1,800g at 4°C for 15 min in the swinging bucket centrifuge (Eppendorf, USA).
- The nuclei were washed twice by centrifuging at 1,800g, at 4°C with wash buffer.
- After the final wash the pellet was suspended in 1ml of HB without β-Mercaptoethanol.

3.2.3 Nuclei staining with DAPI
The integrity of the isolated nuclei was analyzed by DAPI staining as shown in figure 3.1. The nuclear fraction was stained for 15 min with 0.1 µg/mL 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) in 0.1 M potassium phosphate buffer (pH 7.4) and
then washed twice with phosphate buffer saline (PBS). For microscopy, a small volume of suspension was placed on a slide, covered with a cover glass and the images were captured with or without UV filter.

3.2.4 Embedding the nuclei in agarose plugs
- One percent low-melting point (LMP) agarose was prepared in HB without β-Mercaptoethanol and was stored in 45°C water bath.
- The nuclei were pre-warmed at 45°C and mixed with an equal volume pre-warmed 1% LMP agarose using a cut-off-pipette tip.
- The mixture was then aliquotted into ice cold plug molds on ice. After the agarose completely solidified, the plugs were transferred into 5-10 vol of lysis buffer (0.5M EDTA, pH 9.0-9.3, 1% Sodium Lauryl Sarcosin and 0.1% proteinase K).
- The buffer was added just before transferring the plugs to lysis buffer.
- The plugs were incubated for 24-48 h at 50°C with gentle shaking, washed once with 0.5M EDTA, pH 9-9.3 for 1 h at 50°C, once with 0.5M EDTA, pH 8.0 for 1 h on ice and was finally stored in 0.05M EDTA, pH 8.0 at 4°C.

3.2.5 Vector Preparation
plndigo BAC cloning vector linearised at BamHI recognition site and dephosphorylated was obtained by Epicenter (USA).

3.2.6 Partial digestion and size selection of HMW DNA
- Agarose plugs were cut into 4 pieces and Bam HI enzyme was added (5.0- 8.0 units) to half a plug and the digestion time kept was 10 minutes at 37°C.
- Each digestion was carried out in a 200µl total volume and the reactions were stopped by adding 20µl of 0.5M EDTA, pH 8.0, on ice.
- Partially digested DNA was analysed on a LMP (low melting point) agarose gel in 0.5 X TBE (0.45M tris, 0.45M boric acid, 10mM EDTA, pH 8.0) by PFGE (Pulse field gel electrophoresis) using the following conditions: Voltage 6V/cm, temperature 140°C, pulse time 10-60s, 2 state run, ramping linear and the total run time was 30hrs, pump settings were kept at 70-80 (CHEF, BIORAD, USA).
• DNA fragments ranging from 150-300kb were excised from the gel and were directly used for second size selection in 1% agarose gel with pulse time 5-15s and run time 20hrs. Rest all conditions were same as first size selection.

• Agarose slices ranging from 150-250 kb were excised and were equilibrated for 10-15 minutes on ice in TE (10mM Tris-HCL, 1.0mM EDTA, pH 8.0) with two times change of buffer after 15 min., the slices were melted at 68°C for 5 min. and digested with 1U of gelase (Epicentre, USA) per 100mg of LMP for 45 min. at 45°C.

• The enzyme was heat-inactivated at 70°C for 5 min. and the DNA was quantified on 1% agarose gels.

3.2.7 Ligation

• Ligation of genomic DNA into the pIndigo BAC vector was done with Fast-link DNA ligase available with the Copy control BAC cloning kit (Epicentre, USA).

• The ligation was carried out in a 75μl volume in which about 100ng partially digested and size selected chickpea genomic DNA fragments were ligated to 25ng of digested and dephosphorylated pIndigo BAC vector (molar ratio of 4:1 with excess of genomic DNA) with 2U of Fast-link DNA ligase and incubated overnight at 16°C.

• The ligation reactions were desalted by using agarose cones: agarose0.5g + glucose0.9g + 50ml water were mixed together and the solution was allowed to cool down at 50°C, 800μl of the solution was dispensed in 1.5ml microcentrifuge tubes for each DNA solution.

• A 0.5ml microcentrifuge tube was placed on the molten agarose-glucose solution pressed lightly and was allowed to solidify.

• After removing the 0.5 ml tube there was a concave pit in the solidified agarose-glucose. The agarase digested DNA was transferred to this pit and was incubated on ice for 1hr.

• The salt from the DNA would diffuse into the agarose glucose. The desalted DNA was taken into fresh microcentrifuge tubes and was directly used for transformation reactions.
3.2.8 Transformation

- The vector used for transformation was pIndigo vector (Epicenter, USA) as shown in Figure 3.3.

- The pIndigo vector (epicenter) was introduced in electrocompetent cells EPI300 (Epicenter, USA) by electroporation using a cell porator and Voltage booster system (Biorad, USA).

- A 2μl of ligation mixture was added to 50 μl of electrocompetent cells in 0.2cm gap electrode gene pulser cuvettes (Biorad, USA) for a single electroporation. The cell porater settings were as follows:
  
  | Voltage | 2.0K |
  | Capacitance | 25μF |
  | Resistance | 200 Ω |

- After electroporation the cells were transferred to 975 μl SOC solution (Invitrogen, USA) and incubated at 37°C with shaking at 200rpm for 1hr. The cells were spread on 50 ml LB agar plates (140mm) containing chloramphenicol (12.5 μg/ml), 80 μl X-gal (20 mg/ml) and 8 μl isopropylthio-β-thiogalactoside (200mg/ml) grown at 37°C for 16hr and stored at 4°C.

- White colonies transformed were streaked and transferred to 384 well microtiter plates containing 60 μl of LB freezing buffer (36mM of K2HPO4, 13.2 mM of KH2PO4, 1.7mM of citrate, 0.4mM of MgSO4, 6.8mM of (NH₄)Cl, 4.4% glycerol, 12.5μg/ml chloramphenicol, LB per well manually, incubated at 37°C for 24hr and stored at -80°C.

- The 384 well microtiter plates having the BAC library (CaWBL) were further replicated in two copies using 384 pin replicators (Epicenter, USA).

3.2.9 Isolation of recombinant DNA and insert analysis

- Clones were streaked onto LB plates containing 12.5 μg/ml of chloramphenicol. Two hundred clones were randomly selected, picked up and grown in 4ml of LB with antibiotic (12.5 μg/ml of chloramphenicol) at 37°C for 16hr.

- Alkaline lysis method, used to isolate the plasmid DNA follows as:
• 4.0ml of the overnight culture was pelleted down by centrifuged at 13000g for 1-2 min.
• 200μl of sol-I was added to the recovered pellet and vortexed the samples vigorously.
• 200μl of sol-II was added and tubes were inverted down 2-3 times to mix the solution (it induces lyses).
• 300μl of sol-III was added to each tube and tubes were inverted 2-3 times.
• Centrifuged at 13000g for 10 min at RT.
• The supernatant than transferred to fresh microfuge tube with the help of 1ml pipette.
• 540μl of Isopropanol was added to each tube to the recovered supernatant, mix it by inverting the tubes 4-6 times.
• Samples were centrifuged at 13000g for 10 minutes.
• Decant the Isopropropanol and gave a brief spin to remove Isopropanol
• Pellet was wash with 70% ethanol.
• Pellet was air dried at 37°C for 10 minutes.
• Pellet was resuspended in 20μl TE Buffer and samples were mixed by gentle tapping.
• Plasmid DNA was loaded in to 0.8% agarose gel and DNA was quantified.
• The isolated DNA samples were digested with BamHI (NEB,USA) for 4hours and then loaded on a 0.8% agarose gel. The gel was subjected to PFGE at 6V in 0.5 X TBE for 16hr using a 5-15s ramped switch time.
• The average insert size of the library was estimated to be 160kb by comparison with a PFGE marker (Biorad, USA) electrophoresed parallely in the same gel.

3.2.10 Screening of BAC library
From our molecular mapping data STMS9 available at genetic distance of 0.2 cM and STMS10 at 1.0 cM was identified as a closely linked marker to foc1. Primary screening was done with the superpooled BAC clones multiplexing these two primers.

The BAC library composed of 110 plates corresponding to 42,240 total BAC clones.
• BAC clones from a 384 well plate were pooled in one 96 well plate in such a way that eight clones from each 384-well plates were pooled in each well of 96-well plate, as shown in the figure 5.

• In this way a total of 55 superpools were prepared corresponding to 110, 384 well plates.

• Primary screening was done with superpooled BAC clones of 55, 96-well plates.

• For primary screening, lysis of the pooled clones was done in the following way:
  • With the help of a 384-pin replicator, the BAC clones from the 384 well plates were transferred on to the omniplates (nunc, USA) having LB+MgSO₄ and chloramphenicol and were kept in 37°C for over night.
  • For pooling 8 clones from the 384 well plate in individual wells of 96 well plate, 50 μl of the PCR-Lyse solution was added to each well of 96 well plate using colony fast-screen kit (PCR screen, epicenter, USA).
  • With a sterile toothpick, a portion of a colony was picked from the corresponding omniplate of the 384-well plate and the cells were deposited at the bottom of the well of 96 well plates having 50 μl of the PCR-Lyse solution. The process was repeated using a fresh toothpick for each colony chosen.
  • The pooled 96 well plates were covered and vortexed vigorously until the colony picked was completely resuspended.
  • These pooled 96 well plates were incubated at 99°C for 5 minutes in a thermocycler or water bath. The PCR-Lyse Solution facilitates very efficient release of PCR-ready DNA from the cells and inactivates endogenous nucleases.
  • Each of these pooled 96 well plates were vortexed briefly and chilled on ice for 2 minutes.
  • 2 μl of the PCR-ready DNA from each well of these pooled 96-well plates were transferred to another 96 well plate PCR plate.
  • A plate PCR was performed in 20 μl volume using pre-established conditions as given below:
STMS9 + STMS10 primers  -  1 + 1 µl
10 X buffer  -  2 µl
dNTPs  -  0.5 µl
Taq polymerase  -  0.2 µl
Milli Q water  -  14. µl

Total volume - 20 µl

• The PCR was performed with the following program

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step1</td>
<td>94 °C</td>
<td>3 min</td>
</tr>
<tr>
<td>Step2</td>
<td>92 °C</td>
<td>30 Sec</td>
</tr>
<tr>
<td>Step3</td>
<td>54 °C</td>
<td>30 Sec</td>
</tr>
<tr>
<td>Step4</td>
<td>64 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>Step5</td>
<td>Go To Step 2 &amp; Repeated for 34 time</td>
<td></td>
</tr>
<tr>
<td>Step6</td>
<td>72 °C</td>
<td>10 Min</td>
</tr>
<tr>
<td>Step7</td>
<td>4 °C</td>
<td>1 Hour</td>
</tr>
<tr>
<td>Step8</td>
<td>End</td>
<td></td>
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</tbody>
</table>

• 4 µl of the gel loading solution was added to each well of the PCR plate.

• The 96 well plate PCR products were loaded onto 2% agarose gel, along with lambda Hind III ladder and were run till the bands resolved clearly.

• The remaining 48 µl of the PCR-ready DNA can be stored at 4°C for up to 1 month in the event that subsequent PCR reactions need to be done.

• Individual BAC clones from the 384 well plates corresponding to the positive BAC clone 96 well plates in the primary screening were now taken for secondary screening.

• The individual clones of 384 well plates corresponding to the positive superpools from primary screening were picked from freshly plated 384 colonies in omniplates (as discussed earlier).

• The individual colonies were picked and lysed in 10 µl of lysis buffer in a 96 well plate and the plate was lysed as in primary screening.
2 μl of individual BAC clones in each well of 96 well plates were further aliquoted into 96 well PCR plates and a PCR cocktail with individual primers (STMS9, STMS10) was made.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>STMS10 primers</td>
<td>1 μl</td>
</tr>
<tr>
<td>10 X buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.2 μl</td>
</tr>
<tr>
<td>Milli Q water</td>
<td>14. μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

The PCR program was same as in the primary screening.

Insert analysis of the positive clones:

- The secondary PCR positive clones were streaked onto LB plates containing 12.5 μg/ml of chloramphenicol and grown in 4ml of LB with antibiotic (12.5 μg/ml of chloramphenicol) at 37°C for 16hr.
- The clones were subsequently isolated by alkaline lysis method, as discussed earlier and were digested with BamHI enzyme for 4hours in a total volume of 30 μl and then loaded on a 0.8% agarose gel.
- Clones were analysed on PFGE gel at 6V in 0.5 X TBE for 16hr using a 5-15s ramped switch time.

### 3.3 Results

#### 3.3.1 Isolation of High Molecular Weight (HMW) DNA

HMW DNA isolation and its quality are a prerequisite for BAC library preparation. The nuclei were isolated using the universal nuclei isolation protocol developed by Zhang et al (1995) to prepare megabased sized genomic DNA. The nuclei isolated were stained with DAPI, observed scored and photographed under fluorescence microscopy as shown in figure 3.1. The nuclei isolated were estimated to be 10^7 nuclei per ml. Nuclei were intact and were nicely spaced as shown in the figure. They were then embedded in low melting point agarose plugs and HMW DNA was purified in the agarose.
Fig 3.1: Isolated nuclei analyzed by staining with 4',6'- diamidino-2-phenylindole hydrochloride (DAPI).
3.3.2 Partial digestion and pulse field gel electrophoresis

The digestion of nuclei embedded in the agarose plugs with BamHI was standardized using a range of enzyme units and varied digestion time. The best results were seen at enzyme concentrations 6.5, 7.0, 7.5 and 8.0U at digestion time of 10min. The plugs were loaded on to PFGE along with 50kb PFGE ladder, that showed a significant peak of partially digested DNA wherein the partially digested DNA clustered around 150-300kb as shown in figure 3.2. In order to eliminate small molecules (less than 100kb) from HMW DNA fragments, 150- 300kb region was excised from the first size selection gel and reloaded onto PFGE gel for second size selection. Different size ranges that is, 150-200 and 200-300kb, from the first size selection was loaded onto different wells of PFGE gel. The HMW DNA was seen to cluster around 150-250kb which was excised and purified subsequently.

3.3.3 Ligation and transformation

As the BAC vector is a key factor in transformation, BamH1 digested and dephosphorylated plIndigo vector (epicenter) was tested on gel as in figure 3.3. Agarose blocks excised from second size selection were further purified and digested with gelase enzyme overnight. A 4hr digestion was also tested but it did not yield enough DNA which could be taken for ligation reactions. The gelase digested samples of excised agarose blocks were loaded onto 1% agarose gel and were quantified for ligation. The insert DNA was quantified and was found to be approximately 20-30ng/μl. Bam H1 digested Chickpea genomic DNA was ligated with plIndigo vector in a total volume of 100-150 μl. Ligation in less volume that is about 70 μl did not yield good transformation results. A total of 5 ligations were taken for constructing the BAC library.

The ligated products were transformed in electrocompetent cell, EC100 and EPI300 (Epicenter,USA) with control plasmid pUC19. Transformation using EPI300 cells in a 0.2cm gap electrode cuvettes gave 300-500 colonies whereas EC100 cells gave only 50-100 colonies. All transformations produced around 95% recombinant white colonies. Therefore all the ligation products were transferred to EPI300 cells.
Fig 3.2: First Size Selection-LMP Agarose Plug having Nuclei of Chickpea in gel digested with different conc. of BamHI enzyme.
Fig 3.3: Diagrammatic representation of cloning of CaBAC
3.3.4 BAC library characterization

The CaBAC library consisted of 42,240 clones and was arrayed in 110, 384-well microplates. Isolating and analyzing randomly selected clones from different ligations showed that the library had an average insert size of 160 kb, with a range from 150 kb to 200 kb as shown in the figure 3.4 and 3.5 respectively. Empty clones were not observed in the library thus CaBAC library represented 10-fold genome coverage, taking into consideration estimated average insert size of 150kb and the genome size of chickpea as 750MB.

3.3.5 Screening of the BAC library

The BAC clones from 384 well plates were pooled and lysed in 96 well plates as shown in figure 3.6. Each well of the 96 well plate had 8 clones from 2, 384 well plate. Pooled clones from 96 well plates were lysed. A 96 well plate PCR was performed using 2 μl of lysed clones. As the PCR program used with our both the primers STMS9 and STMS10 were same, multiplexing was done in the primary screening as shown in figure 3.7(A). For screening, BAC clones from 384-well plate were pooled and transferred to 96 well plates. All the clones from 110, 384 well plate were pooled into 55, 96 well plates and were used for primary screening. Out of the 110 (384-well plates) 4 plates CaWBL81, CaWBL82, CaWBL83 and CaWBL84 showed positive bands in the primary screening. In these plates that is in 192(96X2) pooled clones, about 50 pooled clones showed strong positive bands rest of the bands were very light. In secondary screening all the 192 pooled clones were individually screened with individual primers as shown in figure 3.7(B). Here the annealing temperature for STMS9 was altered to 54 °C and extension temperature at 64 °C for getting a better result. In total 18, BAC clones were positive in the plates CaWBL81 and CaWBL82 among 1536 individual clones. Out of the 18 positive BAC clones 4 clones were positive with both STMS9 and STMS10, and rest 14 clones were positive with either only STMS10 or STMS9.

3.3.6 Analysis of the BAC positive clones

All the 18 positive clones were isolated, digested with BamH1 and run on PFGE. Four of these eighteen clones that were positive with STMS9 and STMS10 were digested also with BamH1 and were separately run on PFGE as shown in figure 3.8. The insert size of all the 4 clones was approximately 200kb.
Fig 3.4: Isolation of randomly selected CaBAC run on 0.8% Agarose gel
Fig 3.5: A. Digestion of randomly selected CaBAC clone. B. Distribution of insert sizes from randomly selected CaBAC clones. 1 to 9 representing BAC clones showing different insert sizes, I represents insert and V represents vector band.
Fig 3.6: Pooling method of BAC clones for screening. Well no. A-1 of 96 well plate was represented by well no. A-1 to A-4 & I-1 to I-4 of 384 well plate.
Fig 3.7: PCR screening of CaBAC clones. (A) Primary screening PCR of 96-well Pooled Plate multiplexed with (STMS9 & STMS10). (B) Secondary screening PCR with primer STMS9.
3.4 Discussion
Positional cloning is a promising method for isolating and studying genes for which only the locus derived phenotypes is known. For this YACs and BACs are the tools of choice for generating high resolution genetic maps and identifying candidate genes.

For the analysis of plant genomes by PFGE and especially for BAC cloning, an important step is the preparation of HMW DNA. To prepare HMW DNA from plant tissues, the cell walls must first be removed before the cells are embedded in agarose. Earlier widely used methods for the preparation of HMW DNA involved the isolation of protoplasts using cell wall hydrolyses (Cheung and Gale, 1990; Ganal and Tanksley, 1989; Honeycut et al., 1992; Van Daelen et al., 1989; Wing et al., 1993) which is extremely time-consuming and costly. Thus we prepared HMW DNA using the nuclei isolation protocol by Zhang et al., 1995. It is known that the intactness of the nuclei prepared by the liquid nitrogen method was much higher than prepared by the blending method. Once nuclei are isolated, a supporting matrix in the form of either agarose plugs or microbeads is used to embed these nuclei. A large number of nuclei are lost during preparation of microbeads in comparison to agarose embedding, since it is difficult for the restriction enzymes to penetrate the nuclei embedded, in agarose plugs we had cut plugs containing nuclei into small pieces to increase the surface-volume ratio which will make it easier for the restriction digestion enzymes to penetrate. The washing steps of the plugs having HMW DNA was important as it yields a high quality and stable DNA which does not degrade after months of storage at 4°C. The digestion time of HMW DNA plugs were standardized at 37°C for 10 minutes. Higher incubation time till 15-20 minutes did not yield better results. The PFGE analysis using a contour clamped homogenous electric field (CHEF) apparatus was used to analyse the partial digestion of the HMW DNA plugs as it can be used to separate large DNA fragments. The BamHI concentration used to digest the HMW DNA in agarose plugs ranged from 3.5-8 Units (3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8) 3.5-6 U did not give any bands whereas best results were seen in 6.5 and 8.0 U. In the first size selection, PFGE conditions the run time and the pulse time were standardized to 24hrs and 10-60s respectively. Rest of the conditions that is the ramp time, voltage and the pump settings were constant throughout. As a small BAC molecule has a higher probability of being transformed into an E. coli cell than a larger BAC molecule, it is necessary to remove small DNA fragments of sizes less than 100kb from HMW DNA. This may reduce the
Fig 3.8: Positive BAC clones with both STMS9 & STMS10 primers digested with Bam HI run on 1% LMP Agarose
insert size of the BAC library. Use of a second round of size selection has proven effective for eliminating most small DNA fragments and has resulted in the successful construction of BAC library having larger insert sizes. So, in our study after excising 150-300kb bands from first size selection, the bands were further resolved in PFGE, with a change in pulse time of 5-15s and run time of 14 hours. The PFGE in this program was run horizontally with more number of wells fused so that different sized fragments within 150-300kb could be added. On excising, the quantity of agarose also is less in a horizontally run gel, which would give a better gelase digestion. Further from the second size selection 150-250kb fragments were selected for agarase digestion. Previous results from earlier systems have shown that recombinant BACs larger than 350kb have not been recovered. The reason behind inability to recover larger BACs could be the larger size of the molecule that can be delivered into E.coli by electroporation (Rajesh, coyne et al., 2004)

BAC cloning vectors for HMW DNA insert libraries allow the cloning of inserts upto 300kb in size (Biren B.et al.,1999). The BAC vector used here is pIndigoBAC-5 (epicenter, USA) available commercially in a ready to use form. This BAC cloning vector is 7506bp, linearised at a unique restriction enzyme site BamHI and dephosphorylated. High levels of background colony formation are a common problem among BAC vectors which interferes with ligation and transformation reactions. This happens by incomplete linearization of the vector since supercoiled DNA transforms bacteria at much higher efficiencies than linear DNA, and vector thus leaving self-ligatable vector for recircularisation when treated with DNA ligase. Complete dephosphorylation of BAC preparations also renders any copurifying contaminating DNA (that is E.coli genomic DNA) unclonable and thus it would not contribute to background colony formation.

In the present study 25ng of vector ligated with about 100-150ng of insert DNA in a 50-75 µl of ligation reaction yielded around 300-500 colonies after transformation. One of the essential criteria for getting a good transformation is the use of fresh ligation reactions. Storage of ligations at -20°C, even for a few days could cause a significant reduction in BAC clone recovery. On using re-frozen ligation mixtures the majority of the clones were lost.

Successful construction of a BAC library depends on both a high transformation efficiency and high proportion of desirable BAC insert sizes. BAC library construction
involved electroporation to introduce the ligated DNA into *E.coli*. Apart from insert size, efficiency of transformation (cfu/μg of DNA) is affected by many factors such as buffer components, temperature (Antonov *et al.*, 1993) and the electroporation conditions determined by users including voltage gradient, resistance and capacitance (Tekle *et al.*, 1991). In addition, the genetic background of host cells (Hanahan *et al.*, 1991), post-pulse treatment (Dower *et al.*, 1988), the topological form (Neumann *et al.*, 1982) and treatment of DNA samples (Kobori *et al.*, 1993) are some other factors, which contribute to the efficiency observed.

The transformation efficiency of the commercially available electrocompetent cells guarantees at least $10^{10}$ transformants/μg with control plasmid (pUC19). Here we have used 2 commercially available electrocompetent cells-EC100 and EPI300 (epicenter, USA).

Transformations with EPI300 cells in 0.2cm gene pulser cuvettes gave 300-500 colonies per ligation, whereas in the same cuvettes with EC100 cells, it gave 100-200 colonies. Transformations also yielded better results in 0.2cm cuvettes and its respective electroporation conditions as compared to 0.1 cm cuvettes.

Global physical mapping and sequencing of a complex genome require readily usable DNA large inserts, high integrity, and complete genome coverage (International human genome mapping consortium 2001; International human genome sequencing consortium 2001) We have constructed a BAC library containing a total of 42,240 clones with an average insert size of 150kb that is equivalent to 10X genomes of chickpea, providing a greater probability of obtaining a single-copy sequence from the library. To facilitate Chickpea genomics a BIBAC library that has 23,780 clones equivalent to 3.8X genomes of chickpea (Rajesh *et al.* 2004) and two libraries a BAC and a BIBAC containing a total of 38,016 clones equivalent to 7.0X genomes of chickpea (Lichtenzveig J., *et al.*, 2005) have been reported earlier. The PCR based screening process adopted in this study has proven to be efficient and specific. By screening superpools followed by single BAC clones, positive BAC clones can be rapidly identified. Here we had multiplexed our earlier identified STMS primers STMS10 and STMS9 since the PCR program for both were same. On multiplexing we had to perform a total of 55 PCRs in 96 well plates for 42,240 total clones in primary screening. Multiplexing with some of the reactions gave quiet an intense band in the 2% agarose gels as compared to screening with individual primers, which indicated
some of the BAC clones were positive with both the primers. In the secondary screening where 192 pooled BAC clones were individually screened with STMS10 and STMS9 primers, a total of 32 PCRs were performed. Out of the 18 positive clones, 4 clones L1, M1, N1, O1 were positive with both the primers in plate number CaWBL82. The clone H1 was positive with only STMS9 in the same plate. In the plate CaWBL81, 11 clones I13, J13, K13, L13, M13, P13, A14, E14, I14, K14, M14 were positive with only STMS10 and in the same plate clones F14 and J14 were positive with STMS9. The clones that were positive from the whole library were from same or nearby rows of the consecutive plates. This indicates that the clones were from the same ligation and so on digestion with BamHI there should be very less difference in the insert sizes of the clones. An advantage of this screening strategy is the specificity of the PCR compared with standard colony hybridization method. Using relatively long primer sequences (25 bases) and an annealing temperature of 62°C, presence or absence of a single strong PCR band have been observed for all the clones. In contrast to colony hybridization methods PCR screening methods with STMS primers have less chance of false signals.

Thus here we report construction of a 10X coverage Chickpea BAC library. The library can be used to accelerate different aspects of genome research in chickpea including studies of the structure and organization of multigene families and comparison of specific regions of the genome with other closely related legume species in addition to map based cloning of agronomically important genes. The sequencing and molecular characterization of PCR positive BAC clones with the tightly linked molecular landmarks for *Fusarium* loci would be used to clone the R- gene loci in the near future.