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Research Article

Agrobacterium Mediated Delivery of Multiplex CRISPR/Cas9 System in Cotton for Cotton leaf curl virus Disease Resistance

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Abstract: In Pakistan, cotton crop contributes 23% to the GDP and its massive export provides 60% of the total profit in trading business. Unfortunately, the Gemini viruses destroy the cotton crop at an alarming rate. Now, the CLCuVs causes the Cotton Leaf Curl Virus Disease in cotton crop and reduce its productivity. This viral attack on cotton plant resulted 5 billion US dollars loss to the Pakistan. Well, in that regard, some conventional methodologies were used like the plant breeding and specific RNA editing. At the same time, biotechnology introduced some very attractive techniques which carry the massive potential for the eradication of this disease. These techniques include the ZFNs, TALENs and CRISPR/Cas9. In my research, CRISPR methodology was adopted because of its marvelous efficiency of site specific mutagenesis. The conserved locations of the rep gene of the different CLCuVs were identified as the target sites. These specific sites provided the information for the establishment of the 3 gRNAs. The single expression vector pHSE-401 having the multiple guided RNAs and 1Cas9 were cloned. The specific cotton variety coker-312 was used. The transformation of the vector was performed by hypocotyls excision of the cotton plant and delivery infection was done by agrobacterium EHA-105 Strain. Then, 1000 infectious hypocotyls were shifted on the MSB having respective antibiotics and then, on the regeneration media which converted the hypocotyls edges into the callus. Only two transgenic calli were obtained, the percentage of transgenic calli was 0.02% which were screened by PCR and run on gel. The bands of 200 bp confirmed the presence of the CRISPR/Cas9 construct in cotton calli.

Keywords: CRISPR/Cas9, Multiplex vector, 3gRNA and Cas9, Agrobacterium, Transformation, Callus.

1. INTRODUCTION

The economy of Pakistan majorly relays on the sector of agriculture. Its percentage in GDP is 19.82% but the participation of the agriculture sector dropped from 50% in 1950 to the about 21% in 2014. The money which is earned by the agriculture products, labor force of forestry, farming of the livestock and the crops transformation feed the 64% of the village population of Pakistan. According to statistics, 66% of Pakistan people have employment in agriculture sector. In the decade of the 20th century, the growth rate of the agriculture products was 2.5% annually. In the agriculture package of 1997, the yield of the crop was developed up to 5.9%. To increase the productivity of the agriculture, the annual production was increased from 0.2% in the 2010 and the 2.9% rise happened in 2015 (Anonymous, 2014-2015).

Without any doubt, the progress of the country depends on the development of the sector of the agriculture. The rural population of the Pakistan uses the cotton crop as an earning source. Approximately, in 80 countries, the cotton crop provides the labor opportunity to the workers. Total 90% of the financial aid is provided by the upland cotton. 25 million metric tons of fiber was produced over the 34.4 million hectares land (Anonymous, 2008).

In the 1996, United States produced the genetically modified cotton which had the Bt gene. After introducing the Bt gene in the cotton plant, just 25% of the pesticides were utilized (Pannetier et al., 1997). A pivotal role of the genetic engineering has been observed in the development of the insect resistance and herbicide resistance characteristics in the cotton plants. Begomoviruses introduced severe types of diseases which damage the economy of Pakistan. Begomoviruses complex with the alpha and beta satellites molecules cause the CLCuD in cotton crop and reduced its productivity. CLCuD is a major threat for cotton crop (Sattar et al., 2013).

Different types of cotton leaf curl viruses are as under:

- Cotton leaf curl Alabad virus (CLCuAIV)
- Cotton leaf curl Kokhran virus (CLCuKOV)
- Cotton leaf curl Bangalore virus (CLCuBAV)
- Cotton leaf curl Multan virus (CLCuMUV)
- Cotton leaf curl Rajasthan virus (CLCuRAV)

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Thickening of the vein, reduction downward or upward leaf area, puckering and decline in the growth rate of the cotton are the symptoms of the CLCuD (Sattar et al., 2013).

2. RESEARCH OBJECTIVE

- Induction of the infection of Agrobacterium in hypocotyls of cotton crop by transformation
- Development of the transgenic callus of cotton plant
- PCR screening of the callus to identify the multiplex CRISPR/Cas9 construct

3. MATERIALS AND METHODS

The present research work was conducted in Cotton Biotechnology Laboratory (CBL), Center for Advanced Studies in Agriculture and Food Security (CAS-AFS), using the standard protocols of the laboratory.

3.1 Synthesis of Primers for gRNA

According to the sequence of the gRNA, primers were designed by CHOPCHOP primer designing tool. Primers had the restriction sites and produced the only sticky ends. The Oligos annealing was done through PCR, oligos were annealed by fluctuating the temperature of the system. After every minute, the temperature was decreased from 95°C to the 4°C by decreasing 10 degree.

3.2 Confirmation through digestion

The pHSE-401 plasmid was restricted with the restriction enzymes (*XbaI, EcoRI*) and then, run on the agarose gel. Restriction digestion has the purpose to obtain two fragments. These were almost 11738bp and 4916bp. The sizes of these specific fragments confirmed the plasmid of pHSE-401 (Green and Sambrook *et al*., 2012).

3.3 Reagents and materials

- Restriction enzymes (*XbaI and EcoRI*).
- Plasmid (pHSE_401).
- 10x Buffer.
- Water (d_3H_2O).

3.4 Protocol

All the following reagents were added in an Eppendorf tube with such concentration.

Restriction enzymes	=	1µl
Plasmid (pHSE- 401) DNA	=	2µl
10x buffer	=	1µl
D_3H_2O	=	6µl
Total	=	10µl

3.5 Running of Agarose Gel (Green and Sambrook, 2012)

- Agarose
- 6x sample loading buffer
- 1x TAE buffer
- 1kb DNA ladder
- Ethidium Bromide

3.6 Preparation of 50x TAE Buffer

- Glacial Acetic acid = 57.1ml
- Tris–base = 242grms
- 0.5 M EDTA = 100ml

3.7. 1x TAE Buffer preparation

For preparing the 100ml volume, 98ml of d_2H_2O and 2ml of 50x TAE buffer were used.

3.7.1 Preparation of agarose gel

In a clean 250ml flask, 100ml of 1x TAE was added and 1grm of agarose gel powder was included to make 1% of gel (W/V).

- The gel solution was kept in microwave until the solution became transparent.
- The solution was kept outside the oven to about 60°C.
- Now, 3µl (EtBr) Ethidium Bromide was added into the gel.
- In a casting tray, the hot liquid was poured and the comb was kept by inserting the sides into the notches.
- After the polymerization of the gel, the comb was taken out from the gel to expose the wells for the sample.

3.7.2 Preparation of the loading buffer

- Glycerol in water 30% (v/v)
- Bromophenol Blue 0.25% (w/v)
- Xylene Cyanol 0.25% (w/v)

3.8 Agarose Gel Loading

- In every 20µl reaction mixture, 4µl of 6x loading buffer was added.
- On gel, samples were loaded and then, order was recorded.

3.9 Gel Running

- In a chamber, the gel was kept.
- Electric supply was provided to the chamber of electrophoresis.
- Voltage was set at 80V.
- The samples migrated from the negative electrode to the positive electrode.
- Electricity source was removed.
- The lid of the tank was taken away.
- As EtBr was added, then blue strain showed the movement of DNA directly.
- In gel documentation machine, gel was placed (Bio-Rad) and samples were compared with the ladder DNA.

3.10 Miniprep technique for Plasmid Isolation

3.10.1 Reagents and Materials.

Antibiotics (Kanamycin, Tetracycline, Rifampicin) for the isolation of plasmid.

- Colonies of E.coli
- LB media (500 ml)
- Ethanol (70%, 100%)
- D_3H_2O , containing 20µl (m) RNA ase
- Alkaline solution I
- Alkaline solution II
- Alkaline solution III

3.11 Synthesis of L.B Media

- Yeast extract =5grms
- NaCl =10grms
- Tryptone =5grms

3.12 Preparation of Re-suspension solution

- Tris-Chloride (pH 8.0) =25mM
- Glucose =50mM
- EDTA (pH8.0) =10mM

After synthesis, solution I was autoclaved and stored at 4°C (Green and Sambrook, 2012).

Synthesis of alkaline lysis solution II (Lysis solution) (Green and Sambrook, 2013)

- SDS =1% (w/v)
- NaOH =0.2N
- Synthesis of alkaline lysis solution III (Green and Sambrook, 2012)
- Glacial acetic 11.5mL
- Water 28.5mL
- Potassium acetate 5M (60mL)

3.13 Cells preparation (Green and Sambrook, 2012**)**

- One colony of the mutated bacteria is inoculated in 10ml LB media having Kanamycin, Rifampicin and Tetracycline culture was kept at 37°C with fast shaking.
- The mutated bacteria in LB media was taken 1.5ml only and poured in Eppendorf tubes. Then, it was spun for 30 seconds at 15000rpm at the 4°C temperature.
- .The supernatant was discarded and the remained part was utilized for the next stage.
- By dynamic vortexing, the deposited bacterial cells were kept again in 100µl of ice-cold alkaline lysis solution I.
- In every solution, 200µl of freshly synthesized alkaline lysis solution II was appended. Tubes were covered and all the contents were mixed by rapidly inverting the tubes approximately five times.
- Then, used 150µl of ice col alkaline lysis solution III and it was intensified and inverted for many times.
- At 4°C, the bacterial solution was spun at the high speed for 5 minutes.
- The supernatant was transferred to a new tube,
- In supernatant, 100% ethanol was added kept at -20°C for half an hour.
- The essence was spun at the 4oc for two minutes only in a new tube aqueous layer was transferred for next step, pellet was proceeded.
- In pellet, 70% ethanol was added and closed tube was upended for multiple times.
- The deionized distilled water having RNA are was used for dissolving the DNA and at 20°C, it was stored.

3.14 Cotton transformation and regeneration of plant

- Cotton (Coker-312) seeds were sown in peat moss and covered with aluminum foil for 2 to 3 days.
- On daily bases, watered the plants.
- Hypocotyls of cotton were appeared after 6 days.
- The hypocotyls sterilization.

3.15 Process of sterilization

- Commercial 12% bleach is available, 1% or 1.5% is used mostly for cotton.
- 8.33ml of 12% bleach and prepare volume up to 10ml.
- 5ul of tween 20 is used.
- The sterilization solution is added into the cotton's hypocotyls.
- In shaker, shaking should be done for 15 minutes.
- For five times, washing with D_2H_2O water.
- Drying of hypocotyls with the filter paper.

3.16 Agrobacterium culture Preparation

- For the purpose of transformation.
- The *Agrobacterium tumefaciens* strain EHA-105 was used. This strain had pHSE -401 plasmid transformed.
- The growth of the vector occurred in LB media at 28°C in the shaking incubator.
- Centrifugation of the culture was done at 4°C at 15000rpm.
- Pellet was re-suspended in the MGL for the infection of the explants.
- The hypocotyls were sliced with sharp blade and then, they were inoculated with the agrobacterium suspension for approximately 30 minutes.
- Sterile filler paper were used and then, dried them carefully.
- What man filter paper were used for the transfer of the dry hypocotyls segments.
- Shift the hypocotyls on the plates which carry the co-cultivation media.
- For induction of the callus, after two or three days, shifted them to selection media.
- The callus developed on the edge of the hypocotyls of cotton.

3.17 Preparation of LB Media

- 5g/L extract of Yeast
- 10g/L of NaCl
- 5g/L of Tryptone

3.18 MGL Preparation

- 5g/L Tryptone.
- 0.1g/L of $MgSO_4$
- 5g/L of NaCl
- 1g/L of Glycine
- 0.25g/L of KH_2 PO_4
- pH 5.8

3.19 MSB Media Preparation

- The Murashige and Skoog Basal medium w/ Vitamins
- 0.1 mg/l Kinetin
- 3% (w/v) Glucose
- 20mg/ L Acetosyringone
- 1g/ L $MgCl_2$
- 0.1mg/ L 2.4-D
- 0.25% (w/v) Phytagel pH 5.8

3.20 Selection on Callus Induction media (CIM)

- 0.1 mg/L 2, 4-D
- 3% (w/v) Glucose
- 0.1 mg/L Kinetin
- 40mg/L Cefotaxime
- 50 mg/L Kanamycin
- 25% (w/v) Phytagel, pH 5.8

3.21 Preparation of chemically competent cells of E. coli

3.21.1 Chemicals, Reagents and Apparatus

- Calcium Chloride
- Potassium Acetate
- Manganese Chloride
- Glycerol
- Rubidium Chloride/ Potassium Hydroxide
- MOPS/ PIPES

3.22 250ml TFBI

- 10mM $CaCl_2$
- 30mM Potassium Acetate
- 100mM Rubidium Chloride
- 50mM Manganese Chloride
- 15% of Glycerol
- Maintain pH to 5.8 by adding the 1M acetic acid
- Filter sterilize through 0.2mm filter paper

3.22.1 Protocol (Green and Sambrook, 2012)

From the LB plate a single colony of E.coli strain was selected and picked and inoculated 2.5ml of LB medium shaking was done at 220rpm at the 37°C in the incubator shaker overnight.

- The culture of agrobacterium was utilized to inoculate 250ml of the LB medium.
- Then, cells were grown in a 1 liter flask for three hours until the $0D_{600}$ increases up to 0.4 – 0.6.
- Through centrifugation, the cells were collected at 5000rpm at 4°C for 5 minute.
- The supernatant was discarded.
- In 0.4 volume of ice-cold TFBI, cells were gently re-suspended.
- On ice surface, the re-suspended cells were incubated for 5 minutes.
- Now, again supernatant was discarded.
- In 0.4 volume of ice-cold TFB2, the cells were gently re-suspended.
- For 30 minutes, the cells were incubated on ice and then, aliquoted into the pre-chilled tubes.
- Rapidly, freeze the tubes by dipping into the liquid Nitrogen and then, store at -80°C.
- Protocol for the preparation of the E.coli calcium chloride competent cells.
- In 50ml falcon tube, inoculate a single colony into the 5ml LB media.
- At 37°C, grow O/N.
- In the next morning, utilize the 1ml to inoculate 100ml of LB in the 250ml bottle.
- For 4-6 hours, shakes at 37°C.
- For 10 minutes, keep the cells on ice (keep cold form now on).
- Now centrifuge the cells and collect the cells in the big centrifuge for just three minutes at 6000 rpm.
- Discard the supernatant and then, gently re-suspend on 10ml cold 0.1m Calcium Chloride. (Cells are very delicate and sensitive to the mechanical disruption, so handle them nicely).
- For 20 minutes, incubate the cells on ice.
- Centrifuge the cells
- Discard the supernatant and carefully re-suspend 5ml cold 0.1 M Calcium Chloride / 15% Glycerol
- Now, dispense in micro tubes (300 µl/ tube)
- Freeze at the -80°C

3.23 E.coli Transformation

- Keep lu of plasmid DNA in a micro tube.
- Carefully, add the 100µl of the competent cells.
- DNA control tube having cells but no DNA is incubated for 30 minutes in ice.
- At 42°C, heat shock it for two minutes.
- Keep them back on ice.
- In tubes, now add 900µl of LB media.
- Incubate for 30 minutes at 37°C.
- In LB Amp or LB carb plates, plate 100-1000µl of the cells.
- In a blood plate, plate 100µl of the NO DNA control (For checking the quality of cells). Now, grow the O/N, the rest is saved in the cold room or even freeze with 15% of Glycerol. This is the case when you do not get the colonies.
- If, there is a need of multiple colonies or the ligation is not quite efficient, then centrifuge the cells for one minute at 8000rpm, discard 900µl supernatant, 100µl left is re-suspend and whole lot is plated.

3.24 Preparation of the agrobacterium competent cells

- Take the 8ml overnight culture of the Agrobacterium and grow it in LB media having 5ug/ml Tetracycline +25 ug/ml Kanamycin +50 ug/m Rifampicin.

- The LB must has the 5g/L NaCl at all the stages.
- Do not add antibiotics in 192ml LB media, inoculate 8ml overnight culture.
- At28°C, shake it until an OD reaches up to 0.5.
- At 4°C, centrifuge at the 4000rpm for fifteen minutes.
- Now use 10mm tris solution for the re-suspension of the pellet.
- At 4°C, centrifuge for 15 minutes at 4000rpm.
- In 20ml chilled LB, re-suspend the pellets and store it in liquid nitrogen. These cells get freeze at -80°C, they can stay competent for approximately for 6 months.

3.25 Agrobacterium transformation

- Take out the agrobacterium competent cells from the -80°C freeze and now leave them on ice for 20-30 minutes.
- Stored agar plates are removed from the 4^{o}C freezer and let them warm up to the temperature of the room and incubated these plates at 37°C in an incubator.
- In a micro centrifuge or falcon tube, mix 1-5 µl of DNA into the 20-50µl of the agrobacterium competent cells, carefully mix by using the slight flicking of the fingers at the bottom of the tube for few times.

3.26 Protocol of freezing

- The competent cell /DNA is incubated on ice for 20-30 minutes.
- Water bath having the temperature of 42°C is used because heat shock the every transformation tube by putting the bottom ½ to 2 /3 of the tube for 30-60 seconds (for most of the time 45 seconds are considered as an ideal period.
- Leave the tubes again on ice for two minutes.
- Add 250-1000 µl of LB media into the bacteria and for 45 minutes, grow them in shaking incubator at the 37°C.
- The agar plate must contain the appropriate selection antibiotics.
- So plate few or all of the transformation onto a 10cm LB agar plate.
- At 37°C, incubate the plates overnight.

3.27 Protocol for plasmid purification (Green and Sambrook, 2012)

- Take the 3ml liquid LB medium having respective antibiotic then, culture the single colony of E.coli and grow overnight at the 37°C temperature.
- The culture of E.coli was centrifuged in 1.5ml (micro) centrifuge tubes for 30 seconds at the 13500rpm.
- In 250µl of the re-suspension solution, re-suspend the pelleted cells now transfer the suspension of the cells to a micro centrifuge tube. Through vortexing or pipetting up and down, the bacteria must be re-suspended thoroughly until where is no any clump remains.
- The 250µl of the Lysis solution is added and mix it properly by investing the tube.
- Invent the tube 4-6 times, so the solution becomes first viscous and them little clear.
- The 350µl neutralization solution is added and then rapidly mix. Invert the tube 4-6 times properly and thoroughly.
- For minutes, centrifuge the debris if pellet cell and DNA of chromosomes.
- The supernatant is transferred to the, gene JET spin column through decanting or by the pipetting.
- Try to prevent the transfer of the white precipitate
- For one minute, centrifuge remove the flow-through and put the column again into the same collection tube.
- The gene JET spin column is washed by the addition of the 500µl of the wash solution I and then, centrifuge for approximately 30 to 60 seconds and then remove the flow through.
- The 500µl of wash solution is added to the gene Jet spin column the dilution is done by adding the ethanol before the use. Centrifuge for 30 to 60 seconds and remove the flow-through. Put the column again into the collection tube.
- The 500µl of the wash solution is used for the washing process.
- Remove the flow, through and then centrifuge for one more extra minute to remove the residues.
- Wash the solution, this step is mandatory for the removal of the residual ethanol in preps of plasmid.
- Take a fresh 1.5ml micro centrifuge tube and shift the Gene JET spin column into this tube. In the center of the Gene JET spin column, Elution Buffer is added to elute the plasmid DNA. Just try to avoid the touching of the pipette tip with the membrane. For 2 minutes incubate at the room temperature and for 2 minutes centrifuge then
- Through the column in trash bin and store this purified plasmid having DNA at 20°C.

3.28 Agarose Gel Preparation Method

- 1g of agarose is weighted.
- In a flask, add this agarose powder into the 100mml of the 1*TAE buffer.
- Heat it in oven for 3 minutes and wait until it dissolved completely.
- For cooling the gel, leave it for few minutes.
- Et Br is added with respect to the final volume of the gel which is 2µl.
- Now put the comb into the tray of gel.
- The gel is poured into the already settled comb tray.
- Solidification is done by leaving it for some time.

3.29 1X TAE Buffer

- Tris Base is 4.84g.
- Glacial Acetic Acid is 1.14ml.

- 2ml of 0.5 M EDTA (pH=8).
- With water, bring the volume up to one Liter.
- Samples loading and running of the Gel.
- Buffer is added for preparing the sample.
- In electrophoresis unit, keep the agarose get tray.
- Gently add the TAE Buffer into the electrophoretic unit just to cover the gel
- carefully remove the combs from the agarose gel.
- Loading the ladder into the first well of the gel.
- The, remembers the number of the well and load your sample. Now, set the voltage between the 80-150V and then run the gel.
- After 30 minutes power off the unit.
- Now, remove the gel gently and observed it under the system of gel documentation.

PCR confirmation

Reagents

Green Master Mix	**6µl**
Reverse primer	1µl
Forward primer	1µl
Water	4µl
Template	1µl

Primers

Forward primer	**GACAAGAAGTACTCGATCGGCCTC**
Reverse Primer	AATGGCCCCTGAACTTAATCATG

PCR profile

Steps	**Temp.**	**Time**	**No. of cycles**
Initial Denaturation	94^0C	5 min	
Denaturation	94^0C	30 sec	
Annealing	54^0C	45 sec	35 cycles
Extension	72^0C	1 min	
Final Extension	72^0C	5 min	

Protocol

- 13µl reaction mixture was added in the PCR tubes.
- After added mixture, PCR tubes were incubated in thermos-cycler.
- PCR product was visualized on 1% agarose gel under gel documentation system.
- 1kb ladder was used to estimate the product size.

4. RESULTS

4.1Map of cloning vector

In this research, multiplex vector pHSE-401 was used. It is the first vector which is brilliant in multiplexing. The map of the pHSE-401 cloning vector was obtained through Addgene website.

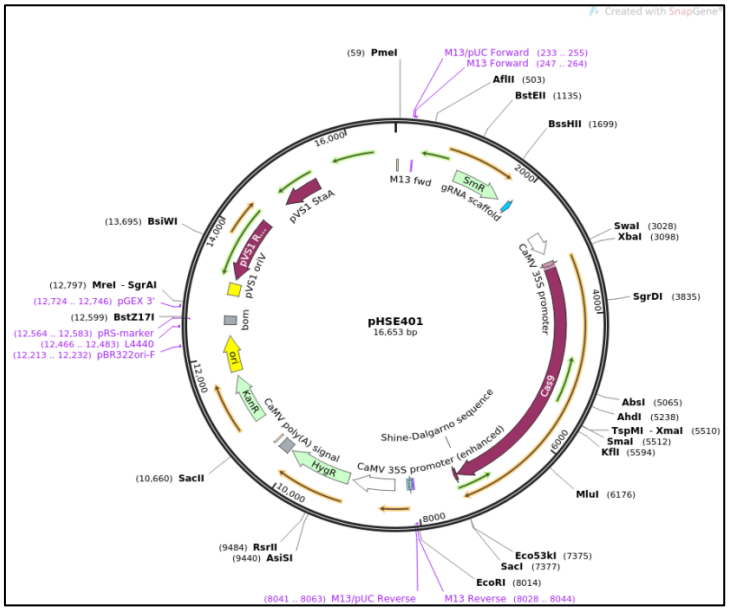

Figure a: The map of the cloning vector pHSE-401.

4.2 Map of the pHSE-401 expression vector

The map of the vector was taken from the Vector Builder. There are the 3gRNAs, 3gRNAs scaffold and 1Cas9 in this pHSE-401 vector. The vector has complete 16935 bp, U6-26p and U6-26t are also the parts of the expression vector. Cas9 which consists of 5645 bp. This vector was transformed into the agrobacterium, kanamycin, rifampicin and tetracycline are the specific antibiotics for this vector.

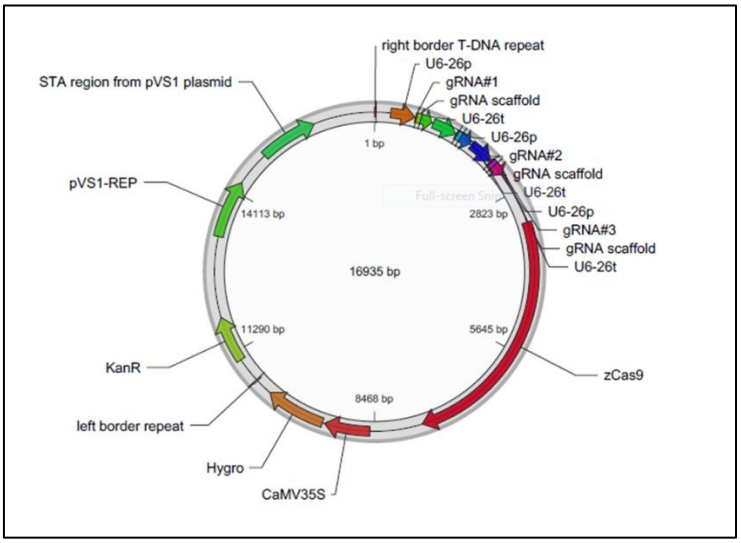

Figure b: Map of pHSE-401 plant expression vector having 3gRNAs and Cas9.

4.3. Transformed E. coli cells and their colonies Preparation

The transformation of plasmid in the Top-10 strain E. coli was done by the heat shock method by using the standard protocol of the laboratory. Multiple colonies of the E. coli were obtained over single plate.

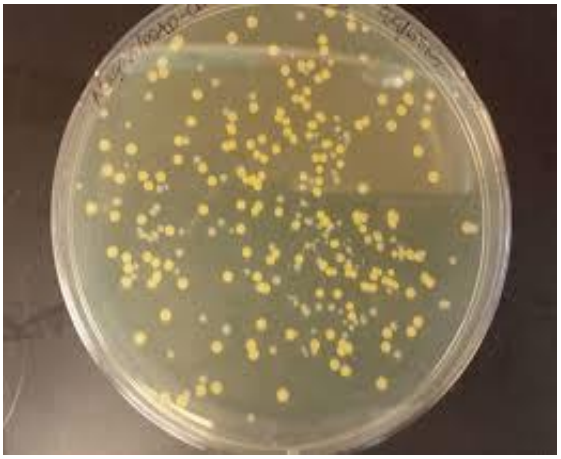

Figure c: Transformation of the vector pHSE-401 in E.coli.

4.4. Formation of the colonies of the transformed Agrobacterium cells

The Agrobacterium cells of EHA-105 strain were transformed with the vector pHSE-401 by the freeze thaw method. The whole procedure was performed on ice and then, kept in liquid nitrogen container. Agrobacterium cells were stored in the glycerol stock and they were spread on the agar plate for making the colonies of the transformed Agrobacterium.

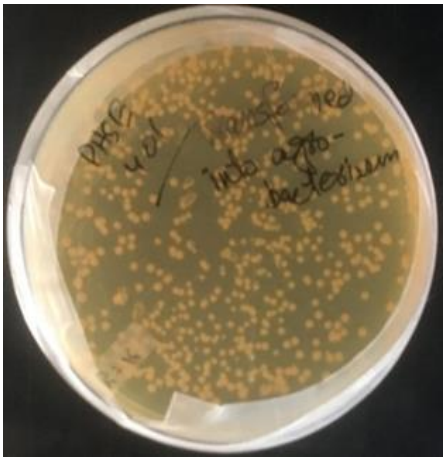

Figure d: Colonies of transformed pHSE-401 into Agrobacterium.

4.5. Plasmid Confirmation

The expression vector Phse-401, containing plant codon optimized Cas9 was obtained by the VectorBuilder. The pHSE-401 plasmid was restricted with the restriction enzymes XbaI and EcoRI and then, run on the 1% of agarose gel. By the restriction digestion process, two fragments were obtained. These were almost 11738bp and 4916bp. The sizes of these specific fragments confirmed the plasmid of pHSE_401.

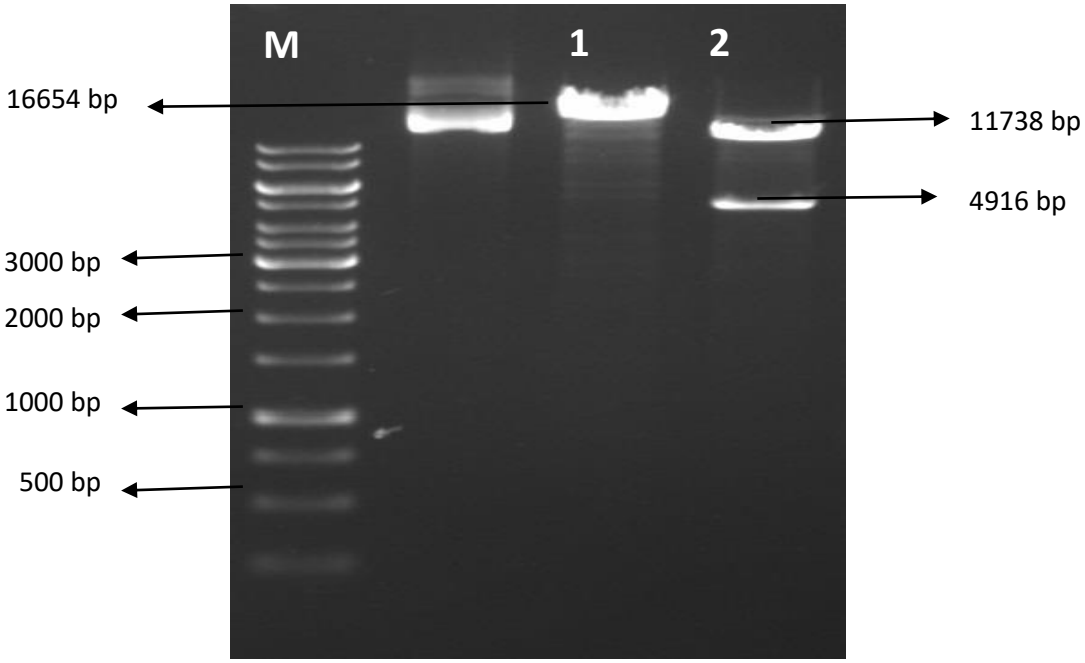

Figure e: pHSE-401 plasmid confirmation. (M) 1kb DNA ladder. (1) Undigested fragment of pHSE-401 plasmid. (2) Digested fragments of pHSE-401 plasmid.

4.6. Preparation of the nursery of cotton plants in laboratory

The linted cotton seeds were taken and the lint was removed with the commercial sulphuric acid due to which lint was completely removed. This step was performed in the fume hood because of the commercial sulphuric acid. Now, the de-linted cotton seeds were sterilized by using the ethanol and $HgCl_2$. The ethanol sterilized the cotton seeds, then these seeds were sown in the peat moss for three days and covered with aluminum foil and the covered pots were uncovered after three days. So, the immature plant came out which became mature after 4 days, these plants are the less mature in age.

Figure f: Nursery development of Coker-312.

4.6.1. Hypocotyls sterilization and excision

All the cotton plants were taken out from the peat moss after seven days and their leaves and roots were separated from hypocotyls and then, hypocotyls were shifted into the falcon having sterilization solution. And after that shake it gently and then, removed the sterilization solution from the petri plate. Now, 1000 hypocotyls were cut into the smaller segments. The hypocotyls were cut into the small pieces with the sharp sterile blade. After cutting into short segments, they were placed on filter paper. Again dry the smaller fragments of cotton hypocotyls with the autoclaved filter paper.

Figure g: Excision and drying of hypocotyls.

4.6.2. MGL addition

Agrobacterium primary and secondary culture was prepared and then, secondary culture was centrifuged at 8000rpm for 05 minutes. The secondary culture was taken, then the pellet formation occurred and supernatant was discarded and the MGL solution was added in the pellet and then, pellet was dissolved. For this step, ice was used and intense shaking was done in this step for the complete pellet dissolving in the MGL solution. This dissolved solution was added into the cotton hypocotyls and waited for 20 minutes and then, the solution was removed.

Figure h: Poured MGL solution having dissolved pellet of agrobacterium.

4.6.3 Shifting of hypocotyls on MSB media

All the hypocotyls were transferred on the MSB media which carried the hormones and kanamycin selection antibiotic and the plates were covered and kept in dark for 2 days for giving suitable environment for agrobacterium infection development in hypocotyls. The purpose of the shifting on MSB is the initiation of the growth in the hypocotyls edges. Darkness is the major requirement for this step. After 48 hours, hypocotyls are shifted to the next media.

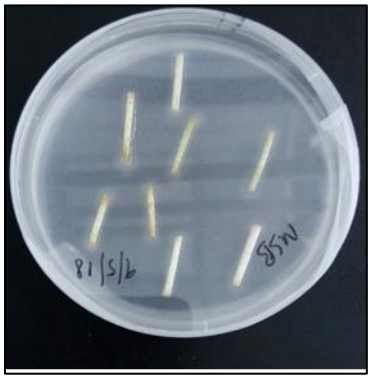

Figure i: Shifting of hypocotyls on MSB media.

4.6.4. Transferring of hypocotyls on CIM media

All the hypocotyls were taken out from MSB media after the 48 hours and then, they were shifted on CIM media. CIM media had the respective antibiotics, glucose, glycine and necessary nutrients which stimulated the growth of the hypocotyls because callus started to appear at the edges of segments. At the edges, after 4 weeks, 2 calli appeared and again hypocotyls were shifted to new CIM plates for further growth.

Figure j: Hypocotyls shifting on Callus induction media.

4.6.5. Formation of callus

After the four weeks, callus started to appear at the edges of the hypocotyls of the cotton crop. The undifferentiated cells started to appear at the sides of the 2 hypocotyls portions. After every four weeks period, new plate of freshly prepared CIM media was used for the proper and efficient growth of the calli. The efficiency of result was 0.02%. Only two transgenic calli were obtained.

Figure k. Callus appeared on excised edges of hypocotyls.

4.6.6. PCR based screening of callus

DNA was extracted from 2 transformed callus grown on selection media. For this method, Mini prep kit was used and DNA was isolated. Then, PCR technique was used by following the standard protocol. For that purpose, primers were designed for gRNA through CHOPCHOP bio-informatics tool and fragment was amplified through this process. After PCR, product was run on 1% agarose gel for getting the band for the identification and confirmation of the gRNA. So, the band of 200 base pairs confirmed the presence of the gRNA in the callus of cotton. This cotton callus was transgenic callus with transformed vector.

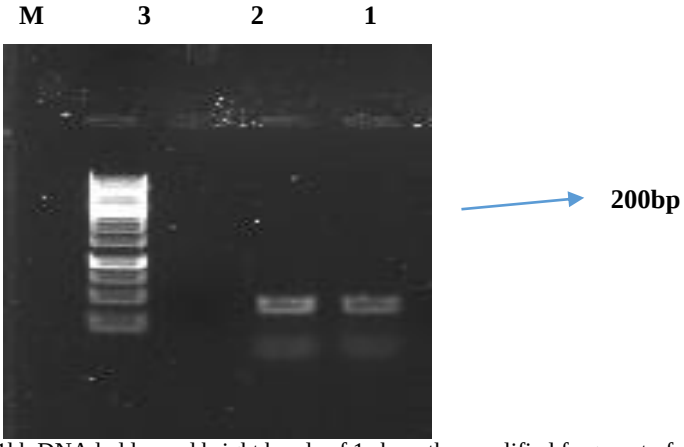

Figure l: Confirmation of gRNA in plants (M) 1kb DNA ladder and bright bands of 1 show the amplified fragment of gRNA.

DISCUSSION

The economy of Pakistan majorly depends upon the cotton products like lint and cotton seeds. Its products play role in the 10% GDP and 55% in the export business. Nowadays, synthetic substance and fertilizers are used. The crop cultivation land is increased which increased the production of cotton. The cotton crop has become susceptible to pests, insects and viruses. Cotton leaf curl virus disease is a dangerous threat for the cotton production because leaves get curly and inverted. There is only DNA A component because the genome of these viruses is monopartite which is equal to the genome of the *Begomoviruses* of family *Geminiviridae*. The complex of these viruses cause the 80% reduction in the cotton production last year. Another option to conventional GMO (transgenic) crop and plant rearing strategies to enhance trim plant is focused on genome designing or genome altering (Khaoula *et al*., 2003).

Through site particular nucleases, the directed transgene reconciliation technique fulfilled in a persuasive and unequivocal way. With the help of these built nucleases we develop perfectly DSBs (2 fold stranded breaks) to focus DNA and absolutely provoke the DNA repair component (Luisa *et al*., 2007). Among these Nucleases, there is Zinc finger nuclease (ZFNs) has a built exhibit of zinc fingers which then, converge in to the synergist region of Fok nuclease, acts about as a dimers to acquaint DSBs (Fenola *et al*., 2002). The second nuclease is TALEN (Transcription activator like effector nucleases consists of DNA restricting area of a TALE joined with non-particular FoK nuclease, form a grouping specific nuclease that was utilized to develop a DSBs. From these two units of grouping specifically, TAL Effector Nuclease attach to the objective DNA and then, engineer a spacer locale of 2-9 bp to permit the dimerization of endonuclease areas and develop DSBs (Jieliang et al., 2003). A new technique of present days has come as a game changer for this reason. This is Cluster regularly interspaced short palindromic repeats (CRISPR) related to Cas endonuclease. This magic has quite recently been uncovered. A little planner which is the guided RNA accomplish the endonuclease Cas9 towards the target DNA and chop the particular grouping vital to the guide RNA (Haft *et al*., 2018).

For Pakistan economy, from the last 30 years, CLCuD has become a critical threat. The rep gene of these viruses is targeted, Multiple techniques like ZFNs, TALENs and CRISPR/Cas9 have been utilized to inhibit this disease by targeting the specific site which is mandatory for the process of- rolling circle replication mechanism in the *Begomoviruses* in the field of genome engineering , for triggering the genome of CABs, CRISPR/Cas9 system has become a gene changer CRISPR/Cas9 system is taken as a most advanced methodology because it has the ability to trigger the different genes simultaneously. There are the multiple gRNAs and Cas9 in single vector (Ayan *et al*., 2019).

In our research, firstly, the designed vector map was taken from VectorBuilder. The vector pHSE-401 is the first vector which is brilliant for multiplexing, it has 3 gRNA and 1 Cas9. All the 3 RNAs perform the role as a guide and help the Cas9 towards its target area, ultimately for the development of the cuts at the respective sites. The sequence of the vector pHSE-401 was confirmed through the restriction digestion method. For this purpose, two restriction enzymes were used. They cut the vector DNA into the two fragments of specific sizes which confirmed the identity of the vector when we run it on the gel. NCBI was used to find the sequence of genome of CLCuK. The vector DNA was isolated through mini prep kit and its concentration was checked by running it on the gel. Gel Documentation image was captured, the clear bands came which showed the good concentration of the plasmid DNA and after this, in Agrobacterium vector was transformed (Naeem *et al*., 2018).

Coker 312 cotton variety was used for transformation because Coker 312 has good regeneration capacity. We have established a rapid and technically efficient transformation system. Well, there

are several methodologies are available but they are quite complex and time consuming. The agrobacterium strain EHA105 was selected because it gave good results in previous cotton studies. For that purpose, cotton seeds were grown in peat moss because studies showed that it has more water retention capacity. After 3 days, immature plants started to appear. When these plants became mature on the 7th day, then they were used for transformation, we used to keep them in dark instead of light because previous studies show that green hypocotyls give excellent and rapid callus forming result.

The cultures of Agrobacterium were prepared, firstly, primary culture was developed and then, secondary culture was formed with respective antibiotics kanamycin, rifampicin and tetracycline, OD_{600} was 0.8-1.0. The agrobacterium culture was centrifuged at 8000rpm. On the day of transformation, 15 hypocotyls were cut with sharp blade and then, used for the purpose of the transformation. The hypocotyls were co-cultivated with the Agrobacterium pellet dissolved in the MGL solution. Then, 1000 hypocotyls were shifted to MSB media, for this, hypocotyls were kept for two days in dark with fully covered aluminum foil. Then, CIM media was prepared, hypocotyls were transferred from the MSB to the CIM media which has glycine, glucose and agar etc. It gave stable environment for agrobacterium infection. We added Plant Tissue Culture and carbenicillin, it gave contamination free media to the hypocotyls and after the process of the shifting, 2calli appeared. There was the 0.02% of calli. After this, PCR Screening was the major step. For PCR Screening, for primer designing, CHOPCHOP bioinformatics tool was used. Two primers were designed, one of them is forward and the other is reverse, these primers were designed for the confirmation of the gRNA. The callus DNA was extracted through mini prep kit. The step of DNA isolation was performed for getting the pure DNA. Now, the PCR protocol was used for PCR Screening, there were 35 cycles run. Then, PCR product was obtained and run on the gel against 1kb ladder, two bright bands of 200 bp were observed and they confirmed the presence of the gRNA. So, it means that CRISPR/Cas9 construct has successfully transformed into the cotton crop. Now, this crop has ability to tackle the CLCuVs by chopping the genome of the virus with Cas9 (Sattar *et al*., 2010).

This study is quite different from other studies because it has the involvement of the multiplex CRISPR vector with 3 gRNAs and 1 Cas9. We have developed an efficient and quite competent derived transformation methodology which has enough potential to protect the cotton crop from CLCuD. The advanced techniques of the CRISPR/Cas9 works in a marvelous way. It targets the replication encoding genes of virus by introducing the cleavage that was the major reason to perform the cotton transformation for developing the resistance against the cotton leaf curl virus disease in cotton plant. Therefore, the CLCuD cascade can be targeted through this techniques by aligning approaches for the induction of resistance along with CRISPR/Cas9 tool. Our method can be used for the resistance development against crop damaging viruses (Zaidi *et al*., 2018).

3. CONCLUSION

Cotton crop provides us food, feed and economical support. In 2006, approximately 34.3 million hectares (Mha) cultivation land was yielding the almost 25 million metric tons (MMT) of fiber. For 20 million farmers among 80 countries of are producing cotton as a cash crop (De Onis et al., 1993). Cotton lint is quit valuable, therefore China (26.40%) then, India (20.5%). After that United States of America (13.9%) and Pakistan (8.5%). Different other countries like Brazil (6.3%), Uzbekstan (4%). Australia (3.8%) and Turkey (3.3%). These countries are considered as the major producing bodies of the cotton lint. Cotton crop is attacked by many pests, insects, bacteria, viruses and fungi. So, these pathogens are the big threats for the cotton crop. In different stages of plant life like in shoot development, feed and food and flowering are also affected through the antibiotics stresses, drought, cold, high temperature and salinity. For the economy of the country, CLCuD is the big danger for the transmission of the virus to the cotton plant, whitefly acts as a vector. The viral genome is targeted at the specific genes included in replication or the life cycles Begomoviruses. For this purpose, multiplex editing with CRISPR/Cas9 system is surely preferred. In this study, pHSE_401 vector was used which has the 3 gRNA and 1 Cas9. This construct was transferred into the agrobacterium and cultures having respective antibiotics were developed. Then, cotton hypocotyls fragments were prepared and interacted with the agrobacterium culture. Then, MSB and CIM media were used. Callus appeared and PCR was done which showed that the CRISPR/Cas9 – 3gRNAs complex was completely transformed into the cotton plants through Agrobacterium, the plants having the CRISPR/Cas9 construct did not show the symptoms of the cotton leaf curl virus disease. The purpose of the study was to inhibit the replication of the cotton leaf curl virus by targeting the viral genome.

REFRENCES

1. Ali, M. (1997). Breeding of cotton varieties for resistance to cotton leaf curl virus. *Pak. J. Phytopathol*, *9*(1), 1-7.
2. Ali, Z., Abulfaraj, A., Idris, A., Ali, S., Tashkandi, M., & Mahfouz, M. M. (2015). CRISPR/Cas9-mediated viral interference in plants. *Genome biology*, *16*(1), 238.
3. Ali, Z., Ali, S., Tashkandi, M., Zaidi, S. S., & Mahfouz, M. M. (2016). CRISPR/Cas9-mediated

immunity to geminiviruses: differential interference and evasion. Sci Rep 6: 26912.
4. Anonymous. (2008). FAO statistics. http://www.fao.org.accessed 7 Jan 2008.
5. Anonymous. (2014-15). Economic Survey of Pakistan. 2014-15. 2:25.
6. Baltes, N. J., Hummel, A. W., Konecna, E., Cegan, R., Bruns, A. N., Bisaro, D. M., & Voytas, D. F. (2015). Conferring resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. *Nature Plants*, *1*(10), 1-4.
7. Baltes, N. J., J. Gill-Humanes, T., Cermak, P. A. Atkins &Voytas, D.F. (2014). NA replicons for plant genome engineering. *Plant Cell. 26*: 151-163
8. Barrangou, R. & van der Oost, J. (2013). CRISPR-Cas systems: RNA-Mediated Adaptive Immunity in Bacteria and Archaea. 82, 237-66.
9. Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., ... & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science*, *315*(5819), 1709-1712.
10. Beerli, R. R., Segal, D. J., Dreier, B., & Barbas, C. F. (1998). Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proceedings of the National Academy of Sciences*, *95*(25), 14628-14633.
11. Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, *409*(6818), 363-366.
12. Bibikova, M., Golic, M., Golic, K. G., & Carroll, D. (2002). Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases. *Genetics*, *161*(3), 1169-1175.
13. Bolotin, A., Quinquis, B., Sorokin, A., & Ehrlich, S. D. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*, *151*(8), 2551-2561.
14. Böttcher, B., Unseld, S., Ceulemans, H., Russell, R. B., & Jeske, H. (2004). Geminate structures of African cassava mosaic virus. *Journal of virology*, *78*(13), 6758-6765.
15. Briddon, R. W., & Markham, P. G. (2001). Cotton leaf curl virus disease. *Virus research*, *71*(1-2), 151-159.
16. Briddon, R. W., Bull, S. E., Amin, I., Mansoor, S., Bedford, I. D., Rishi, N., ... & Markham, P. G. (2004). Diversity of DNA 1: a satellite-like molecule associated with monopartite begomovirus–DNA β complexes. *Virology*, *324*(2), 462-474.
17. Briddon, R. W., Mansoor, S., Bedford, I. D., Pinner, M. S., & Markham, P. G. (1999). Clones of cotton leaf curl geminivirus induce symptoms atypical of cotton leaf curl disease. *Virus Genes*, *20*(1), 19-26.
18. Briddon, R. W., Mansoor, S., Bedford, I. D., Pinner, M. S., Saunders, K., Stanley, J., ... & Markham, P. G. (2001). Identification of DNA components required for induction of cotton leaf curl disease. *Virology*, *285*(2), 234-243.
19. Carroll, D. (2011). Genome engineering with zinc-finger nucleases. *Genetics. 188*: 773-782.
20. Ceasar, S. A., Rajan, V., Prykhozhij, S. V., Berman, J. N., & Ignacimuthu, S. (2016). Insert, remove or replace: a highly advanced genome editing system using CRISPR/Cas9. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1863*(9), 2333-2344.
21. Čermák, T., Baltes, N. J., Čegan, R., Zhang, Y., & Voytas, D. F. (2015). High-frequency, precise modification of the tomato genome. *Genome biology*, *16*(1), 232-246.
22. Chandrasekaran, J., Brumin, M., Wolf, D., Leibman, D., Klap, C., Pearlsman, M., ... & Gal-On, A. (2016). Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Molecular plant pathology*, *17*(7), 1140-1153.
23. Cheng, X., Li, F., Cai, J., Chen, W., Zhao, N., Sun, Y., ... & Wu, X. (2015). Artificial TALE as a convenient protein platform for engineering broad-spectrum resistance to begomoviruses. *Viruses*, *7*(8), 4772-4782.
24. Dalmay, T., Hamilton, A., Rudd, S., Angell, S., & Baulcombe, D. C. (2000). An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell*, *101*(5), 543-553.
25. Damalas, C. A. (2009). Understanding benefits and risks of pesticide use. Res. *Essays. 4*(10), 945-949.
26. Endo, M., Mikami, M., & Toki, S. (2016). Bi-allelic gene targeting in rice. *Plant Physiol*, *170*: 666-677.
27. Fauser F., Schiml, S., & Puchta. H. (2014). Both CRISPR/Cas-bascd nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. *Plant J. 79*: 348-359.
28. Feng, Z., Mao, Y., Xu, N., Zhang, B., Wei, P., Yang, D. L., ... & Zeng, L. (2014). Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. *Proceedings of the National Academy of Sciences*, *111*(12), 4632-4637.
29. Finnegan, E. J., Margis, R., & Waterhouse, P. M. (2003). Posttranscriptional gene silencing is not compromised in the Arabidopsis CARPEL FACTORY (DICER-LIKE1) mutant, a homolog of Dicer-1 from Drosophila. *Current Biology*, *13*(3), 236-240.
30. Fonfara, I., Richter, H., Bratovič, M., Le Rhun, A., & Charpentier, E. (2016). The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature*, *532*(7600), 517-521.

31. Gamborg, O. L., R. A. Miller and K. Ojima. 1968. Nutrient requirements of suspension culture of soybean roots cells. Exp. Cell. Res. 50: 151-158.
32. Gao, J., G. Wang, S. Ma, X. Xie , X. Zhang, Y. Wu, P. Zhao and Q. Xia . 2015. CRISPR/Cas9-inediated targeted mutagenesis in Nicotiana tabacum. *Plant Mol. Biol*, *87*(1-2): 99-1 10.
33. Gao, J., G. Wang, S. Ma, X. Xie, X. Wu, X. Zhang, Y. Wu, P. Zhao and Q. Xia. 2015. CRISPR/Cas9-mediated targeted mutagenesis in Nicotiana tabacum. *Plant Mol Biol*, 87: 99-1 10.
34. Garneau, J. E., M. E. Dupuis, M. Villion, D. A. Romero, R. Barrangou, P. Boyaval, C. Fremaux, P. Horvath, A. H. Magada'n n and S. Moineau. 2010. The CPJSPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature. 468: 67-71.
35. Hull, R., S. N. covey and A. J. Maule.1987. Structure and replication of caulimovirus genomes. *J. cell sci. suppl*, 7: 213-229.
36. Hussain, T. and M. Ali. 1975. A review of cotton diseases of Pakistan. Pak. *Cottons*. 19: 71-86.
37. Ilyina T. V. and E. V. Koonin. 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eukaryotes and archae bacteria. Nucleic Acids Res. 20: 3279-3285.
38. Iqbal, Z., M. N. Sattar and M. Shafiq. 2016. CRISPR/Cas9: A Tool to Circumscribe Cotton Leaf Curl Disease. Front. Plant Sci. 7: 475.
39. Ishino, Y., H. Shinagawa, K. Makino, M. Amemura and A. Nakata. 1987. Nucleotide sequence of iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product, J. Gen. Vir. 76: 2915-2922.
40. Jin, S. X., X. L. Zhang, S. G. Liang, Y. C. Nic, X. P. Guo and C. Huang. 2005. Factors affecting transformation efficiency of embryogenic callus of upland cotton (Gossypium hirsutum L.) with Agrobacterium tumefaciens. Plant Cell Tiss org. 81: 229-237.
41. Jin, S. X., X. L. Zhang, Y. C. Nie, X. P. Guo, S. G. Liang and H. G. Zhu. 2006b. Identification of a novel elite genotype for in vitro culture and genetic transformation of cotton. Biology Plant. 50: 519-524.
42. Jinek, M., K. Chylinski, l. Fonfara, M. Hauer, J. A. Doudna and E. Charpentier. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 337: 816-821.
43. Joung J. K. and J. D. Sander. 2013. TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol. 14: 49-55.
44. Kanchiswamy, C. N. 2016. DNA-free genome editing methods for targeted crop improvement. Plant Cell Rep. .35: 1469-1474.
45. Karginov, F. V. and G. J. Hannon. 2010. The CRISPR system: small RNA-guided defence in bacteria and archaea. Mol. Cell. *37* (l): 7-19.
46. Kheyr-Pour, A. M. Bendahmane, V. Matzeit, G. P. Accotto, S. crespi, and B. Gronenborn. 1991. Tomato yellow leaf curl virus from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Res. 19: 6763-6769.
47. Koonin E. V. and T. V. llyina. 1992. Geminivirus replication proteins are related to the Prokaryotic plasmid rolling circle DNA replication initiator proteins. J. Gen. Virol. *73*: 2763-2766.
48. Kumar, A., J. Kumar and J. Khan. 2010. Sequence characterization of cotton leaf curl virus from Rajasthan: phylogenetic relationship with other members of geminiviruses and detection of recombination. Virus Gen. 40: 282-289.
49. Lai, H., J. He, M. Engle, M.S. Diamond and Q. Chen. 2012. Robust production of virus-like particles and monoclonal antibodies with geminiviral replicon vectors in lettuce. Plant Biotechnol J. 10: 95-104.
50. Laufs J., S. Schumacher, N. Geisler, l. Jupin and B. Gronenborn. 1995. Identification of the nicking tyrosine of geminivirus Rep protein. FEBS Lett. 377: 258-262.
51. Li, Z. S., Z. B. Liu, A. Q. Xing, B. P. Moon, J. P. koellhoffer, L. X. Huang, R. T. ward. E. Clifton. S. C. Falco and A. M. Ciqan.2015. Cas9-guide RNA directed genome editing in soybean. Plant Physiol. 169: 960-70.
52. I,ico, C., Q. Chen and L. Santi. 2008. Viral vectors for production of recombinant proteins in plants. J. Cell. Physiol. 216: 366-377.
53. Makarova, K. S., D. H. llaft, R. Barrangou, S. J. Brouns , E. Charpentier , P. Horvath , S. Moineau , F. J. Mojica , Y. l. Wolf , A. F. Yakunin , J. van der Oost and E. V. Koonin . 201 1. Evolution and clessification of the CRISPR-Cas systems. Nat. Rev. Microbiol. 9(6):467-477
54. Makarova, K. S., N. V. Grishin, S. A. Shabalina, Y. l. Wolf and E. V. Koonin. 2006. A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryoti RNAi, and hypothetical mechanisms of action. Biol Direct. l: 7.
55. Makarova, K. S., Y. I. Wolf', O. S. Alkhnbashi, F, Costa, S. A. Shah, S. J. Saunders, R. Barrangou S. J. Brouns, E. Charpentier, D. H. Haft, P. Horvath, S. Moineau, F. J. Mojica, R.M. Terns, M. P. Terns, M. F. White, A.F. Yakunin, R. A. Garrett, J. Vander Oost, R. Backofen and E. V. Koonin.2015. An updated evolutionary classification of CRISPR-Cas systems. Nat. Rev. Microbiol.13 (11): 722-736.
56. Mali, P., L. Yang, K. M. Esvelt, J. Aach, M. Guell, J. E. DiCarlo, J. E. Norville and G. M. Church. 2013a. RNA-guided human genome engineering via Cas9. Science. 339: 823-826.
57. Mansoor, S. R. W. Briddon, Y. Zafar and J. Stanelye. 2003. Geminivirus disease complexes: an emerging threat. Trends Plant Sci, 8: 128-1 34.

58. Mao, Y. Y., Zhang, Z. Feng, P. Wei, H. Zhang, J. R. Botella and J-K. Zhu. 2015. Development of germ-line-specific CRISPR-Cas9 systems to improve the production of heritable gene modifications in Arabidopsis. Plant Biotechnol J. 14:519-532.
59. Mao, Y., Z. Zhang, Z. Feng, P. Wei, H. Zhang, J. R. Botella and J. K. Zhu. 2015. Development of germ-line-specificCRlSPR-Cas9 systems to improve the production of heritable gene modifications in Arabidopsis. *Plant Biotechnol. J, 14*(2): 519-532.
60. Marraffini, L. A. and E. J. Sontheimer. 2008. CRISP R interference limits horizontal gene transfer in staphylococci by targeting DNA. Science. 322: 1843-1845.
61. Marraffini, L, A. and E. J. Sontheimer. 2010. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat. Rev. Genet. I l (3): 181-190.
62. Mercer, A. C., T. Gaj, R. P. Fuller and C. F. 3rd. Barbas. 2012. Chimeric TALE recombinases with programmable DNA sequence specificity. Nucleic Acids Res. 40: 11163-11172.
63. Nakayama, T., M. B. Fish, M. Fisher, J. Oomen-Hajagos, G. H. Thomsen and R. M. Grainger . 2013. Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in Xenopus tropicalis. Genesis. 51(12): 835-843.
64. Navot, N., E. Pichersky, M. Zeidan, D. Zamir and H. Czosnek. 1991. Tomato yellow leaf curl virus: a whitefly-transmitted geminivirus with a single genomic component. Virology. 185: 151-161.
65. Nekrasov, V., B. Staskawicz, D. Weigel, J. D. G. Jones and S. Kamoun. 2013. Targeted mutagenesis in the model plant Nicoatina benthamiana using Cas9 RNA-guided endonuclease. Nat Biotechnol. 31: 691-693.
66. pannetier, C., M. Giband, P. Couzi, V. L. Tan, M. Mazier, J. Tourneur and B. Hau. 1997. Introduction of new traits into cotton through genetic engineering: insect resistance as example. Euphytica. 96: 163-166.
67. Piatek, A. and M. M. Mahfouz. 2016. Targeted genome regulation via synthetic programmable transcriptional regulators. Crit.Rev.Biotechnol. *37*(4): 429-440.
68. Schiml, S., F. Fauser and H. Puchta. 2014. The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. *Plant J*. 80: 1139-50.
69. schmid-Burgk, J. L., T. Schmidt, V. Kaiser, K. Honing and V. 2013. A ligation independent cloning technique for high-throughput assembly of transcription activator- like effector genes. Nat. Biotechnol. 31: 76-81.
70. settlage, S. B., R. G. See and L. Hanley-Bowdoin. 2005. Geminivirus C3 protein: replication enhancement and protein interactions. *J. Virol*. 79: 9885-9895.