APPENDIX

Appendix 1 DNeasy? Plant Mini Kit Protocol

1. Grind the plant leaves into a white powder.
2. Add 400 ?l Buffer AP1 and 4 ?l RNase A stock solution (100 mg/ml) to a maximum of 100 mg (wet weight) or 20 mg (dried) disrupted plant tissue and vortex vigorously.
3. Incubate the mixture for 10 min at 65?C. Mix 2 or 3 times during incubation by inverting tube.
4. Add 130 ?l Buffer AP2 to the lysate, mix, and incubate for 5 min on ice. Centrifuge the lysate for 5 min at 14000 rpm.
5. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 14,000 rpm.
6. Transfer the flow-through fraction from step 5 into a new tube without disturbing the cell-debris pellet.
7. Add 1.5 volume of Buffer AP3/E to the cleared lysate, and mix by pipetting
8. Pipet 650 ?l of the mixture from step 7, including any precipitated that may have fromed, into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at 8,000 rpm and discard the flow-through. Reuse the collection tube in step 9.
9. Repeat step 8 with remaining sample. Discard flow-through and collection tube.
10. Place the DNeasy Mini spin column into a new 2 ml collection tube, add 500 ?l Buffer AW, and centrifuge for 1 min at 8,000 rpm. Discard the flow-through and reuse the collection tube in step 11.
11. Add 500 ?l Buffer AW to DNeasy Mini spin column, and centrifuge for 2 min at 14,000 rpm to dry the membrane.
12. Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml microcentrifuge tube and pipet 100 ?l Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15-25 ^{o}C), and then centrifuge for 1 min at 8,000 rpm to elute.

Appendix 2 Digestion Reaction of Genomic DNA with *Rsa*I

Reaction mix

40 ?l total genomic DNA
6 ?l 10X restriction buffer
4 ?l *Rsa*I (10U/?l)
10 ?l distilled water
60 ?l total volume

After preparation the reaction mixture was incubated at 37^{o} overnight. Digested products were checked in 1% agarose gel.

Appendix 3 MinElute™ Gel Extraction kit Protocol

1. Add 3 volumes of Buffer QG to 1 volume of PCR products.
2. Add 1 volume of isopropanol to the sample and mix by inverting the tube several times
3. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.
4. To bind DNA, apply the same to the MinElute column, and centrifuge at 13,000 rpm for 1 min.
5. Discard the flow-through and place the MinElute column back in the same collection tube.
6. Add 500 ?l of Buffer QG to the spin column and centrifuge at 13,000 rpm for 1 min.
7. Discard the flow-through and place the MinElute column back in the same collection tube.
8. To wash, add 750 ?l of Buffer PE to the MinElute column and centrifuge at 13,000 rpm for 1 min.
9. Discard the flow-through and centrifuge the MinElute column for and additional min at 13,000 rpm
10. Place the MinElute cloum into a clean 1.5 microcentrifuge tube.

11. To elute DNA, add 10 ?l of Buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.

Appendix 4 Reaction Mix and PCR program for checking Ligation of adaptors

Reaction Mix

55 ?l	digested DNA
7.0 ?l	10X buffer
1.5 ?l	T_4 ligase (10U/?l)
6.5 ?l	adapter mix (10 pm/?l)
70 ?l	total volume

PCR Program

94 °C	3.0	min	
94 °C	30	sec	} 30 cycles
54 °C	30	sec	
72 °C	1.30	min	
72 °C	7.0	min	

Appendix 5 Reaction Mix and PCR program for checking Hybridization of Oligoneucleotide probe

Reaction Mix

1.0 ?1	DNA template
1.0 ?1	10 mM dNTPs
5.0 ?1	the 21 mer oligonucleotide (10 pmol/?1)
2.5 ?1	10X PCR buffer
0.5 ?1	Taq polymerase (5U/?1)
15.0 ?1	ultrapure water
25.0 ?1	Total volume

PCR Program

94 ^{o}C	3.0	min	
94 ^{o}C	30	sec	30 cycles
54 ^{o}C	30	sec	
72 ^{o}C	1.30	min	
72 ^{o}C	7.0	min	

Appendix 6 Reaction Mix and Condition require for Cloning into a plasmid vector

Reaction Mix

3.0 ?1	DNA fragment (~ 50 ng/?1)
5.0 ?1	2X Rapid buffer
1.0 ?1	pGEM-T Easy vector (50 ng/?1)
1.0 ?1	T4 Ligase
10 ?1	Total volume

The reaction was incubated at 16 ^{o}C overnight.

Apendix 7 Preparation of DH5α competent cells

1. Grow a 5 mL overnight culture of bacteria in incubate shaker (150 rpm, 37 ^{o}C).
2. Dilute 1:10 and add SOB medium up to 50 ml, shake at 37 ?C for 5 hr.
3. When cells reach proper density, transfer *E. coli* solution to 1.5 ml tube (1.5 ml/tube) and keep the cells on ice for 10 minutes.
4. Spin down *E. coli* solution at 4000 rpm for 15 minutes at 4 ^{o}C.
5. Discard supernatant and keep cells on ice.
6. Resuspended the pellet in 750 ?l of cold $CaCl_2$.
7. Centrifuge cells at 500 to 1000 g for 5 min at room temperature.
8. Pour off supernatant. Be vary careful not to disturb the diffuse cell pellet.
9. Resuspended the pellet in 100 ?l of cold $CaCl_2$.
10. To store competent cells, add sterile glycerol to final concentration 15%.
11. Keep at -80 ?C

Appendix 8 Heat Shock Transformation Protocol

1. Thaw frozen cells on ice, 10-15 minutes.
2. Add 100 ?l of the cells to ligation reaction.
3. Incubate on ice for 30-45 minutes.
4. Heat shock the cells by transferring the tubes into a 42 ^{o}C water bath for 3.30 minutes
5. Place the tube on ice and incubate for 3 minutes.
6. Add 1 ml of LB medium to each tube. Incubate the tubes at 37 ^{o}C with gentle shaking for 45 minutes
7. Plate the cells using 1/10 dilution and pipette cells onto LB plates. Spread the cells by dragging the cell suspension across the agar surface with spreader back and forth several times.
8. Replace the lid and let the plates stand until all liquid is absorbed into the agar.
9. Plate the cells upside down in a 37 ^{o}C incubator, and incubate them for 15-18 hours.

Appendix 9 Reaction mix and PCR program for colony

Reaction Mix

1.0	?l	colony
2.0	?l	2.5 mM dNTPs
1.0	?l	T7 primer (10 pmol)
1.0	?l	SP6 primer (10 pmol)
2.0	?l	2.5 mM dNTPs
1.5	?l	10X PCR buffer
0.15	?l	Taq polymerase
6.65	?l	ultrapure water
15	?l	total volume

PCR Program

94 °C	4.0	min	
94 °C	1	min	30 cycles
45 °C	1	min	
72 °C	1	min	
72 °C	10	min	
4 °C	forever		

Appendix 10 Plasmid extraction by GenElute™ Plasmid Miniprep Kit

1. Harvest cells by transferring the appropriate volume of the recombinant *E. coli* culture to a microcentrifuge tube and pellet cells at ≥ 12,000 rpm for 1 minute. Discard the supernatant.
2. Resuspend the bacterial pellet with 200 ?l of the resuspend solution. Vortex or pipette up and downto thoroughly resuspend the cells until homogeneous.
3. Lyse the resuspended cells by adding 200 ?l of the lysis solution. Immediately mix the contents by gentle inversion (6-8 times) until the mixture becomes clear and viscous. Do not vortex.
4. Neutralize the cell debris by adding 350 ?l of the neutralization/binding solution. Gently invert the tube 4-6 times. Pellet the cell debris by centrifuging at ≥ 12,000 rpm for 10 minutes.
5. Prepare column by inserting a GenElute Miniprep Binding column into a provide microcentrifuge tube. Add 500 ?l of the Column Preparation Solution to each miniprep column and centrifuge at ≥ 12,000 rpm for 30 seconds to 1 minute. Discard the flow-through liquid.
6. Transfer the cleared lysate from step 4 to the column prepared in step 5 and centrifuge at ≥ 12,000 rpm for 30 seconds to 1 minute. Discard the flow-through liquid.
7. Wash column by adding 750 ?l of the diluted wash solution to the column. Centrifuge at ≥ 12,000 rpm for 30 seconds to 1 minute.
8. Transfer the column to a fresh collection tube. Add 100 ?l of Elution Solution or molecular biology reagent water to the column.

Appendix 11 Reaction mix and PCR program for sequencing

Reaction Mix

2.0	?l	2X ready Reaction Mix
1.0	?l	5X BigDye Sequencing Buffer
3.2	?l	T7 primer (10 pmol)
0.8	?l	Template
3.3	?l	ultrapure water
10	?l	total volume

PCR Program

96 °C	1	min	
96 °C	10	sec	25 cycles
48 °C	5	sec	
60 °C	4	min	
4 °C	forever		