EXPERIMENT 2 – SHOTGUN SEQUENCING OF SCARLET RUNNER BEAN (SRB) GENOME USING MASSIVELY PARALLEL PYROSEQUENCING (OR 454 SEQUENCING)

Purpose: To introduce the concepts of assembly and annotating genomic DNA sequences generated by shotgun sequencing using the next-generation sequencing technique.

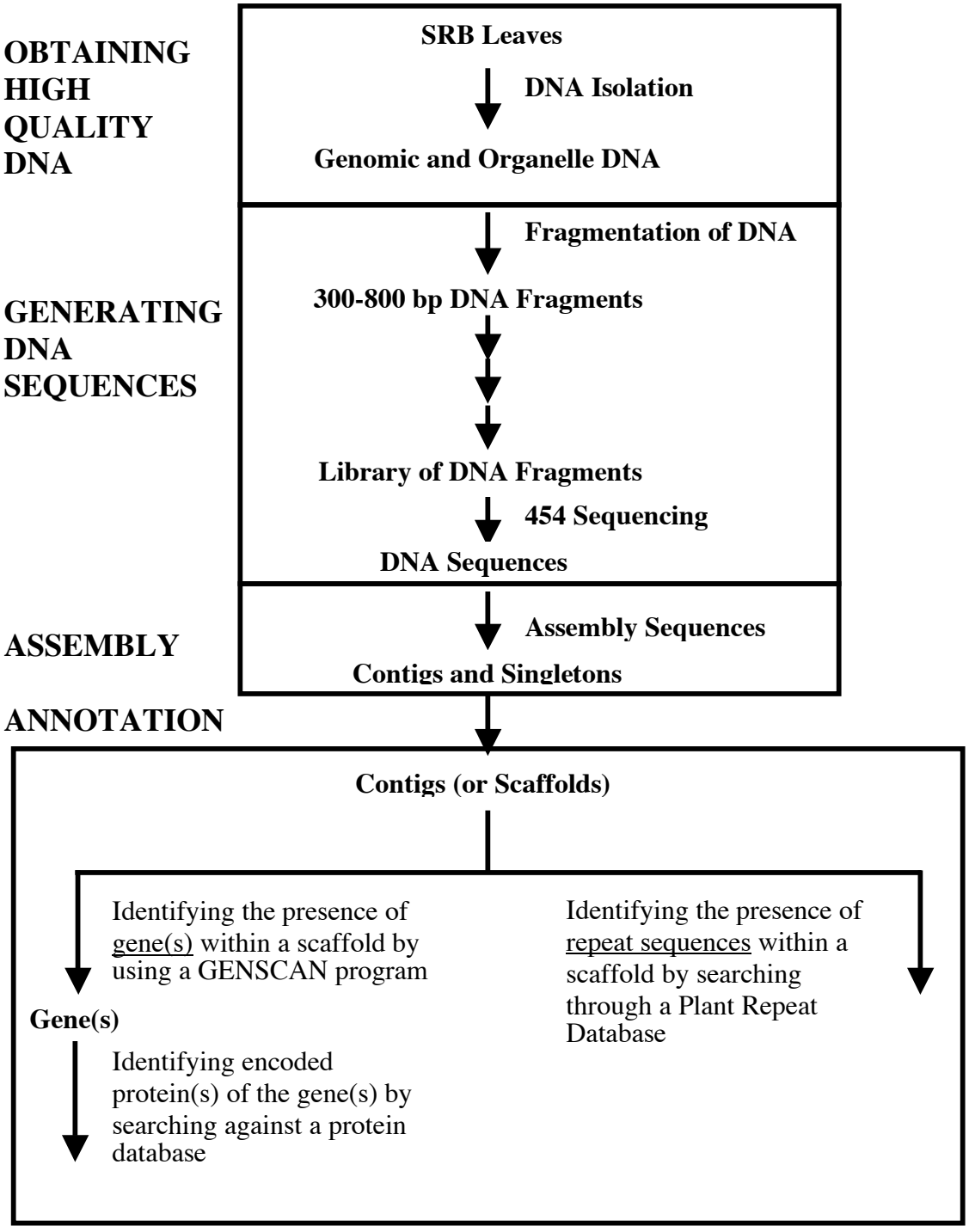
STRATEGY

I. **ISOLATION OF GENOMIC DNA FROM SRB LEAVES USING QIAGEN PLANT DNEASY MINI KIT**

II. **GENERATING DNA SEQUENCES USING 454 SEQUENCING TECHNOLOGY**

III. **ASSEMBLY AND ANNOTATING A CONTIG (OR SCAFFOLD)**

Strategy:


OBTAINING HIGH QUALITY DNA
SRB Leaves
DNA Isolation
Genomic and Organelle DNA
GENERATING DNA SEQUENCES
Fragmentation of DNA
300-800 bp DNA Fragments
Library of DNA Fragments
454 Sequencing
DNA Sequences
ASSEMBLY
Assembly Sequences
Contigs and Singletons
ANNOTATION
Contigs (or Scaffolds)
Identifying the presence of gene(s) within a scaffold by using a GENSCAN program
Gene(s)
Identifying encoded protein(s) of the gene(s) by searching against a protein database
Identifying the presence of repeat sequences within a scaffold by searching through a Plant Repeat Database

ISOLATION OF GENOMIC DNA USING DNEASY PLANT MINI KIT

References:

DNeasy Plant Mini Kit Manual (Qiagen)
454 Sequencing Technology (www.454.com)

Materials and Reagents Needed:

- Liquid nitrogen. *Caution: It is very cold (at least -210°C. Avoid getting frostbite.*
- Styrofoam container for liquid nitrogen
- Aliquots of 14-mL RNase-free tubes containing ~100 mg of frozen powder of SRB leaves stored in a -70°C freezer. *Note: Prepared by Teaching assistant(s).*
- DNeasy Plant Mini kit (Qiagen, Cat. #69104 for 50 extractions) containing AP1 extraction buffer, RNase A, AP2 buffer, AP3/E buffer
- Black ultra-fine sharpies
- 1.5-mL microcentrifuge tubes
- Racks for microcentrifuge tubes
- Pipetman set of P-10, P-20, P-200, P-1000
- Aerosol-barrier PCR tips
- Vortex mixer
- 65°C waterbath
- Gloves (small, medium, large, or extra-large)
- Microcentrifuges
- Kimwipes
- UV Spectrophotometer (Nanodrop)
- Diluted 1-kb DNA Ladder (50 ng/ μL, Invitrogen)
- Agarose
- Microwave
- 1X TAE buffer
- Gel Electrophoresis system
- Power supply
- 10 mg/mL ethidium bromide (EtBr) solution
- Gel Documentation system

PROCEDURE

1. Keep a 14-mL tube containing ~**100 mg** of frozen leaf powder in liquid nitrogen for a few minutes.
2. Set the P-1000 pipetman to **400 µL** (or 4 volumes of the sample weight).
3. Remove the tube from the liquid nitrogen container.
4. Immediately, tap the tube five times against the base of the vortex mixer to loosen the frozen powder. <u>*Caution:*</u> *Do NOT tap the tube too forcefully nor too softly!*
5. Quickly, pipet **400 µL** of buffer **AP1** and dispense it into the tube.
6. Mix the contents by vortexing the tube until the powder completely dissolved in the buffer. *It may take one minute or so to dissolve the powder. The mixture is now call the homogenate.*
7. Transfer the homogenate to a 1.5-mL microcentrifuge tube using the P-1000 pipetman and a pipet tip.
8. Add **4 µL** of **RNase A** (supplied with the kit) to the homogenate. Mix the content by flicking or inverting the tube several times.
9. Incubate the tube in the **65°C waterbath** for **10 minutes**.
10. Spin the tube briefly (5 seconds) to bring the water condensation down.
11. Add **130 µL** (or **1.3 volumes** of the sample weight) of buffer AP2 in the tube. Mix by shaking or inverting the tube. Spin the tube for 5 seconds.
12. Incubate the tube **on ice** for **5 minutes**.
13. Spin the tube at **13,000 rpm** for **5 minutes**.
14. Transfer the lysate into a **QIAshredder** (**light purple**) mini spin column sitting in a 2-mL collection tube. <u>*Note:*</u> *While pipetting the lysate, AVOID touching the pellet at the bottom of the tube.*
15. Spin the QIAshredder column and its collection tube at **13, 000 rpm** for **2 minutes**. *A liquid passed through the column is called a* ***flow-through****.*
16. Meanwhile, write on a lid of a new 1.5-mL microcentrifuge tube your initial and set the tube on a rack.
17. Transfer the flow-through from the collection tube to a newly labeled 1.5-mL tube.

18. Determine a volume of the flow-through using a P-1000 pipetman set at **530 µL**. *Note: Adjust the dial up and down to get the approximate volume.*
19. Record the volume: _____ **µL.** Multiply this volume by **1.5** for step 20 below.
20. Add **1.5 volumes** of buffer **AP3/E** to the flow-through. Mix the contents by pipetting the mixture up and down for 10 times.
21. Transfer ~**650 µL** of the mixture to a **DNeasy** (**clear**) mini spin column in a 2-mL collection tube.
22. Spin the column and its tube at **8,000 rpm** for **1 minute**. Pour off the flow-through solution in a beaker labeled "Waste".
23. Repeat steps 21 & 22 for the remaining volume of the mixture at step 20.
24. Transfer the **DNeasy** mini spin column into a NEW 2-mL collection tube.
25. Pipet **500 µL** of buffer **AW** to the DNeasy mini spin column.
26. Spin at **8,000 rpm** for **1 minute** to wash the membrane in the column.
27. Pour off the flow-through in the beaker labeled "Waste".
28. Wash the column with another **500 µL** of buffer **AW**. Spin at **13,000 rpm** for **2 minutes**. *Inspect the membrane of the column to ensure that the membrane is dry.*
29. Meanwhile, label a new 1.5-mL microcentrifuge tube according to the sample gDNA, your initial, and date.
30. Transfer the DNeasy spin column to the newly labeled tube.
31. Pipet **50 µL** of buffer **AE** or **sterile water** to the center of the membrane.
32. Incubate the column for **5 minutes** at **room temperature**.
33. Spin the tube and column at **8,000 rpm** for **1 minute**. *Caution: Do NOT discard the flow-through solution, which is the genomic DNA solution.*
34. Repeat steps 31-33.
35. Discard the spin column and its collection tube.
36. Close the lid of the microcentrifuge tube containing the isolated genomic DNA solution and put the tube on ice.
37. Determine the total volume of the genomic DNA solution using a P-200 pipetman and an aerosol-barrier PCR P-200 pipet tip. *Note: there is approximately 98* ***µL*** *of the genomic DNA solution. So, you can set the P-200 pipetman to 98.*

38. Determine the concentration of DNA solution using a Nanodrop UV spectrophotometer. Your TA will help you to use the spectrophotometer. Determine the total amount of isolated genomic DNA.
39. Determine the quality of the isolated genomic DNA on a 0.7% agarose/ethidium bromide gel along with the 1-kb DNA ladder. <u>*Note:*</u> *(1) Your TAs will prepare the agarose gel for you.* (2) *Two students will share one agarose gel.* (3) *As little as 60 ng of genomic DNA can be observed on the agarose/EtBr gel.*
    a. Label a new 1.5-mL microcentrifuge tube as "**SRB gDNA**", **your initial, date**.
    b. Pipet 10 μL of the genomic DNA solution into the new labeled tube.
    c. Add 1.5 μL of 6x Loading dye to the DNA solution. Discard the pipet tip. Flick the tube to mix its contents. (Option) Spin the tube for 5 seconds to bring all liquid droplets to the bottom of the tube.
    d. Pipet 10 μL of a ready-to-use 1-kb DNA ladder (50 ng/μL) into a well of the 0.7% agarose gel.
    e. Pipet 11.5 μL of the DNA/loading dye mixture to the next well adjacent to the 1-kb ladder well.
    f. Record the loading pattern of your sample on the agarose gel below:

    g. Pipet 10 μL of 10 mg/mL Ethidium bromide stock solution to the running TAE buffer near the bottom of the gel.
    h. Cover the gel box with its lid.
    i. Connect the electrodes according to their colors to the power supply's plug-ins (example, RED to RED and BLACK to BLACK)
    j. Switch on the power supply and adjust the voltage to ~120 volts.
    k. Run the gel for 1-1.5 hrs.
    l. Record the Starting time: _____ and the Ending time:_____ .
    m. After electrophoresis, turn off the power supply, slide the lid off the gel box, transfer the gel and the gel cast into a plastic container, carry the container to

the gel documentation for picture taken. Your TAs will help you taking the picture of your gel.

n. Place the gel picture in a glassine envelope that is pasted on this page.

o. Analyze the quality of the isolated genomic DNA sample.
   Is the DNA intact or fragmented?
   What is the estimate size of DNA?

40. Store the tube of genomic DNA solution in your assigned white box in the refrigerator.