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Use of Nipponbare BAC Clones for Physical Mapping of an *R* Gene Locus in Rice

Jong-Seong Jeon and Pamela C. Ronald

Summary

Major advances in rice genomics during the last few years have made positional cloning in rice much more efficient. Nipponbare is a model rice genotype being sequenced by the International Rice Genome Sequencing Project Consortium. Here, we describe an efficient procedure of the construction of physical map for positional cloning of resistance gene (*R*) using the Nipponbare genetic resources. This advanced strategy should be useful for the efficient identification of agronomic important *R* genes from many resistant rice genotypes, including wild rice species.

Key Words: Rice; *R* gene; Nipponbare; BIBAC library; saturation mapping; positional cloning.

1. Introduction

Flor ***(1)*** proposed a model based upon the genetic studies using flax and the flax rust pathogen. The "gene-for-gene" model predicts that plant resistance will occur only when a plant possesses a dominant resistance gene (*R*) and the pathogen expresses the complementary dominant avirulence gene (*Avr*). An alteration or loss of the plant resistance gene or the pathogen *Avr* determinant leads to disease on the host as the outcome. The *R* gene products are hypothesized to act as receptors for the products of the avrulence locus. The model holds true for many host–pathogen interactions.

Cloning and characterization of several disease resistance genes in different plant species has revealed common structural features in their predicted protein products, including nucleotide binding site (NBS) and leucine-rich-repeat (LRR) domains, suggesting that common mechanisms for perception and transduction of pathogen signals exist in diverse plant species ***(2)***. In rice, the most serious fungal disease of rice is blast caused by the fungus *Magnaporthe grisea*

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and the most serious bacterial diseases of rice in Africa and Asia is bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Of the four rice disease resistance genes cloned from rice so far, three, *Xa1* ***(3)***, *Pib* ***(4)***, and *Pita* ***(5)***, possess sequences encoding the NBS–LRR domains, whereas one, *Xa21*, codes for an LRR receptor kinase-like protein ***(6)***.

The *R* gene-mediated resistance is known to be an economical method to control losses in the field. The combined presence of *R* loci ensures a mechanism for conferring long-term and durable resistance ***(7)***. Using molecular markers of isolated genes or tightly linked to resistance loci, simultaneous selection of multiple resistance loci, called marker assisted selection, has been facilitated for pyramiding *R* genes in a certain cultivar.

Positional cloning, also called map-based cloning, is the process of identifying the genetic basis of a phenotype, i.e., resistance, by looking for linkage to markers whose physical location in the genome is known. The amount of effort required for map-based cloning of genes in rice has dropped dramatically in recent years. Saturation mapping, a new approach to produce markers in a very small interval of several hundred kilobase pairs, is indispensable for positional cloning.

During the last few years major advances in rice genomics have made positional cloning in rice much more efficient. A high-density genetic linkage map and a YAC-, PAC-, and BAC-based contig map have been constructed for the rice cultivar Nipponbare ***(8)***. More than 110,000 sequence-tagged connectors have been generated by sequencing both ends of every BAC clone ***(9)***. A fingerprint-based contig of BAC clones has been anchored with restriction fragment-length polymorphism markers onto the genetic map ***(10)***. In addition, Nipponbare is the rice genotype being sequenced by the International Rice Genome Sequencing Project Consortium ***(11)***.

Nipponbare appeared to be susceptible to many *M. grisea* strains. Recent studies, however, demonstrated that the genetic resource of Nipponbare lacking an *R* gene can be efficiently used to develop markers required for saturation mapping of the *R* locus ***(12–14)***. Here, we describe in detail an efficient method of the construction of physical map for positional cloning in rice using the Nipponbare genetic resources. First, initial markers linked to an *R* gene are mapped on the high-density genetic map of Nipponbare/Kasalath. Secondly, Nipponbare BAC clones physically spanning the region are identified using the Arizona Genomics Institute (AGI) database (http://www.genome.arizona.edu/fpc/rice/). Third, additional genetic markers for saturation mapping are produced using subclones of the identified BACs. Fourth, a small interval of Nipponbare corresponding to the *R* gene region is delimited by determining recombination breakpoints. Fifth, a physical map of the R locus is constructed using flanking markers and a BIBAC library generated from the *R* gene-containing line.

2. Materials

2.1. Nipponbare BAC DNA Isolation

1. Terrific broth (TB) liquid medium (1.0 L): To 900 mL of double-distilled H_2O (ddH_2O), add 12 g of bacto-tryptone, 24 g of bacto-yeast extract, and 4 mL of glycerol. After sterilizing by autoclaving, allow the solution to cool to less than 60°C, and then add 100 mL of a sterile solution of 0.17 *M* KH_2PO_4 and 0.72 *M* K_2HPO_4.
2. ddH_2O sterile water
3. Chloramphenicol
4. Resuspension buffer: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* ethyelene diamine tetraacetic acid (EDTA), 100 µg/mL RNaseA.
5. Lysis buffer: 200 m*M* NaOH, 1% sodium dodecyl sulfate.
6. Neutralization buffer: 3 *M* potassium acetate, pH 5.5.
7. Miracloth.
8. Isopropanol.
9. 10 m*M* Tris-HCl, pH 8.0.
10. RNase.
11. Phenol:chloroform (1v:1v).
12. Absolute ethanol.
13. 70% Ethanol.
14. Agarose.

2.2. Sublibrary Construction of BAC DNA

1. *Sau*3AI.
2. QIAquick Gel Extraction Kit, Qiagen.
3. pBluescriptII SK(+) (Stratagene).
4. ElectroMAX DB10B cells (Life technologies).
5. CELL-PORATOR (Gibco BRL).
6. Ampicillin.
7. LB medium (per 1 L): 10 g of bacto-tryptone, 5 g of bacto-yeast extract, 10 g of NaCl.
8. SOC medium (1.0 L): Mix 25 g of LB broth, 10 mL of 1.0 *M* KCl stock solution, and 970 mL of ddH_2O. Adjust pH to 7.0 and autoclave. Immediately before use, add 10 mL of 1.0 *M* $MgSO_4$ solution and 10 mL of 2.0 *M* glucose solution.
9. 50X TAE (1.0 L): Mix 242.2 g of Tris base, 57.1 mL of glacial acetic acid, 100 mL of 0.5 *M* EDTA (pH 8.0) stock solution, and add ddH_2O to 1 L.

2.3. BIBAC Library Construction

1. *Hin*dIII.
2. 0.5 *M* EDTA (pH 8.0) solution.
3. Low-melting-point agarose (BMA, SeaPlaque GTG Agarose).
4. CHEF DR II system (Bio-Rad).
5. Low-range PFG marker (NEB).

6. β-Agarase I (1.0 U/μL; NEB).
7. Nitrocellulose filters (Millipore VSWP02500).
8. 10% (w/v) polyethylene glycol (PEG).
9. pBIGRZ vector: Request to Dr Shinji Kawasaki.
10. T4 ligase with 10X buffer (NEB).
11. X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactoside).
12. Isopropylthiogalactoside (IPTG).
13. Kanamycin.
14. Freezing media: 2.5% w/v LB broth, 13 m*M* KH_2PO_4, 36 m*M* K_2HPO_4, 1.7 m*M* sodium citrate, 6.8 m*M* $(NH_4)_2SO_4$, and 4.4% v/v glycerol. Autoclave and allow media to cool to less than 50°C. In a laminar-flow hood, add $MgSO_4$ stock solution to a final concentration of 0.4 m*M*. Immediately before use, add antibiotics solution to each liter of freezing media.

3. Methods

3.1. Identification of Nipponbare BAC Clones Encompassing Initial Markers

1. To construct the high-resolution map of an *R* gene, initial co-segregating markers linked to the *R* gene should be identified by the bulked segregant analysis combined using randomly amplified polymorphic DNA ***(15)*** and/or amplified fragment-length polymorphism methods ***(14)*** using a small population of 50 individuals.
2. To pinpoint the Nipponbare genomic region corresponding to the R locus, sequence the identified initial co-segregating markers, open the AGI WebBSS URL (http://www.genome.arizona.edu/fpc/rice/), enter the query sequences, and blast your sequence against Nipponbare sequenced clones (*see* **Note 1**).
3. If the **step 2** procedure fails to identify a positive hit(s), carry out a colony hybridization experiment using your markers as a probe with two (*Hin*dIII and *Eco*RI) BAC libraries of Nipponbare that are provided by the Clemson University Genome Institute (CUGI; http://www.genome.clemson.edu/). This experiment may allow you to identify a large physical region consisting of Nipponbare BAC clones encompassing the markers (*see* **Note 2**).
4. Go to the WebFPC (http://www.genome.arizona.edu/fpc/rice/WebAGCoL/WebFPC/) and click the contig including BAC clone(s) matching your sequences.
5. Choose 6 to 20 (depending on the distance of the initials markers) of the contiguous Nipponbare BAC clones to develop internal markers that flank your gene. Details of the contig with respect to total number of BAC clones are accessible at http://www.genome.arizona.edu/fpc/rice/ (*see* **Notes 3** and **4**).
6. The Nipponbare genomic resources are provided upon request.

3.2. Development of Markers for Use in Saturation Mapping

3.2.1. Isolation of BAC DNA

1. Inoculate the *E. coli* strain (Nipponbare BAC clone) into 100 mL of TB liquid medium supplemented with antibiotics chloramphenicol (12.5 mg/L) and grow at 37°C for 12 to 16 h with vigorous shaking.

2. Harvest the bacterial cells in a blue cap tube by centrifugation at 1500*g* for 10 min.
3. Suspend the bacterial pellet in 10 mL of resuspension buffer.
4. Add 10 mL of lysis buffer, mix gently but thoroughly by inverting several times, and incubate at room temperature for 5 min.
5. Add 10 mL of neutralization buffer, mix immediately but gently by inverting several times, and incubate on ice for 15 min.
6. Centrifuge the reaction at 1500*g* at room temperature for 20 min.
7. Transfer the supernatant solution (approx 25 mL) containing plasmid DNA into new tube by filtering over miracloth promptly.
8. Add two-thirds volume (approx 17 mL) of isopropylalcohol and shake the tubes gently.
9. Centrifuge at 1500*g* at room temperature for 15 min.
10. Allow the pellets to dry at room temperature.
11. Dissolve 500 μL of 10 m*M* Tris-HCl (pH 8.0) with RNase (20 μg/mL) and transfer the solution into an Eppendorf tube.
12. Add 500 μL of phenol:chloroform (1 v:1 v) and mix gently.
13. Centrifuge the mixtures at full speed for 5 min and transfer the supernatant (approx 450 μL) into a new tube.
14. Add 800 μL of absolute ethanol, mix well, and centrifuge at full speed for 5 min.
15. Wash DNA with 500 μL of 70% (v/v) ethanol.
16. Dry the pellets and resuspend in 100 μL of 10 m*M* Tris-HCl (pH 8.0).

3.2.2. Sublibrary Construction of BAC DNA

1. Perform a series of partial restriction digests of each BAC clone with *Sau*3AI by using different amount of enzyme and the digestion time. Once the optimal conditions for producing fragments of 0.2 and 2.0 kb are determined, perform a mass digestion using several clones.
2. Separate the digestion onto 0.8% agarose gel containing 1X TAE buffer.
3. Cut the 0.2- to 2.0-kb fragments and elute DNA fragments using QIAquick Gel Extraction Kit (Qiagen), a commercial gel extraction kit.
4. Ligate the fragments with a *Bam*HI-digested and dephosphorylated pBluescriptII SK(+) or a suitable cloning vector for approx 4 to 12 h.
5. Transform the ligates into ElectroMAX DB10B cells (Life Technologies) by using CELL-PORATOR (Gibco BRL) with wet ice.
6. Remove cells from microelectroporation chamber and immediately add to 1.0 mL of SOC medium.
7. Shake gently (37°C) for 1 h.
8. Spread 10 to 100 μL on LB plates with 100 μg/mL ampicillin.
9. Incubate overnight at 37°C.
10. Randomly pick up 150 recombinant white clones, culture in LB liquid media with 100 μg/mL ampicillin, and isolate plasmids from the overnight cultured cells by a minipreparation procedure ***(16)***.

3.2.3. Development of Polymorphic Markers

1. Amplify cloned inserts by PCR using T3 and T7 primers under the following incubation condition: 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

2. With several restriction endonucleases, for examples, *Eco*RI, *Bam*HI, *Hin*dIII, or etc., digest genomic DNAs isolated from resistant and susceptible parents used in the generation of mapping population.
3. Separate the digestion on 0.8% agarose gel, and transfer DNAs onto a nylon membrane according to Sambrook et al. *(16)*.
4. Perform DNA gel-blot hybridization using the PCR products as probes and the digested DNAs as targets according to Sambrook et al. *(16)*.
5. Identify polymorphic markers which show a clear polymorphism between the resistance donor and the susceptible recipient genotypes at the DNA gel-blot analysis (*see* **Note 5**).
6. Determine the sequences of the newly generated markers by using T3 and T7 primers (*see* **Note 6**).
7. To use the markers most efficiently in the analysis of a large population, they should be converted to PCR-based markers. Design PCR primers from the sequence information of new markers using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).
8. To develop the PCR markers, amplify DNA fragments of both parents using marker-specific PCR primers at an appropriate annealing temperature, usually between 56 and 58°C.
9. Determine directly the sequence of both fragments by using PCR primers.
10. Select first the sequences containing a different restriction enzyme digestion pattern between parental genotypes to develop cleaved amplified polymorphic sequence (CAPS) markers (*see* **Note 7**).
11. To confirm that the newly developed CAPS markers show a polymorphic band patterns, digest about 10 μL (approx 200 ng PCR product) with appropriate restriction enzymes for an hour at 37°C, separate the digestions on approx 1.0% to 2.0% agarose gel, and examine the digested band patterns.

3.3. Saturation Mapping Using Newly Developed Markers

1. Isolate genomic DNA from leaves (approx 100 mg) of all growing plants according to Chen and Ronald *(17)*. At a final step, dissolve the final DNA products in 100 μL of 10 m*M* Tris-HCl (pH 8.0; *see* **Note 8**).
2. Perform PCRs using marker-specific primers in a 30-μL reaction using 1 μL (approx 50 ng) of genomic DNAs.
3. Digest each 5 μL of the PCRs in a 30-μL reaction for 1 h with selected restriction enzyme producing DNA polymorphism between both parents, and separate a half volume of the digestion on an approx 1.0 to 2.0% agarose gel (*see* **Note 9**).
4. Determine genotypes of 50 susceptible individuals at loci of all developed PCR markers by repeating **steps 2** and **3** (*see* **Note 10**).
5. Construct a genetic linkage map of the region containing your gene using Mapmarker software *(18)*. Use all segregation dataset generated from the analysis including the initially established cosegregating markers' data set.
6. Present map distances in cM between markers according to Kosambi function *(19)*.
7. A prescreening strategy to identify plants with rare recombination events around the *R* gene is useful using the closest flanking markers (*see* **Note 11**). Without the

analysis of phenotypes of all plants, this experiment can identify more recombinants between both markers. Enlarge the mapping population to approx 2000 individuals until a small physical *R* gene region of less than 200 kb is narrowed down by repeating **steps 1** to **6** (*see* **Note 12**).

8. Confirm phenotypes of all identified individuals displaying the rare recombination events in the progeny from each line by infecting rice pathogen, for example, *Magnaporthe grisea* or *Xoo*. This will distinguish heterozygous from homozygous for the resistance donor genome, enabling one to constructing an accurate map.
9. Construct a final high-resolution map of the *R* gene using all dataset by repeating **steps 5** and **6**.

3.4. Construction of BIBAC Contig Spanning the R Gene Locus

3.4.1. BIBAC Library Construction

1. Isolate high-molecular-weight DNA from young leaves of 3-wk-old rice plants carrying the *R* gene by the CTAB method described in Murray and Thompson (*[20]* *see* **Note 13**).
2. Digest high-molecular-weight DNA partially with *Hin*dIII in a 50-μL reaction. To determine the conditions that yield a maximum percentage of fragments between 25 and 40 kb, perform a series of partial restriction digests by using different amount of enzyme and the digestion time
3. Add 5 μL of 0.5 *M* EDTA to stop the reaction.
4. Load into a 0.8% low-melting agarose gel and separate in 1X TAE buffer by a pulsed-field gel electrophoresis (PFGE) device (CHEF DR II system, Bio-Rad) at 160 V using a 6-s initial and 6-s final switch time for 16 h at 14°C.
5. Cut the purified DNA from the low-melting-point agarose slice containing 25 to 40 kb (*see* **Note 14**).
6. Record the weight of the agarose slice on a balance and transfer the slice to a sterile Eppendorf tube.
7. Incubate in 2 vol of 1X β-Agarase I buffer on ice. Repeat this process five times. This step removes electrophoresis buffer from the gel slices which possibly could interfere with ligation.
8. Decant the final wash and place the tubes in a 70°C water bath for 5 min or until all of the agarose has become liquid.
9. Quickly transfer the tubes to a 42°C water bath and add β-Agarase I to each tube so that there is 1 U of enzyme for every 200 μL of molten low-melting-point agarose (*see* **Note 15**).
10. Gently swirl the contents of each tube and incubate the tubes at 42°C for an hour.
11. To concentrate the DNA solution, perform dialysis for approx 3 h against 30 mL of 10% PEG solution in a 90-mm Petri dish using a particular nitrocellulose filter (Milipore; cat. no. VSWP02500 filter, pore size 0.025 μm).
12. Place insert DNA (approx 50 ng), the *Hin*dIII-digested and dephosphorylated pBIGRZ vector (approx 10 ng), and T4 DNA ligase (10 U) with buffer in 0.5-mL microcentrifuge tubes (*see* **Note 16**). Gently mix and do not vortex as this may shear the insert DNA. The pBIGRZ vector *(21)* is available from Dr. Shinji Kawasaki (*see* **Note 17**).

13. Incubate the ligation reactions at 16°C overnight.
14. Place ligation reaction tubes in a 65°C water bath for 20 min to heat-denature the enzyme.
15. To desalt the ligated DNA, place the ligation reaction on the Milipore VSWP filter as described in **step 11**.
16. Using pipet tips, transfer all of the desalted ligation reactions into a single 0.5-mL microcentrifuge tube.
17. Transform the ligation mix by electroporation using a Cell-Porator (Gibco-BRL) into ElectroMAX DB10B cells (Life Technologies).
18. Remove cells from micro-electroporation chamber and immediately add to 1.0 mL of SOC medium.
19. Shake gently (37°C) for 1 h.
20. Spread approx 10 to 100 µL on LB plates with 100 µg/mL Kanamycin, 240 mg/ L X-gal, and 80 mg/L IPTG (*see* **Note 18**) so that approx 500 clones grow in a Petri dish.
21. Incubate overnight at 37°C.
22. Pour 4 mL of the freezing media per Petri dish and collect all colonies (approx 500) to form a BIBAC pool (*see* **Note 19**). Keep the pool at –70°C.
23. Repeat **steps 18–23** until 200 BIBAC pools are ready (*see* **Note 20**).

3.4.2. Screening of BIBAC Library for Construction of BIBAC Physical Contig

1. Fifty-microliter stock solution of each 200 BIBAC pools are cultured in 100 mL of kanamycin-containing TB media for overnight, and isolate total BIBAC plasmids as described in **Subheading 3.2.1.**
2. A pooling system for a PCR-based procedure is prepared as follows: equivalent amounts (10-µL aliquot) of DNAs purified from 10 pools are mixed to make a super pool, producing 20 super pools (*see* **Note 20**).
3. Perform a first round of PCR using each 10 ng of DNA of 20 super pools and marker-specific primers in a 30-µL reaction (*see* **Note 21**).
4. Separate a part (10 µL) of the PCR on 1.0% agarose gel and identify a super pool(s) showing a positive hit.
5. In a second round of PCR, screen the individual 10 pools making up the super pool using the same PCR condition by repeating **steps 3–5**.
6. Finally, screen more than 2000 individual clones of the identified pool by a colony blot hybridization Sambrook et al. ***(16)*** using the PCR products amplified in the second PCR as probes.
7. Culture the isolated clone in Kanamycin-containing TB media and purify BIBAC plasmid DNA as described in **Subheading 3.2.1.**
8. Determine both BIBAC end sequences using T3 and T7 primers.
9. Develop the BIBAC end sequence-specific primers for use subsequently in chromosome walking (*see* **Notes 22** and **23**).
10. Repeat **steps 3–10** until the genomic region of the *R* gene is completely covered by BIBAC clones (*see* **Note 24**).

4. Notes

1. AGI sequenced the ends of Nipponbare BAC library (92,160 clones) as part of an international effort to sequence the rice genome.
2. The filters from a *Hin*dIII BAC (OSJNBa) library of Nipponbare consist of 36,864 clones/set with an average insert size of 130 kb and corresponding to 10 genome equivalents. An *Eco*RI BAC (OSJNBb) library has an average insert size of 120 kb and 55,296 clones.
3. Most contigs consist of more than 100 BAC clones and at least 30 developed markers.
4. Nipponbare BAC contigs should provide new markers located on the Nipponbare/Kasalath linkage map (http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html), including 3267 markers. In the other way to identify Nipponbare genomic resources encompassing your markers, open the INtegrated rice genome Explorer (http://rgp.dna.affrc.go.jp/giot/INE.html), which is a database integrating the genetic map, physical map and sequencing information of rice genome, and look for BAC/PAC/YAC clones with markers.
5. The clones that are monomorphic between the parents, those with comparatively weak hybridization signals or those with multiple bands are considered inappropriate for further analysis.
6. The clones encoding a "nucleotide binding site plus leucine-rich repeat" (NBS–LRR) motif are included first. Select polymorphic markers evenly distributed or homologous to known resistance genes.
7. To avoid an error caused during PCR amplification, sequence a mixture of PCR product, instead each PCR product cloned into T-vector. In case some markers cannot be converted to CAPS markers, the amplified corresponding regions of both markers from parents are directly compared the sequence of the PCR products. This can identify a few nucleotide differences between parents.
8. The rapid DNA minipreparation method developed by Chen and Ronald ***(17)*** is suitable for avoiding cross-contamination and for PCR applications.
9. Using a higher percentage of agarose gel helps produce a clear difference between relatively small sizes of digested DNAs.
10. Fifty susceptible individuals are suitable for construction of an initial fine map. Because the score of susceptibility is more reliable than resistance because of escapes from the inoculum, first analyze all susceptible plants to make an accurate map. If skewed recombination events are expected at the locus, use of a second mapping population can be a better way.
11. When limited quarantine facilities are used for blast inoculations, the prescreening strategy is useful.
12. The recombinant lines are further analyzed using internal markers that are additionally developed later.
13. Nipponbare does not carry the *R* gene, therefore construction of a BIBAC library from the resistance donor cultivar is necessary.
14. Use the low-range PFG marker (NEB) as size standards. The marker lanes are cut and stained with ethidium bromide. Make small incisions in the marker gel slices

at 25 to 40 kb on a UV light box. Reconstruct the gel by placing the unstained gel beside the marker gel slice, and cut the 25- to 40-kb DNA-containing gel block. Never expose the genomic DNA to UV light as this will cause a break of size-selected genomic DNA.

15. β-Agarase I (NEB) works best on gels made with Tris-acetate buffer. For gels made with Tris-borate buffer, doubling the required amount of β-Agarase I is recommended.
16. If a 5:1 ratio of insert to vector does not produce a satisfactory outcome, change the ratio of insert to vector to improve the results. To use a high quality of BIGRZ vector, purify the vector by the CsCl-ethidium bromide gradient centrifugation method ***(16)***. Heat-killing HK Thermolabile phosphatase (Epicentre Technologies) easily and completely is useful. Avoid a damage of the vector DNA during dephosphorylation to prevent an appearance of false-positive clones.
17. The BIGRZ vector is capable of about 30 kb average insert. It usually yields a high transformation efficiency of rice. A BIBAC library consisting of relatively smaller insert sizes may be sufficient or even preferable for certain applications, for examples, constructing a small contiguous genomic region and complementing the clones into wild-type (i.e, susceptible) rice plants.
18. Use of a higher amount of X-gal facilitates to distinguish blue colonies from white ones.
19. After incubating test plates overnight, determine the titer of the transformation reaction and the percentages of white and blue colonies. If more than 80% of the colonies are white, select randomly 20 white colonies and perform a *Hin*dIII digestion to determine an average size of inserts. A high percentage (more than 40%) of blue clones indicates possible problems during vector preparation, ligation, or transformation.
20. In rice, the library should carry more than 100,000 clones with an average DNA insert size of 30 kb, a 5X genome equivalent. This strategy is efficient for screening a large library with small sizes of inserts. The alternative strategy of picking more than 100,000 individual clones is time-consuming and laborious.
21. To span the physical region containing the *R* gene, use four markers for the first library screening.
22. The sequenced information of each end of the isolated BIBAC clones is used to generate PCR products to screen the library again to extend the physical region. By repeating this approach, identify additional clones spanning the region.
23. In case some BIBAC-end sequences are homologous to transposable elements, they cannot be used as probes for BIBAC library screening due to their repetitive nature. To fill out the interval, subclones isolated from the *Sau3*AI shotgun library of the BIBAC clone are utilized to find the linking clone as described in **Subheadings 3.2.2.** and **3.2.3.**
24. A small missing region can be simply amplified and confirmed by its sequence analysis.

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