# Chapter 3

# Materials and Methods

The present investigation of Genome editing through CRISPR-Cas9 for Improvement of Beta Carotene in Groundnut [*Arachis hypogaea* (L.)] was carried out at the Department of Biotechnology, Atmiya university and Junagadh Agricultural University, the detailed information about materials used methods adopted for the present research work is elaborated in this chapter.

## 3.1 Carotenoids Extraction and Estimation

### 3.1.1 Preparation of Carotenoids Standards and Standard Curve

The carotenoid standards were purchased from Sigma-Aldrich, US and CaroteNature GmbH, Switzerland. The standards were dissolved into respective solvents, and dilutions were prepared following the manufacturer's instructions. Dilutions were made using tetrahydrofuran (THF) and were injected into High-Performance Liquid Chromatography (HPLC).

### 3.1.2 Carotenoids Extraction

**Solutions used:**

1. **Extraction buffer:** Diethyl ether (DEE) (7): methanol (MeOH) (3) + butylatedhydroxytoluene (BHT) (0.1%)
2. **Pre-wash solution:** Methanol (40%) + magnesium carbonate ($MgCO_3$) (0.5 %)
3. **Wash solution:** Saturated sodium chloride (NaCl) (2): 10% anhydrous sodiumsulfate ($Na_2SO_4$) (1)
4. **Mobile phase I:** MeOH (95%) + HPLC-grade water (5%)
5. **Mobile phase II:** Methyl tert-butyl ether (MTBE)

### 3.1.3 High-Performance Liquid Chromatography (HPLC)

Carotenoids were separated using a YMC C30 column (YMC, Kyoto, Japan) equipped with an autosampler (Waters, 2767) and photodiode array (Waters, 2998) in

an HPLC system (Waters, Milford, MA, USA). A gradient was encoded into the mobile phases I and II. For calibration, comparison of retention times, and quantification, carotenoid standards (lutein, β-carotene, and α-carotene) were employed. Data analysis and quantification were done at 450 nm using Mass Lynx ™ software.

## 3.2 Glassware, Plasticware and Accessories

All Glassware used during this study were bought from Borosil, India whereas plasticwares from Tarson, India. The disposable single use tissue cultured petri dishes bought from HiMedia were gamma irradiated sterilized.

### 3.2.1 Chemicals

All chemicals such as, MS medium, plant growth regulators (PGRs), vitamins, antibiotics, Luria Bertani etc, used during experiment were of analytical grade and purchased from HiMedia, India.

### 3.2.2 Cleaning and Sterilization of Glassware and Accessories

All Glassware, plasticwares and experiment aids used were cleaned by soaking them overnight into solution of potassium dichromate and hydrochloric acid/ sulphuric acid. Oily or greasy stains were removed by washing with HiClean solution, followed by washing with running tap water, there after thoroughly rinsed with RO water to remove salt adhered, and dried in a hot air oven at 70°C. The accessories like forceps, scalpel, blade holders, etc., were cleaned with 95% ethanol.

### 3.2.3 Sterilization of Equipment

Since plant tissue culture manipulation and transformation required ambient environment and aseptic handling, all the equipment were sterilized with appropriate sterilizing agent.

To avoid any cross contamination from research aids such as test tubes, glass bottles, petri plates, forceps, scalpel blade holder, milliQ water, Whatman filter paper etc. were autoclaved at 121°C, for 20 min before brought into the culture room. Heat labile research aids such as micro pipettes were sterilized by wiping with 95% ethanol.

### 3.2.4 Sterilization of laminar airflow cabinets

All the aseptic transfer or manipulation works like sterilization of seeds, preparation and inoculation of explants, subculture, revival of Agrobacterial strains, transformation, etc. were carried out in a sterile laminar flow cabinet. Before starting any hygienic operation, the UV lamp provided within the hood switched on for 30 minutes prior to use followed by the airflow, and swabbing of working platform and walls with 95 per cent ethyl alcohol to ensure total sterile conditions. Before taking the materials into the laminar airflow cabinet, they were sprayed with 95 % ethyl alcohol. In case of glassware, the mouth of the culture bottles, culture tubes, petriplates, etc. was flamed before and after the use. All works inside LAF were performed by wearing surgical gloves and mask. Before starting the experiment, the hands were sterilized with Hicare hand sanitizer. While transfer of seeds or explant to the culture bottles/plates/tubes, the instruments were dipped in absolute alcohol and flame sterilized.

### 3.2.5 Sterilization of materials used in the transformation work before and after

All transformation required axenic handling of desirable microbes and avoid of cross contamination so, to maintain such environment after every tissue culture and transformation work, each and every material and equipment used were sterilized by spraying with 95% ethanol followed by autoclaving at 121°C for 20 min. Used media was autoclaved before disposing. Any spillage/overflow of bacterial culture was swabbed with 1N NaOH to denature the recombinant nucleic acid.

### 3.2.6 Sterilization of chemicals and growth regulators

The heat labile chemicals like, growth regulators, antibiotics were filter sterilized in the sterile laminar airflow chamber with the 0.22 µm pore size Millipore filtration membranes while other than heat liable chemicals were sterilized by autoclaving at 121ºC for 15 to 20 min.

## 3.3 Tissue Culture Media

MS basal medium (Murashige and Skoog, 1962) augmented with various PGRs was used for the study.

### 3.3.1 Preparation of Stock Solutions of MS Salts and PGR

MS media salt stocks solutions of macronutrients, micronutrients, iron and vitamins were prepared separately and kept in separate screw cap amber reagent bottle. Stock solutions of macronutrients (Stock-A) was prepared at a strength of ten times the final concentration (10X) required to make a liter of the nutrient media, micronutrients, iron and vitamins (Stock-B, Stock-C and Stock-D) were prepared at a strength of 100 times the final concentration (100X) required to make a liter of the nutrient media. Each chemical was dissolved separately in small quantities of milliQ water. The components of each stock were mixed and the final volume was made up using autoclaved milliQ water.

#### 3.3.1.1 Macro-nutrient Stock: Stock-A (10X)

All the Macro-nutrient components of MS salts (Appendix I) were dissolved in 500 ml autoclaved milliQ water on a magnetic bead stirrer to dissolve completely and evenly. Final volume was made up to one liter and stored in reagent bottle in a refrigerator at 4ºC.

#### 3.3.1.2 Micro-nutrient Stock: Stock-B (100X)

Micro/or minor nutrient chemicals (Appendix I) were dissolved in 500 ml autoclaved milliQ water on a magnetic bead stirrer and the final volume was made up to one liter and stored in reagent bottle in a refrigerator at 4ºC.

#### 3.3.1.3 Iron stock: Stock-C (100X)

The chemical components of Iron stock (Appendix I) were dissolved separately in 100 ml autoclaved milliQ water on a magnetic bead stirrer. $Na_2EDTA.2H_2O$ and $FeSO_4.7H_2O$ were dissolved in separate beaker, boiled for minutes to mix well. Final volume was made up to 500 ml and boiled for a few minutes until solution turned clear. The solution was cooled to room temperature and stored in amber reagent bottle at 4ºC in a refrigerator.

### 3.3.1.4 Vitamins stock: Stock-D (100X)

All the chemical components of vitamins (Appendix I) were dissolved in 100 ml autoclave MilliQ water on a magnetic bead stirrer and the final volume was made up to 500 ml and stored in reagent bottle at 4ºC in a refrigerator.

### 3.3.1.5 Carbon source

HiMedia supplied analytical-grade sucrose, which was utilized as the carbon source in MS medium. Using a magnetic bead stirrer, new sucrose was added to the MS media at a concentration of 3% w/v each time, and the medium was then filled to the final volume.

### 3.3.1.6 Gelling agent

In all regeneration and transformation works tissue culture tested grade of agar was used as gelling agent. For tissue culture agar was used at a concentration of 0.8% (w/v), whereas for growth/revival of bacterial strains it was used at a concentration of 1.5% (w/v). For seed germination and rooting of tissue cultured plants 0.4% of agar was used.

## 3.4 Growth regulator stocks

All growth regulator stock solutions used in the experiment were made independently at a concentration of 1 mg/ml, covered in aluminum foil, and kept in the refrigerator at 4 °C.

### 3.4.1 BAP stock solution (1 mg/ml)

To prepare 1 mg/ml 6-Benzyl amino purine (BAP) stock solution 100 mg powder form of the BAP dissolved in around 2.0-3.0 ml of 1N NaOH, when powder get dissolved completely volume was made up to 100 ml with MilliQ water under the sterile laminar air flow chamber. Finally, homogenously dissolved BAP solution was filter sterilized (0.22µm) and kept in refrigerator at 4ºC, hormone was shaking well every time before use in MS media, if any precipitation observed fresh stock solution was prepared.

### 3.4.2 IBA stock solution (1 mg/ml)

100 mg of the growth regulator powder was dissolved in 2.0–3.0 ml of 1N NaOH to create the indole-3-butyric acid (IBA) stock solution. The volume was then increased to 100 ml using a MilliQ water filter that had been sterilized (0.22µm) in a laminar air flow chamber and stored in a refrigerator at 4°C.

### 3.4.3 GA stock solution (1 mg/ml)

Gibberellic Acid (GA) stock solution was prepared by dissolving 100 mg of the growth regulator powder in 100 ml with MilliQ water filter sterilized (0.22µm) under the laminar air flow chamber and kept at 4°C in refrigerator.

## 3.5 Antibiotic stocks

### 3.5.1 Kanamycin stock solution (100 mg/ml)

500 mg of kanamycin powder was dissolved in 2.0–3.0 ml of MilliQ water to create a kanamycin stock solution, which was then topped up at 5 ml. The kanamycin solution was kept at 4°C after being filter sterilized (0.22µm) in a laminar air flow chamber.

### 3.5.2 Cefotaxime stock solution (100 mg/ml)

Cefotaxime stock solution was prepared by dissolving 1000 mg (1gm) of the cefotaxime powder in 2.0-3.0 ml of MilliQ water and the final volume was made up to 10 ml, sterilized by filtration under (0.22 µm) the sterile laminar air flow chamber and stored at 4°C.

## 3.6 Preparation of Plant Tissue Culture Medium

Half of the volume of MS media needed was added to a clean beaker filled with MilliQ water, and the appropriate amounts of the different stock solutions (Appendix I) were measured and added. A magnetic bead stirrer was used to add and dissolve the necessary amount of sucrose. once every component—aside from the gelling agent—has been added and well combined. Using a digital pH meter and stirring, 1N NaOH/ 1N HCl was added to bring the medium's pH down to 5.7–5.8. A measuring cylinder

was then used to make up the remaining volume of the solution. After adding the necessary amount of agar (0.8%) to the medium, it was well mixed by boiling. The medium was autoclaved for 15 minutes at 121°C and 15 pounds of pressure to sanitize it. Heat liable PGRs were added in media after filter sterilization under laminar hood, other than head liable PGRs are autoclaved along with MS media.

### 3.6.1 Cultural Conditions

The plant tissue culture experiments were conducted under well controlled conditions of the culture room. The culture room maintained at 25º ± 2ºC, uniform light (cal 1000 lux) provided by fluorescent tubes for a light/dark cycles of 16/8 hour. All the aseptic manipulations were carried out under horizontal laminar air flow chamber. Before any work to start laminar flow was pre-sterilized with ultraviolet light provided inside for 30 minutes and wiping working platform with absolute alcohol. Sterilized instruments were used for inoculation. Defined precautions were taken to maintain aseptic conditions inside the cabinet. Laminar flow was fumigated with potassium permanganate and formaldehyde (37%) with interval of 15 days and culture room once in two months.

### 3.6.2 Preparation of media bottles/plates/tubes

After autoclaving the media was brought into the culture room and cooled up to the temperature tolerated onto back side of hand. Outer surface of media bottle sprayed with 70% ethanol and then took into the pre-sterile laminar hood. Required plant growth regulators, antibiotics, etc, were added by filter sterilization then poured into the desirable culture vessels. About 10 ml of the medium was poured into 150 x 25 mm sterile culture tube or 50 ml into sterile tissue culture bottle and magenta boxes or 40 ml into 110 X 40 mm tissue culture sterile petri dishes. Before and after pouring the media, mouth of the screw cap bottle containing the media was flamed in order to prevent microbial contamination. Cap/lid of the culture vessel kept opened till the media get solidified to prevent accumulation of vapor. The media containing vessels bottles/plates/tubes were stored in culture rooms at 25± 2ºC. Inoculation of seeds or explants was performed within one week ensuring that the containers are free from microbial contamination.

### 3.6.3 Luria Bertani (LB) Medium

LB medium was used for the revival and preparation of fresh cultures of original and transformed *Agrobacterium tumefaciens* strains containing the p201N vector, gRNA and cas9 construct. The LB powder was dissolved in required volume of MilliQ water, 1.5% (w/v) tissue culture grade agar was added and sterilized by autoclaving at 121ºC for 15 min. Rifampicin/ Kanamycin and Rifampicin were added into sterilized molten or liquid LB by filter sterilization.

## 3.7 Optimization of protocol for *in vitro* regeneration of Groundnut GGJ 20

The seeds extracted from ripe, dried pods were rinsed for five minutes under running water. The seeds were moved to a sterile flask in the laminar airflow cabinet and surface sterilized by adding 70% ethanol to it. For 30 seconds, the seeds were washed. After the ethanol was removed, 0.1% HgCl2 was added, and the mixture was gently stirred for three minutes. The $HgCl_2$ solution was disposed of after three minutes, and the seeds were washed three to four times with sterile distilled water (Radhakrishnan *et al.,* 2000). In the laminar hood, the testa of the sterilized seeds was aseptically extracted using forceps and a sterile scalpel. After dividing the seeds into two halves, or cotyledons, the embryo that was joined to the cotyledons was removed, a process known as de-embryonation. Direct use of the de-embryonated cotyledons as explants for culture was made. A sharp surgical blade was used to gently remove each leaf from the bunch that was present at the embryonic axis. De embryonated cotyledonary were cut in half with a scalpel blade and carefully placed on a 110 x 40 cm petri plate that contained full strength MS basal media supplemented with different amounts and combinations of plant development regulators (Table 3.1, 3.2 & 3.3).

### 3.7.1 Multiplication and elongation of shoots

The regenerated shoots were transferred to fresh shoot multiplication medium for multiplication and elongation of shoots by keeping the combination and concentration of growth regulators as mentioned in (Table 3.1 & 3.2).

### 3.7.2 Root regeneration from *in vitro* developed shoots and development of complete plantlets

To acquire full plantlets, the regenerated shoots from De embryonated cotyledon explants were moved to root induction media that contained MS basal medium along with varied concentrations of different auxins (NAA) (Table 3.3).

### 3.7.3 Hardening and acclimatization of *in vitro* regenerated plantlets

To acquire full plantlets, the regenerated shoots from De embryonated cotyledon explants were moved to root induction media that contained MS basal medium along with varied concentrations of different auxins (NAA) (Table 3.3).

**1. Washing of roots**

Following appropriate root growth in vitro, the plantlets were removed from the tubes without causing any harm to their root system. To get rid of the clinging media, the roots were gently cleaned under running tap water. To prevent wilting after being transferred to potting mixture, plantlets were maintained in a beaker filled with Hoagland solution after the medium was removed.

**2. Preparation of potting mixture**

Potting mixture was prepared by mixing sandy soil and cocopeat in equal proportion. The mixture was sterilized by autoclaving at 121ºC for 20 minutes at 15 pound per square inch in autoclavable bag. After sterilization the plastic glasses were filled ¾ parts with potting mixture.

**3. Planting in potting mixture pots**

The *in vitro* regenerated plantlets after washing with water transferred to potting mixture. The root portion was placed inside the mixture gently and covered with same. After transfer of the regenerated plantlets into potting mixture they were watered with 0.5% bavistin solution and covered with polythene bags to maintain the relative humidity. Plantlets were sprayed with Hoagland solution once a day to fulfill high relative humidity and nourishment. Plantlets were placed inside net house for further development. After 20 days the polythene bags were removed and kept in shade area.

Few weeks later plants were exposed to direct sunlight for two hours a day. This treatment was continued for two more weeks. Lastly the plants were placed in natural environment. At this stage leaves were dark green than it was before and stem showed secondary thickness.

## 3.8 Guide RNA designing and synthesis

The requirement to accomplish minimal editing at unwanted places (off-target editing) and extremely efficient editing at the desired region (on-target editing) presents a problem in designing the best guide RNA (gRNA) sequences for CRISPR systems. Computer forecasts are nearly always selected because of their speed and affordability, even if laboratory validation should ideally be utilized to detect off-target activities. Therefore, a number of investigations have looked at gRNA-DNA interactions to learn more about how CRISPR complexes choose their genetic targets. CHOPCHOP (https://chopchop.cbu.uib.no/) makes use of these advancements to provide an intuitive online interface that assists users in creating the best gRNAs. Numerous CRISPR applications, including as gene knock-out, sequence knock-in, and RNA knock-down, are supported by CHOPCHOP.

To design guide RNA for targeted editing of **lycopene epsilon cyclase** (*LCYE*) gene, sequences of LCYE was retrieve from NCBI). The actual size of gene is 5821 bp. Primers were designed using NCBI Primer blast to amplify LCYE genes with PCR, to select a genomic target (Table 3.4). PCR products were purified, sequenced and result was analyzed. Based on the sequencing result 19bp gRNA (Table 3.5) was designed from online gRNA designing tool (https://chopchop.cbu.uib.no/) upstream to PAM sequence (5'-NGG). CHOP-CHOP is a web-based tool to design gRNA for CRISPR, it simplifies the sorting process fora CRISPR guide sequence in a DNA sequence input. Optimally the gRNA sequence should be 21 base pair (bp) excluding PAM sequence however it can be around 18- 25bp. Larger gRNA (25bp) has less efficiency whereas smaller has maximum off- target probability, in that view 19bp gRNA was designed. Guide RNA-Cas9 plasmid construct was ordered by Addgene and gRNA synthesized by IDT (Integrated DNA Technologies.

## 3.9 Plasmid isolation

As directed by the manufacturer, the culture obtained from Addgene was re-streaked on a LA plate using 50 mg/l kanamycin. Single colonies were put on LB broth for plasmid isolation following colony development. The manual alkaline lysis procedure was used to isolate the plasmid. Cells were pelleted in a 2 mL microcentrifuge tube by centrifuging them repeatedly for 5 minutes at 10,000 rpm and discarding the supernatant. After dissolving the pellet in 200 µL of solution I (Table 3.7) and gently vertexing, 400 µL of freshly made lysis solution II (Table 3.7) was added. The mixture was well mixed by inverting, and it was then incubated at room temperature for three minutes. 300 µL of solution III (Table 10) was added, and the mixture was incubated for 10 minutes at 4°C. Equal volumes of chloroform and isoamyl alcohol (24:1) were added, and well mixed. Centrifuge the material for 20 minutes at room temperature at 12,000 rpm. In a new centrifuge tube, collect the top layer of aqueous material, add an equal amount of cold isopropanol, flip the tubes, and incubate for 15 minutes at -20°C. Centrifuge the material for 20 minutes at 4 °C and 12,000 rpm. After discarding the supernatant, 400 µL of 70% cooled ethanol was added. Centrifuge the material for 10 minutes at 4 °C and 10,000 rpm. The particle was allowed to air dry while the supernatant was discarded. 30 µL of autoclaved distilled water was used to dissolve the pellet, which was then left at room temperature for 15 min.1 µL of RNase added to the plasmid and incubated it at 37 ºC for 1 hour.

#### 3.9.1.1 Agarose gel electrophoresis

Samples of isolated plasmid DNA were electrophoresed on an agarose gel. TAE buffer (1X) and molecular grade agarose (Himedia, India) were used to create the 0.8% gel (Table 3.8). After thoroughly boiling the agarose in the microwave and allowing it to cool to room temperature, ethidium bromide was added, well mixed, and then poured onto a gel casting tray to prevent air bubbles. The comb was put into the gel made of agarose. Following full gel solidification, the plasmid DNA product was combined with 1X gel loading dye and injected onto the agarose gel wells in the electrophoresis tank that had been dipped in TAE buffer (1X). Electrophoresis was used to resolve the gel for 45 minutes at a steady 60 volts. To observe the electrophoresed agarose gel, a gel documentation system (Bio-Rad,USA).

#### 3.9.1.2 Digestion of CRISPR/Cas9 p201Nvector

To linearize the vector, the p201B CRISPR/Cas9 was digested. With a final reaction volume of 20 µl, digestion was carried out using the SwaI restriction enzyme (Table 3.9). Three microliters of plasmid, one microliter of enzyme, and two microliters of the cut smart buffer comprised the reaction mixture; the remaining volume was filled with sterile water. Overnight, the reaction mixture was incubated at 37$^{o}$C. Agarose gel electrophoresis was used to resolve the digested sample, and a gel documentation equipment (Biorad-USA) was used to take pictures. By comparing the bands on a 1 kb DNA ladder, the size of the restriction enzyme-digested product was calculated (Thermo Scientific, USA). (Figure 4.7)

## 3.10 Gel purification of the digested product

### 3.10.1 PCR product purification

PCR product purification was performed using Qiagen QIAquick kit. QIA quick protocol is designed to purify single or double stranded DNA fragments from PCR. From this kit fragments ranging from 100 bp to 10 kb can be purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

**Preparation**

Ethanol (96–100%) added to Buffer PE before use as mentioned on the bottle. 1:250 volume of pH indicator I was added to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH ≤7.5, because adsorption of DNA to the membrane is only efficient at pH ≤7.5.

**Procedure**

1. Five times the PCR product (500 µl of Buffer PB to 100 µl of PCR sample) was added to the PCR sample.
2. QIAquick spin column was placed in a provided 2 ml collection tube.
3. To bind DNA, the PCR sample was put onto the column and centrifuged for one minute at 13,000 rpm.
4. Flow-through coming from the column was discarded and QIAquick column

placed back into the same tube.

5. To wash, 750 µl Buffer PE was added to the QIAquick column and centrifuge for 1 min at 13,000 rpm.
6. After discarding the flow-through and placing it back in the same tube on the QIAquick column, the column was centrifuged for one minute at 13,000 rpm.
7. QIAquick column placed in a clean 1.5 ml microcentrifuge tube.
8. The column was centrifuged for one minute after 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O was added to the center of the QIAquick membrane in order to elute DNA. 30 µl of elution buffer was added to the center of the QIAquick membrane to boost the concentration of DNA. The column was then let to stand for one minute and centrifuged for one minute.

### 3.10.2 NEBuilder® Assembly of CRISPR vectors using ssDNA oligos

This protocol is a follow-up to the one reported in Jacobs *et al.,* 2015. It is superior in several ways: new gRNAs are created and inserted into final CRISPR vectors in a single cloning step, hands-on time is minimal, and multiple gRNAs can be created in a single reaction. Restriction enzymes are only used for vector preparation.The NEBuilder assembly mix contains three enzymes; 5' exo-nuclease, DNA polymerase, and ligase. DNA fragments with 15-40 bp overlaps on their ends are mixed together with assembly mix and are recombined to form a single molecule. The 5' exo-nuclease chews back 5' ends, allowing complementary ends to anneal. The DNA polymerase fills in the gaps, and the ligase seals the nicks, producing a single molecule. This approach allows ssDNA oligos to be used as well. The primers listed are for the p201 Cas9 vectors (Addgene 59175-59178). Four DNAs are mixed together, each with 20-bp overlaps; U6 promoter, gRNA ssDNA 60-mer oligo, scaffold, and p201 vector. The U6 and scaffold DNAs are made by PCR and the p201 vector is digested. Pools of oligos can be used in a single reaction, thus reducing the cost per gRNA. I have observed even distributions of inserts from a single reaction. (Table 3.9 &3.10).

## 3.11 *Escherichia coli* growth Condition

*Escherichia coli* strain DH5α was utilized for cloning. The E. Coli DHα blank strain was cultured for the whole night at 37 °C in Luria broth media with 50 mg/l

kanamycin. A loop was used to streak the culture on Luria agar with kanamycin, and the plate was then incubated at 37 °C. After isolating a single colony, 25% glycerol stocks were made and kept at -80 °C until needed.

### 3.11.1 Competent cell preparation and transformation of *E. coli* Strain DH5α and *Agrobacterium* Strain 4404 (Sambrook *et al.* 1989)

1. From the master plate of *E. coli* strain DHα single colony was picked with the help of inoculation needle and streaked onto LB agar plate and incubated at 37°C overnight.
2. 50 mL of LB broth was inoculated with a single colony from the freshly produced E. coli plate, and the mixture was then incubated at 37°C for the whole night on an incubator shaker set at 150 rpm. Since E. coli grows quickly, 500 milliliters of sterile LB were made ahead of time in case the desired OD was exceeded by the cells.
3. Next day OD was measured at 600nm and kept between (0.4-0.6).
4. Without disturbing the pellet, the culture was aliquoted into a 15 ml Falcon tube, left on ice for 20 minutes, centrifuged at 3,000 g for 10 minutes at 4°C, and the supernatant was disposed of (the cells were maintained cool throughout the procedure).
5. Pelleted cells were re-suspended in 30 mL of ice cold 0.1 M CaCl2 and incubated on ice for 30 mins.
6. After the 30 mins of incubation cells were centrifuged at 4ºC for 10 mins at 3,000 g (2500 rpm).
7. Supernatant was discarded (by pipetting) and suspended in 8 mL cold 0.1M CaCl2 containing 15% glycerol. 100 mL of the competent cells transfer into (1.5 mL) Eppendorf tubes placed on ice followed by snap freeze in liquid nitrogen. Cells were stored at -80ºC for further use in transformation for up to ~6 months.

## 3.12 *E. coli* transformation Materials

*E. coli* competent cells p201B Plasmid DNA as control LB agar selection plates Microcentrifuge tubes Incubator Shaker Laminar air flow.

**Procedure**

1. The competent cells were taken out of the -80ºC freezer and left on ice for ten minutes to fully defrost.
2. One to Five micro liter (10pg-100ng) of plasmid was added, mixed gently by tapping 4-5 times and incubated on ice for 30 mins.
3. After 30 min of ice incubation *E. coli* and plasmid aliquot was placed in 42°C water bath for exactly 30 seconds for heat-shock. Following the heat shock cells were placed on ice for two min.
4. One micro liter of pre-warmed LB medium was added into the tube containing cells followed by incubated on incubator shaker (150rpm) at 37ºC for 1 hr to outgrowth of bacterial cells.
5. After 1hr of incubation cells were spread (20-200 µl) onto a selective plate containing Kanamycin, using sterilized plating beads to spread evenly.
6. Plates were incubated for 16-18 hours at 37°C and inspected for isolated colonies.
7. Transformed *E. coli* strain DHα was conformed through colony PCR for desirable insert.

## 3.13 *Agrobacterium* competent cell preparation

1. The Agrobacterium strain LBA 4404 glycerol stock was removed from the freezer at -80°C and allowed to thaw. A loop-full culture was then streaked on fresh LB-Rifampicin plates and cultured for two days at 28°C.
2. Meanwhile a 250 ml screw cap conical flask was autoclaved.
3. Stock solutions of 20 mM $CaCl_2$ and 150 mM NaCl, was stored at 4°C.
4. From fresh Agrobacterium plates single colony was picked and inoculated in 20 ml of LB-Rifampicin broth in the 250 ml flask inside laminar Air Flow Chamber.
5. The inoculated flask was placed inside incubator shaker on 150 rpm forovernight at 28°C, until the O.D. reaches ~0.5 to 0.6.
6. The culture was aliquoted into 50 ml oakridge tube and spin for 15 minutes at 3,000 rpm at 4°C.
7. The supernatant was decanted and cells were chilled on ice for 10 minutes.
8. Pellets were resuspended in a total of 100 ml of 150 mM NaCl and combine in a single bottle. Spined for 15 minutes at 3000 rpm at 4°C and pellet was

resuspended in 10 ml of 20 mM $CaCl_2$.

9. Cells are aliquoted in 1.5 ml Ependorff tubes snap freeze in liquid nitrogen and stored at -80ºC.

***Agrobacterium* Electroporation DNA**

**Preparations**

The plasmid construct bought from Addgene was in lyophilized form, which is diluted with molecular grade water (RNA and Protein free) to get final concentration of 0.4 -1 µg/ml.

**Materials**

Disarmed *A. tumefaciens* Strain (LBA4404) Binary vector p201B Storage LB broth (40 ml glycerol to 60 ml of LB broth) LB media plates Autoclaved 10% Glycerol Molecular grade water.

**3.13.1 Electroporation**

1. Agrobacterium electrocompetent cells taken from -80°C freezer thawed on ice immediately before use.
2. Into a centrifuge tube 1 ml LB broth was added.
3. Forty micro liters of the electrocompetent cells and 1-5 µl of plasmid DNA was mixed together in TE buffer to an ice-cold 1.5 ml tube. Gently mixed by tapping the tube several times.
4. Pulsed with 16.7 kv/cm with 6-ms time constant.
5. Cells were transferred to the LB broth in a 1.5 ml tube. Remaining cells were resuspended in the microchamber with 750 µl of LB broth using a sterilized micro pipette to remove as many cells as possible.
6. Transformed cells were incubated at 28°C for 4 hours on incubator shaker (150rpm).
7. After 4 hrs 100 µl of transformed cells were spread on selective LB plate using glass bead spreader.

## 3.14 Colony PCR

Colony PCR was performed to quickly screen for plasmid inserts directly from *E. coli* and Agrobacterial colonies. The advantage of this method is that, large number of colonies can be screened at a time without purifying DNA from all of bacteria. This technique especially used for screening colonies after transformation with recombinant plasmids and targeted mutagenesis.

**Materials**

Sterile 200-µl thin-walled PCR tubes Micro tips Micro pipette, Thermal cycler. Reaction Mixture for PCR given in Table 3.12.

**Procedure**

1. In each PCR tubes 10 µl autoclaved MilliQ water was dispensed then half of the single isolated colony was dissolved in tubes (performed in laminar air flow).
2. Forward and reverse primers pipetted in each tube.
3. Master mix for colony PCR was prepared (for each tube 11 µl) by pipetting each ingredient except DNA and primers followed by brief vertexing Next master mix was dispense in each tube, capped, vortexed and centrifuged (short spin) placed inside thermocycler well and run was started as per PCR condition (Table: 3.13).

## 3.15 Genetic transformation in Groundnut GJG20

Genetic transformation studies in Groundnut GJG20 were carried out using Agrobacterium-mediated gene transfer technique.

### 3.15.1 Development of carotene edited plantlets from cotyledon explants

Development of transgenic plantlets was carried out as follows:

### 3.15.2 Bacterial strain and plasmid

Genetically engineered disarmed *Agrobacterium tumefaciens* strain LBA 4404 containing p201B was used for plant genome editing experiment. The Cas9, kanamycin and hygromycin phosphotransferase (*hpt*) gene is driven by 35S promoter whereas

sgRNA is by U6 promoter. Transformed *Agrobacterium* was selected on kanamycin in combination with Rifampicin.

### 3.15.3 Maintenance and growth of *Agrobacterium* cultures

The Agrobacterium strain LBA 4404 carrying p201N vector having Cas9-gRNA construct was maintained on the semi solid luria bertania (LB) medium supplemented with 50 mg/L kanamycin and 30mg/L Rifampicin. Culture was revived every month by streaking the mother culture on fresh LB medium and growing the bacteria for two days at 28ºC under dark conditions. These plates were stored at 4ºC.

Agar was excluded when Luria Bertani broth was prepared. The media was prepared by dissolving required amount of LB powder into MilliQ water. The media was then autoclaved at 121ºC at 15 lbs pressure for 15 minutes. After autoclaving, the media was taken to sterile laminar airflow and cooled to 50-60ºC to which 50 mg/L kanamycin and 30 mg/L Rifampicin was added. Media container was opened in front of burner and poured in petri plate or another vessel. Petri plate was not covered until media get solidified, else water droplets got accumulated which can leads to contamination.

### 3.15.4 Pre-culturing of cotyledon explants

The best shoot regeneration medium optimized was selected for the plant regeneration studies in Groundnut GJG20 with respect to the cotyledonary node explant, which has highest shoot regeneration frequency. After surface sterilization, the cotyledonary node explants were cut and inoculated on shoot regeneration media to study the effect of pre-culturing on transformation frequency. explants were pre-culturing for 48 hours and then subsequently co-cultured with fresh cultures of transformed Agrobacterial cells.

### 3.15.5 Preparation of *Agrobacterium* culture for co-cultivation

Single colony of transformed Agrobacterium was picked from LB plate, inoculated to 50 ml LB broth containing 50 mg/L kanamycin and 30 mg/L Rifampicin (as selection so, that transformed *Agrobacterium* can only grow in the medium) in a sterile screw cap conical flask (500ml) kept in incubator shaker overnight at 28ºC under dark conditions. The absorbance (OD) 1.0 at 600 nm was chosen and appropriate

dilutions were made according to the experimental requirement by adding plain LB broth (with 50 mg/L kanamycin and 30 mg/L Rifampicin) without culture and directly used for infection.

### 3.15.6 Co-culturing

Explants which respond well and look healthy on to pre-culturing media were selected for co-culturing. Swelling and thickening of pre-cultured cotyledon explants was sign of healthiness of explant. Ex-plants were immersed in the freshly prepared Agrobacterial suspension in 30 min with occasional shaking during incubation. The explants were blotted on autoclaved sterilized Whatman No. 1 filter paper and transferred on co-culturing media as that of same pre-culture medium for 72 hrs. After exposure for different experimental durations, co-cultured explants were washed with liquid MS medium supplemented with cefotaxime, blotted on sterile tissue paper and transferred to a selection medium containing 3.0 mg/l BAP for regeneration. Cultured maintained under a 16 h light/8 h dark cycle at 28°C.

### 3.15.7 Transfer of cotyledon explants on the selective shoot regeneration medium after co-cultivation

After 72 hrs. of co-cultivation the cotyledon explants were transferred to the fresh selective shoot regeneration medium, for inhibition of further agrobacterial growth (MS basal medium + 25 mg/l BAP + 500mg/l cefotaxime). The cultures were kept in a culture room at 25± 2ºC for further growth and differentiation. Observation was made regularly for contamination and differentiation. The explants were sub-cultured on the fresh selective shoot regeneration medium every 15 days according to cultured condition.

### 3.15.8 Multiplication, elongation and selection of carotene transgenic shoots

Regenerated carotene transformed Groundnut shoots were multiplied on the multiplication medium containing same strength of PGRs i.e. MS (basal medium) + 3.0 mg/l BAP+ 1.0 mg/l GA+ 5mg/l kanamycin + 500 mg/l cefotaxime. The shoots were maintained on the same medium for elongation.

### 3.15.9 Development of roots in carotene edited shoots of Groundnut GJG20

Regenerated carotene shoots obtained from cotyledonary node explants were transferred to the root regeneration medium containing (MS basal medium+ 1.0 mg/l NAA 500 mg/l cefotaxime) for root induction to get complete plantlets.

### 3.15.10 Hardening and acclimatization of *in vitro* developed carotene edited plantlets

*In vitro* regenerated carotene edited plantlets were transferred on potting mixture for hardening and acclimatization. The survival and establishment of the plantlets were studied after transplanting the plantlets in the potting mixture.

## 3.16 Molecular Analysis of Carotene Edited Plantlets of Groundnut GJG20

### 3.16.1 Isolation and purification of genomic DNA from the carotene transformed and non-transformed (control) plantlets

Genomic DNA of carotene edited and non-edited (control) Groundnut GJG20 plants were isolated by modified CTAB method (Doyle and Doyle, 1990).

**i) Solutions used for DNA extraction**

- **a)** 10% (w/v) CTAB (N-cetyle N, N, N-trimethyl ammonium bromide): 10g of CTAB was dissolved in 70ml of MilliQ water at 65ºC and then final volume was made to 100ml.0.5 M EDTA (pH 8.0): 93.05g of ethylene diamine-tetra acetate was added to 400 ml of MilliQ water. The final volume was made to 500ml.
- **b)** 5 M NaCl: 146.1g of sodium chloride was dissolved in 400ml of MilliQ water and the final volume was made to 500ml.
- **c)** 1 M Tris-HCl (pH 8.0): 60.58g of tris HCl was dissolved in 300ml of MilliQ water. The final volume was then made to 500ml.
- **d)** Chloroform: isoamyl alcohol (24:1): 720µl of chloroform and 30µl of isoamyl alcohol were mixed by vortexing. The resulting solution was kept at 4ºC in amber bottle.
- **e)** Isopropanol (chilled)

**f)** 70% Ethanol

**ii) DNA extraction buffer**

**a)** 100mM Tris base (pH 8.0)

**b)** 20mM EDTA (pH 8.0),

**c)** 1.4M NaCl,

**d)** 2.0 % (w/v) CTAB

**e)** β- mercaptoethanol

**Procedure**

1. About 1.0g of tissue was ground to a fine power using liquid nitrogen in a mortar and pestle.
2. After fully homogenizing the powdered leaves in one milliliter of heated (about 65 degrees Celsius) DNA extraction buffer, they were transferred to a 2.0 milliliter centrifuge tube. Ten microliters of β-mercaptoethanol were then added, and the mixture was gently inverted four or five times.
3. The tubes were incubated for one hour at 65 degrees Celsius in a water bath. After an hour, the tubes were taken out of the water bath and centrifuged for ten minutes at 10,000 rpm.
4. To emulsify, an equal volume of chloroform: isoamyl alcohol (24:1, v/v) was added and well mixed by inversion.
5. Emulsified mixture was spun down by centrifugation at 10,000 rpm for 10 minutes at room temperature.
6. The supernatant was pipetted out gently without disturbing the interphase to another 1.5 ml centrifuge tube.
7. Equal volume of isopropanol was added, mixed by gentle inversion and stored at -20ºC for 2 hrs.
8. To get pelleted DNA, it was centrifuged for 10 minutes at 10,000 rpm after two hours.
9. Supernatant was removed and DNA pellate was washed with 70% ethanol by centrifugation at 10,000 rpm for 10 minutes.
10. Supernatant was decanted carefully and DNA pellet was dried in oven at37ºCfor 15 minutes.
11. 50µl of TE buffer was used to dissolve the DNA pellet, which was then kept in

a freezer at -20ºC.

**iii) DNA quantification and dilution**

To determine the precise amount of DNA, DNA quantification is essential for molecular biology research. The amount of DNA was measured at the primary level using gel electrophoresis on 0.8% agarose with a 90 volt current and visualization using a UV transilluminator. The ratio of absorbance at 260 and 280 nm (A260/A280) was used to evaluate the approximate purity of double standard DNA. The quantity of DNA in the sample is directly correlated with the amount of UV light that the DNA solution absorbs at 260 nm. For pure double-standard DNA, this ratio is 1.8. A260/A280 ratios above 1.8 indicate RNA contamination, whereas those below 1.8 indicate protein contaminants in the sample.

**iv) Dilution of DNA**

After quantification of DNA sample at 260 nm absorbance it was equated by the thumb rule that if absorbance at 260nm is one then concentration of DNA in the sample must be equal to 50µg/ml. After quantifying DNA sample was diluted with autoclaved MilliQ water to bring the concentration to 25µg/µl.

**3.16.2 Confirmation for the integration of CRISPR Cas9 gRNA construct**

PCR amplification and analysis of the edited plants were performed using plasmid's component specific designed primers (forward and reverse) (Table 18). Polymerase chain reactions were performed in a thermo cycler (Veriti 96-Well Thermal Cycler).

Using Taq DNA polymerase, the polymerase chain reaction (PCR) is an in vitro technique for amplifying a particular nucleic sequence. and short stretch of oligonucleotides (primers) which are specific to the DNA to be amplified. DNA amplification through PCR involves repeated rounds of DNA synthesis, which is based on the three steps *i.e*. Denaturation (separation of double helix DNA into single strands to form template strand), annealing (delimiting each original strand for new strand synthesis) and extension (actual synthesis of new DNA strands) from the reaction of dNTPs, primers and Taq polymerase.

**Procedure**

After adding DNA and primers to each PCR plate well, the following ingredients were added in order to create the master mix: water, PCR buffer, dNTPs, and Taq DNA Polymerase. Each well was filled with the vertexed master mix, which was then covered with PCR plate sealing film. After a few seconds of spinning on an MPS1000TM mini-PCR plate spinner, the PCR plate was carefully inserted inside the thermocycler and the run was initiated in accordance with the PCR conditions (Table 3.14).

### 3.16.3 Gel electrophoresis and visualization of amplified products

1. **Solutions/ reagents for agarose gel electrophoresis**
   a) **Ethidium bromide:** 10mg/ml ethidium bromide (EtBr) was prepared by dissolving 100mg of ethidium bromide to 10ml of autoclaved MilliQ water. After the EtBr dissolved completely, transferred into brown/black colored bottle (EtBris photoactive and carcinogenic chemical) stored at room temperature.
   b) **6X loading dye:** Loading dye was prepared by dissolving 25mg of bromophenol blue, into 30 per cent glycerol in water. Kept on shaking condition for 2 hrs so that solution mixed homogenously and stored at 4ºC till use.
   c) **50X Tris acetate EDTA Buffer:** 57.1 ml of glacial acetic acid and 242 g of Tris base were placed in a container. 200 ml of water and 100 ml of 0.5M EDTA were added, and the mixture was swirled on a magnetic stirrer until it appeared clear. After adjusting the capacity to one liter, the autoclave was used to sanitize it.
   d) **Agarose gel:** PCR The result, which was produced by putting 1.2g of agarose in 100ml of 1X TAE buffer and boiling it for two to three minutes (until a clear solution formed), was run on a 1.2% agarose gel. When the temperature on the back of the hand reached 40 to 50 degrees Celsius or was tolerated, 20 microliters of ethidium bromide was applied. The gel casting tray with the welling comb was filled with this solution.

**2. Running conditions**

On a 1.2% agarose gel, PCR-amplified DNA products were electrophoresed. After filling the electrophoresis unit with 1 X TAE buffer, the gel and casting tray were immersed in the same solution, and the comb was taken out. Each sample received 2 µl of loading dye, which was pipetted up and down to mix it, and then placed into each well without creating any bubbles. Agarose gel was loaded in the following order: 100– 1000 bp ladder, edited plant PCR products, non-transformed plant PCR products, and non-template control (NTC), which did not include a DNA template. About 15 µl of the 25 µl PCR product was put into each well, and it was electrophoresed at 90 volts with a continuous current until the tracking dye reached the other end.

**Viewing the gel and photography**

The gel containing amplified DNA products were viewed under UV trans-illuminator. Photograph of gel was taken in Syngene G:BOX gel documentationsystem and stored in the computer software (Gene sys) for further studies. The plant which showed amplified DNA in PCR were marked as positive transgenic plant, whereas, the samples not showing amplified DNA for a particular gene were marked as non-transformed plant. Plasmid DNA containing U6 and Cas9 genes was used as +ve control and genomic DNA of *in vitro* regenerated plant of soybean used as –ve control.

## 3.17 Nucleotide sequencing and Bio-informatics analysis

All PCR-conformed transformed and control plants had their genomic DNA extracted. Using gene-specific primers, the lycopene epsilon cyclase gene was amplified by PCR. The resulting products were purified and qualitatively examined using electrophoresis on a 1.2% gel. To find the mutation in the targeted genes, the purified products were sequenced. Following receipt of the nucleotide sequencing data, BioEdit software was used for analysis and quality control.

## 3.18 Observation recorded

**Regeneration of explants**

The number of explants that regenerated into numerous shoot buds out of the total number of explants cultivated will be used to determine the regeneration percentage of explants.

**Rooting**

Every sprout that is resistant to antibiotics will be moved to the root induction medium. The number of shoots that developed roots out of all the shoots that were sent to rooting media will be used to compute the rooting percentage.

$$\text{Rooting \%} = \frac{\text{Total No. of Shoot that produced healthy roots}}{\text{Total No. of Shoots transfered to rooting medium}} X\ 100$$

**Hardening percentage**

It will be determined by dividing the total number of plants moved to pots by the number of plants that survived in pots.

$$\text{Hardening \%} = \frac{\text{Total No. of Plant Survived in pots}}{\text{Total No. of plants transfered to pots}} X\ 100$$

**PCR screening**

The number of plants that tested positive for PCR out of all the plant samples that were analyzed will be used to determine the percentage of PCR-positive plants.

$$\text{PCR positive \%} = \frac{\text{Total No. PCR positive plans}}{\text{Total No. of plant samples used for PCR screening}} X\ 100$$

## 3.19 Statistical analysis

A statistical analysis was conducted on ex-plants that demonstrated a superior response to hormone concentration and combination on the growth of shoots and roots. Seven treatments were used for root growth and seven for shoot regeneration. Three bottles, plates, or test tubes were used for each treatment, and the design was entirely randomized with three replications. Variance analysis was performed on the data.

**Table 3.1** Different combinations and concentrations of BAP used in MS medium for shoot regeneration from De embryonated cotyledon explant of GGJ-20

| Sr No | Medium composition | | |
|---|---|---|---|
| | **Media Code** | **Basal MS Medium** | **BAP (mg $l^{-1}$)** |
| 1 | SM-0 | Basal MS Medium (control) | |
| 2 | SM-1 | MS | 2 |
| 3 | SM-2 | MS | 5 |
| 4 | SM-3 | MS | 10 |
| 5 | SM-4 | MS | 15 |
| 6 | SM-5 | MS | 20 |
| 7 | SM-6 | MS | 25 |

**Table 3.2** Different combinations and concentrations of BAP with GA used in MS Medium for shoot regeneration from De embryonated cotyledon explant of GGJ- 20.

| Sr No | Medium composition | | | |
|---|---|---|---|---|
| | **Media Code** | **Basal MS Medium** | **BAP (mg $l^{-1}$)** | **GA (mg $l^{-1}$)** |
| 1 | SE-0 | Basal MS Medium (control) | | |
| 2 | SE-1 | MS | 0.5 | 0.1 |
| 3 | SE-2 | MS | 1.0 | 0.2 |
| 4 | SE-3 | MS | 1.5 | 0.3 |
| 5 | SE-4 | MS | 2.0 | 0.4 |
| 6 | SE-5 | MS | 2.5 | 0.5 |
| 7 | SE-6 | MS | 3.0 | 1.0 |

**Table 3.3** Composition of root regeneration media with various concentrations of NAA

| Sr No | Medium composition | | |
|---|---|---|---|
| | **Media Code** | **Basal MS Medium** | **NAA (mg $l^{-1}$)** |
| 1 | RI-0 | Basal MS Medium (control) | |
| 2 | RI-1 | MS | 0.1 |
| 3 | RI-2 | MS | 0.5 |
| 4 | RI-3 | MS | 1.0 |
| 5 | RI-4 | MS | 1.5 |
| 6 | RI-5 | MS | 2.0 |
| 7 | RI-6 | MS | 2.5 |

**Table 3.4** Primer sequence of LCYE gene

| Sl. No | Name of the primer | Primer Sequence 5' -3' | Tm (ºC) |
|---|---|---|---|
| 1 | F- LCYE - Peanut | CCATTTTTATTGGGCGTTCTTA | 59 |
| 2 | R-LCYE- peanut | TTGCTACCCTCAACAATCCTTT | 59 |

**Table 3.5** gRNA sequence information

| Target Sequence:5'-3' | PAM | Specificity | Chromosome |
|---|---|---|---|
| GAATTCTGCAGCTTGACATTG | AGG | 97.6 | 13 |

**Table 3.6** Chemical composition of Luria Bertani medium

| Components | Amount (g/l) |
|---|---|
| Yeast extract | 5 g/l |
| Tryptone | 10 g/l |
| Sodium chloride | 10 g/l |
| Agar | 16 g/l |
| pH | 7.0 |

**Table 3.7** List of plasmid isolation alkaline stock solutions

| | Stock concentration | Working concentration |
|---|---|---|
| Solution I | Glucose (0.5M) | 25 Mm |
| | Tris HCl (1M) (PH 8.0) | 10 mM |
| | EDTA (0.5M) (PH 8.0) | 50 mM |
| Solution II | 10 % SDS | 1% |
| | 1N NaOH | 0.2 N |
| Solution III | Glacial acetic acid | 11.5 % |
| | 5M potassium acetate | 3 mM potassium acetate |

**Table 3.8** 50X TAE buffer stock solution preparation

| Reagent | Volume |
|---|---|
| Tris base | 242.0 g |
| EDTA 0.5M (PH 8.0) | 100 ml |
| Glacial acetic acid | 57.1 ml |
| D/W | Make up 1 litter |

**Table 3.9** Reaction set up for restriction enzyme digestion of binary vector p201B

| Reagent | Volume |
|---|---|
| p201B Plasmid | 3 µl |
| SwaI Enzyme | 1 µl |
| Cut smart buffer (10X) | 2 µl |
| H20 | 15 µl |
| Total volume | 20 µl |

**Table 3.10** NEBuilder cloning reaction

| Reagent | Volume |
|---|---|
| p201 vector (14,349 bp) | 1 µl |
| MtU6 amplicon (377 bp) | 1 µl |
| Scaffold amplicon (106 bp) | 1 µl |
| ssDNA oligo (60-mer) | 1 µl |
| 2x Mix | 5 µl |
| Total | 10 µl |

**Table 3.11** PCR conditions for NEBuilder cloning reaction

| Temperature | Time |
|---|---|
| 50 ºC | 60 min |
| Cool down to 25 ºC at 0.1 ºC/sec | |

**Table 3.12** Reaction mixture for colony PCR

| Sl. No. | Particulars | Quantity requires |
|---|---|---|
| **1.** | Bacterial colonies (as template DNA) | Half of the single isolated colony |
| **2.** | 10x PCR buffer | 2.0 µl |
| **3.** | 15mM $MgCl_2$ | 0.5 µl |
| **4.** | 2.5 Mm dNTPs | 0.1 µl |
| **5.** | 5 U/µl Taq DNA Polymerase | 0.3 µl |
| **6.** | 30 µM Primer 1 (Forward) | 1.0 µl |
| **7.** | 30 µM Primer 2 (Reverse) | 1.0 µl |
| **8.** | Autoclave MilliQ water | 10 + 8.1 µl |
| **9.** | Total | 25 µl |

**Table 3.13** Colony PCR reaction condition

| Stage | Temperature | Time | Cycle |
|---|---|---|---|
| Pre-Heating | 95 ℃ | 3 min. | 1 Cycle |
| Denaturation | 95 ℃ | 30 sec. | |
| Annealing | 60 ℃ | 30 sec. | 30 Cycle |
| Extension | 72 ℃ | 55 sec. | |
| Final Extension | 72 ℃ | 4 min. | 1 Cycle |
| Final Hold | 4 ℃ | Hold | |

**Table 3.14 Primers Sequence of Cas9 and U6 Promoter to conform transferred groundnut.**

| Name of the primer | | Primer Sequence 5' 3' | Tm | Size |
|---|---|---|---|---|
| U_6 | F | ACCATAGCACAAGACAGGCG | 55 | 136 |
| | R | TGGCCCACTACGAAATGCTT | 50 | |
| Cas9 | F | AGATGATCGCCAAGAGCGAG | 55 | 439 |
| | R | ATCCCCAGCAGCTCTTTCAC | 55 | |

**Table 3.15** PCR reaction mixture for amplification of Cas9 and U6 promoter

| Sl. No. | Particulars | Quantity requires |
|---|---|---|
| **1.** | DNA template | 2.0 µl |
| **2.** | 10x PCR buffer | 2.0 µl |
| **3.** | 2.5 Mm dNTPs | 0.1 µl |
| **4.** | 5 U/µl Taq DNA Polymerase | 0.3 µl |
| **5.** | 30 µM Primer 1 (Forward) | 1.0 µl |
| **6.** | 30 µM Primer 2 (Reverse) | 1.0 µl |
| **7.** | Autoclave MilliQ water | 18.1 µl |
| **8.** | Total | 25 µl |

**Table 3.16** PCR reaction condition for amplification of CAS9 and U6 promoter

| Stage | Temperature | Time | Cycle |
|---|---|---|---|
| Pre-Heating | 95 ℃ | 3 min. | 1 Cycle |
| Denaturation | 95 ℃ | 30 sec. | |
| Annealing | 60 ℃ | 30 sec. | 30 Cycle |
| Extension | 72 ℃ | 55 sec. | |
| Final Extension | 72 ℃ | 4 min. | 1 Cycle |
| Final Hold | 4 ℃ | Hold | |