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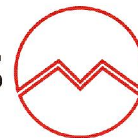

Role of CRSIPR-Cas9 system in plant improvement

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Abstract: The system of CRISPR/cas9 was firstly seen in bacteria and archaea and was found useful in degradation of the exogenous substrates. It is optimized continuously and there is dramatic expansion in its applications. It has revolutionized the field of plant biology. Transgenic techniques have been used to understand basic plant biology and also crop improvement. Using the CRISPR/CAS9 system in genome editing can modify the plants genome.
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Introduction

In the current situation, increasing population is the most critical challenge faced by human is the production and security of food. Besides growing population rate, extreme weather increases the biotic and abiotic stress and reduced the availability of agricultural land. Development of technologies that can contribute in crop improvement and can increase production of crop to some extent. Transgenic techniques have been used to understand basic plant biology and also for crop improvement. Using the CRISPR CAS/9 system in genome editing can modify the plants genome. This system uses single-guide RNAs for genome editing making it simple powerful for targeted gene mutagenesis deletion, insertion and replacement as well as transcriptional regulation (Cong *et al.,* 2013). Genome edited plants have additional advantage over transgenic plants as they carry edited DNA of desired trait. This is the most unique and innovative technique for the breeding of plants and it was developed in 2012 by the team of scientists stated at California University, Berkeley. It has attracted many of the scientists in the recent years as the food production is becoming a ground for research. The areas under CRISPR are gene editing which is DNA free, homology directed repair (HDR) and transient silencing of genes (Mushtaq *et al*., 2020; Yaqoob *et al*., 2020; Sergei *et al.,* 2015). The system of CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) was firstly seen in archaea and bacteria and was found to be useful in degradation of the exogenous substrates. It is optimized continuously and there is dramatic expansion in its applications. It has revolutionized the field of plant biology (Ahmad *et al*., 2020; Wang *et al.,* 2019).

It has been divided into 3 major types which are I, II and III. Today, the research in plants is most concerned with type II of this system. It required RNase III (Ribonuclease III), crRNA (CRISPR RNA), tracrRNA (transactivating crRNA) and CAS-associated 9 protein for editing of the targeted genes. It is known that by the fusion of crRNA to tracrRNA forms the single guide RNA (sgRNA) has the same role like that of crRNA-tracrRNA hybrid (Belhaj *et al.,* 2013). It is been widely used in the rapid development and improvements in the alteration of metabolic pathways of plants, quality of crops and drugs production. It has tremendously impacted the fields of molecular biology and bioengineering in which scientists have searched some CRISPR-derived systems, as Cpf1, dCas9 nickase, fCas9 and other systems of nucleases. It is naturally occurred gene editing tool and it is present in bacteria for immune defence when any virus attacks them. Studies have shown that CRISPR-CAS9 is the great tool for gene editing as compared to others like ZFNs and TALENs (Carroll, 2011). This is showing the developments in CRISPR genome editing through the course of years from year 2013 till now and it is now the talk of the time because population growth and persistent drought leading to food shortages (Ali *et al*., 2020; Khalil *et al*., 2020ab; Danish *et al*. 2020).

Genome Editing and Nucleases

Modern Genomics involves genome editing that is the engineering of DNA nucleases in a particular way that it shows both specific along with non-specific binding domains of DNA. Genome editing is basically a procedure through which targeted gene is precisely

cleaved by engineered nucleases, therefore HDR and NHEJ repairs the existing breaks. Comparative studies among first generation and second generation techniques show efficacy of second generation technique as CRISPR-CAS9 system (reliable and efficient for genome editing) is used in this technique while first generation techniques depicts a lengthy and time taking procedure that uses mega and zinc FNs along with effector nucleases. Second generation techniques can achieve a specific target and is economical. In recent past scientists used zinc finger nucleases for gene editing purpose in plants as well as in animals. Modern studies reveal that ZFNS is not a reliable source for genome editing as it is less specific in nature and it has more non target sites than the actual target sites. CRISPR-CAS9 system is considered as genome editing's new generation as it has replaced other systems such as zinc finger nucleases as well as TALENS (Zhang *et al*., 2016). CRISPR-CAS9 is already existing tool for gene editing purpose in microbes such as bacteria that is used for immune response against virus (Yaqoob *et al*., 2020). This system has other benefits such as it can help to enhance crop's yield and quality, also it can help plant to tolerate stress imposed by the environment. Targeted mutagenesis is a process that usually involves gene knockouts, base substitutions, gene editing at multiple sites and regulation of certain gene transcriptions in plants and in this process CRISPR-CAS9 system became a meaningful tool. Productivity and quality of certain crops is affected by a range of stressors (biotic, abiotic) (Masood *et al*., 2020; Razzaq A *et al*., 2019). SSNs (sequence specific nuclease) are an amazing tool that is used to meet modern world challenges in genome editing in plants. For successful genome editing in plants as well as in animals CRISPR-CAS9 is widely used editing tool. Therefore, in comparison to other gene editing tools (zinc finger nucleases and TALENS) CRISPR-CAS9 is an efficient and reliable system to be used for genome editing (Xiao *et al*., 2014).

NHEJ and HDR

Double-strand break repair machinery is activating by the cleavage of double-stranded DNA by Wild-type Cas9 nuclease site, specifically. The target sequence is disrupted due to indels as non-homologous end joining resulted in absence of a homologous repair template. By the provision of a homologous repair template, knock-ins and precise mutations can be made. This results in exploitation of homology directed repair pathway, alternatively. A site specific single-strand nick is made by mutated Cas9. To introduce a staggered double-stranded break, two sgRNA can be used which can then undergo homology directed repair. Allowing specific localization, with various effector domains Nuclease-deficient Cas9 can be fused as in example, repressors, fluorescent proteins and transcriptional activators (Jiang *et al.,* 2013).

Zinc Finger Nucleases, TALENs, Mega nucleases

To attain desired recognition of target, engineering of TALENS was conducted through the modification of domain repeats of transcription effectors. Modified TALEN's have the ability to identify stretches of 18-20bp and zinc finger nucleases can recognize stretches of only 14-20bp. It is a complicated procedure to design TALENs as there is a need of thymidine base at the start position that is of repetitive nature and enormous size. TALENs are highly specific to target binding sites as compared to ZFNs. In plants such as rice and tobacco TALENs were used for genome editing (Zhang *et al*., 2017). Above mentioned techniques allow us to make any virtual change in genome such as insertion as well as deletion and replacement of gene along with any targeted sequence change in DNA.

So, genome editing technique is consistent for improving yield to achieve the growing demand of world's existing food famine and environmentally safe agriculture scheme. Mega nucleases are the first tools used for genome editing that are naturally found in all aspects of life and play an important role in genome engineering. The second category is Zinc Finger Nucleases and an another category is TALENs which are derived from pathogenic bacterium and the last category of engineered endonucleases derived from bacterial acquired immune system called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/CAS9). Both mutagenesis and gene replacement are stimulated locally by Double Stranded Breaks (Carroll, 2011). Mega nucleases are divided into five families the most important family is LAGLIDADG. Some mega nucleases form homodimers and form palindrome and false palindrome target, while others are monoemic and identify non-palindrome sequence. The existence of complex network interaction between DNA and mega nucleases so, its code has not been identified.

The probability of a genome detection site for each enzyme is very low e.g if the length of detection site of an enzyme is 18 bp so the human genome must be 20 times larger than the value so that there is an identifier for the enzyme to be targeted. The goals include the creation of plants with characters that confer the resistance of abiotic and biotic stresses and with valued compositional properties. These techniques are consistent for improving the yield to achieve the growing demand of world's existing food famine and to launch environmentally safe agricultural scheme, more specific, productive, eco-friendly and cost effective. A significant concern in these technologies is the occurrence of 'off-target' activity and induced mutations which may impede gene

activity studies and functional analysis. Moreover, the 'off-target' activity results in either not reported or unknown produce non-quantifiable cellular signaling, difficult to detect and physiological effects. In past few years, many methods have been developed to identify undesired mutations and off-target effects. Minimizing 'off-target' activity and improving target specificity offers better application of GE technology in crop improvement and plant biology. Mega nucleases are the first tools they are naturally found and the second category is Zinc Finger Nucleases which are made up from series of finger prints on a DNA identifier integrated with Fak1 catalytic domain and the third category is Transcription Activated-Like Effector Nucleases nucleic acids is DNA derived from pathogenic bacterium derived from Xanthomonas and emerged into the second category and the last and latest category of engineered endonucleases is CRISPR/CAS9 which is derived from bacterial immune system (Bibikova *et al.,* 2003; Tahir *et al*., 2020).

Criteria for selection of an endonuclease

In order for an engineered endonuclease to be widely used in targeted genomic editing, it must have two main criteria: A) Identify a long sequence of DNA in order to prevent the creation of toxicity caused by off-target cuts. Because off-target cuts cause changes in unwanted locos and unpredictable phenotypes. B) Identification and cutting in a defined sequence is easy to design. The four main categories of doxy-endonucleases include: megranucleases, endodonucleases, zinc fibrils, endo-nucleases, transcription-promoting transcription factor and CRISPR / cas systems, which are considered as advanced genome engineering tools and each has disadvantages and advantages (Yin *et al*., 2017; Tahir *et al*., 2020).

Requirements of CRISPR cleavage methodology

1. Synthetic 20 nucleotides short sequence gRNA that can bind to DNA

2. Cleavage of 3-4 bases after protospacer adjacent motif by Cas9 nuclease enzyme and it has 2 domains which are HNH domain and Ruv-C like domain which has cleavage activities.

(Jiang *et al.,* 2013)

Mutagenesis in wheat using CRISPR/Cas9

The most widely grown crop in the world is common wheat (*Triticum aestivum* L.). It is considered as the staple and major component of diet in Pakistan. It has genome size of 17 Gb and is complex polyploid. It has more than 80% of repititive sequences in the genome. This poses challenges for analyses of its genes and their subsequent functions. The broad-spectrum resistance is shown by the use of CRISPR/Cas9 system by introducing *TaMLO* mutants in the disease of powdery mildew (Wang *et al*., 2014). With no detectable transgenes, mutants were generated in tetraploid durum wheat and hexaploid bread wheat by editing of genes. A study was carried out through simultaneous modification to generate *Taedr1* wheat plants using CRISPR/Cas9 technology in three homologs of *Taedr1* plants, and the wheat *EDR1* which were showing resistance to powdery mildew and there was cell death noticed by induction of mildew (Liang *et al.,* 2017). In the same manner, by CRISPR/Cas9 technology, the reduction in amount of α-gliadins in the seed kernel provides durum and bread wheat lines with the reduction of immunoreactivity for the consumers which are intolerant to gluten (Howells *et al.,* 2018).

Biolistic Method of Transformation

CRISPR/Cas9 system uses the method of biolistic transformation because it is difficult to transform wheat genetically. By using the biolistic particle delivery method, there can be produced multiple-copy insertions. The expression levels of the Cas9 and sgRNA protein can be increased using this method (Wang *et al.,* 2018).

Gene-Trageting through CRISPR-Cas9 system

In common wheat, three genes were targeted which are *DA1* gene, the *Pinb* gene, and granule-bound starch synthase gene (GBSS or waxy). The two major genes, puroindoline b (*Pinb)* and puroindoline a (*Pina*) were involved in controlling the hardness of grain. The texture of the wheat endosperm become hard if there is any mutation in Pinb or it lacks Pina. The location of Pinb gene is considered to be on 5DS chromosome in common wheat. The quality of wheat flour is influenced by the composition of starch in the grain. The granule-bound starch synthase (GBSS or waxy) synthesize amylose (Zhang *et al.,* 2018). By restricting the period of cell proliferation, the function of negative regulator is performed by DA1 which is an ubiquitin receptor for organ and seed size regulation. The thousand-kernel weight and wheat yield can be increased by using genome-edited wheat lines which have DA1 loss of function. The DA1, Pinb, waxy gene are the potential genes which can be used for manipulation and editings in the size-related and grain quality traits. This is becoming a promising biotechnological technique to improve the quality of wheat (Bortesi *et al.,* 2014; Tahir *et al*., 2020).

Phenotypical changes associated to gene editing

In many plants as wheat, sorghum, rice, maize, *Nicotiana benthamiana*, and *Arabidopsis,* it has been noticed that gene editing is more effectively demonstrated in Cas9/sgRNA system. It is feasible to use the Cas9/sgRNA system for the modification of endogenous genes as marker genes are involved in the phenotypical changes after disruption. In stable transformed T0 plants by Cas9/sgRNA, it has been demonstrated that gene editing is present in number of

limited cases which includes rice plants and *Arabidopsis.* In T1 generation plants mostly in somatic cells, researchers found that Cas9/sgRNA-triggered gene modifications could be found in the most recent two studies using *Arabidopsis.* For the modified gene, only 22–50% of T2 plants were homozygous. In the T3 generation, mutations were stably inherited in both of the cases. The targeted gene disruption is created in rice by the Cas9/sgRNA system (Woo *et al.,* 2015). The modified genes are inherited in stable pattern and this is now being researched in depth for further understandings. The systems were not constructed and designed for complex gene editing to express multiple guide RNAs which was leading to insertions and/or micro-deletions at a single locus. In controlling plant gene expression, the function of large *cis*-regulatory domains where more complex genome rearrangements are allowed by Cas9/sgRNAs (Li *et al.,* 2019; Tahir *et al*., 2020). The development of plant is controlled by the specific noncoding RNA genes and in plant chromosomes, closely clustered genes are controlled by the potential studies of Cas9/sgRNAs. In a precisely targeted manner, there is requirement of removing or replacing entire large regions of chromosomes. When ZFN genes were transiently and chemically induced, using two pairs of Cas9/sgRNAs to simultaneously cleave and remove a chromosome segment has been reported between two widely spaced target sites for deletion of chromosomal segments. The exceptionally simple and productive Gate way-based Cas9/sgRNA vectors are used for quality altering. In the rice genome, for single or multiplex quality alterations intermediate vector can be utilized to develop up to four sgRNA qualities. It is shown that most transformed T0 plants contain alterations which are di-allelic (heterogeneous or homogeneous) and that genes which are modified can be hereditarily isolated away from sgRNA transgenes and Cas9 and dependably transmitted through following ages in a Mendelian manner (Fauser *et al.,* 2014; Tahir *et al*., 2020).

Transient use of CRISPR/Cas9 in roots of tomato

In roots of tomato, there was reported the transient use of CRISPR/Cas9. A CRISPR/Cas9 transgene was induced by *Agrobacterium rhizogenes* in so-called hairy root at desired loci, there was introduction of mutations. There was no regeneration of transgenic plants yet. For the first time the CRISPR/Cas9-induced mutations through resultant generations in heritability as well as the stable transgenic lines of tomato of these mutations. It was reported that the CRISPR/Cas9 system has a high off-target rate in the animal field (Fantini *et al.,* 2013). There is shown a high specificity in plants by CRISPR/Cas9 as several recent studies have shown. This issue in plants which is concerned with off targeting need to be addressed in a systematic manner but possible impacts can be minimized by several approaches as in example the algorithms which have been developed recently that have selected the least predicted off targets with CRISPR/Cas9 sgRNAs for plant genomes. To choose a few sgRNAs against a locus, in different regions of the gene to generate multiple independent alleles that has non overlapping off targets can be used for reverse genetics studies in the desired locus is caused by mutations in any rather than by an off target in an observed phenotype. The mutations which are back crossing are wild-type to that of interest in progenitor lines in genetics of plants is considered as a standard practice due to which all the potential off-target effects are mitigated (Bashir *et al*., 2020; Cermak *et al.,* 2015; Tahir *et al*., 2020).

Contents of β-Carotene and Lycopene increased by CRISPR/Cas9-Mediated Gene Editing

A study was conducted under which 24 transgenic tomato plants were classified into mutant groups of 5 to determine the carotenoids level where the mutants are quadruple, triple, double, and even single ones included which were Lycopene-1 to 5. For further classified and appropriate analysis, the selection of representative transgenic lines was done. For determination of contents of β-carotene and lycopene with the use of HPLC, different mutant groups of tomato fruits at Br+7 of ripening were separately sampled. According to the commercial standards of β-carotene and lycopene, levels of these two most important components of tomato fruit were measured (Nishizawa *et al.,* 2015).

Salinity Tolerance in rice and other plants

The world production of rice is affected by salinity which is one of the most important abiotic stress. For control of salinity, salinity-tolerant cultivars' cultivation is the most environmental friendly and cost effective approach (Asif *et al*., 2020; Iqra *et al*., 2020; Khan *et al*., 2020; Nazir *et al*., 2020; Hoang *et al*., 2016). There have been rarely reported different application of CRISR/Cas9 in elite rice cultivars improvement but in the recent years this technique has been used widely for the genome editing pf the target-site. The salinity tolerance in rice can be improved by targeting the OsRR22 gene with the help of engineered Cas9-OsRR22-gRNA expression vector in rice. In an experiment, it was observed that form out of 14 T0 transgenic plants, 9 were identified with mutations. At the target site, six types of different mutations were shown by these plants during sequencing. The transmission of these mutations to the next generations was completely successful. In the T1 generations, with the help of segregation, there were obtained some mutant plants without transferred DNA (T-DNA) (Zhang *et al*., 2019). The traits of agronomy and salinity tolerance were also examined in two T2

homozygous mutant lines. At the stage of seedling, T2 homozygous mutant lines have shown high tolerance for salinity as compared to the plants which were wild-type. There were shown no significant agronomic traits between plants which are wild-type and homozygous mutant lines. This has shown a promising future of drought resistance through CRISPR/Cas9.

CRISPR/Cas9-Based Resistance against RNA viruses of plants:

RNA viruses can't be cleaved or targeted directly because there is a limitation that by targeting RNA viral genomes directly, only DNA viruses can be targeted through guide RNA-Cas9 system. But, now this concept has been changed as RNA can be cleaved by programming Cas9, and this is done by cleavage of RNA sequences. This cleavage is programmed and mediated by Type III-B CRISPR-Cas system; these sequences guide RNA due to their complementarility (O'Connell *et al.,* 2014). These variants of Cas9 can be used to cleave and target viruses of RNA origin in the cells of plants. The resistance to *Turnip mosaic virus* (TuMV) is shown by the mutant plants of *A. thaliana due to* translation initiation factors (Sanfacon, 2015; Tahir *et al*., 2020). For generating novel genetic resistance to the potyvirus TuMV, use of CRISPR/Cas9 technology resulted in deletion of a host factor, *eIF (iso)4E. T*his factor is highly required for the survival of the viru*s* (Bashir *et al*., 2020; Pyott *et al*., 2016). At complementary target sites, dsDNA of virus is targeted by gRNA-Cas9 complex and via double strand breaks viral genome is cleaved.

Genome Editing mediated by CRISPR/Cas9 in Plants

CRISPR/Cas9 plays a significant role as a genome editing tool in agriculture with certain limitations. Non specific off-targets, inefficient cargo vectors and HR along with the construction of sequences such as PAM are the basic limitations of this technique. To compete the above mentioned challenges certain advances steps are taken to modify the genome editing procedure (Ran *et al.,* 2013).

Cargo-Delivery Vector

In plants accurate genome editing by cargo-vectors is not possible due to certain limitations. These limitations are eliminated by using CRISPR/Cas9 cargo vectors that are DNA based (Makarova *et al.,* 2015).

CRISPR/Cas9 DNA for stable expression of gene

To deliver CRISPR/Cas9 DNA in plants certain vector delivering approaches such as biolistic as well as transformation that is mediated by *Agrobacterium* are used. In plant genome foreign DNA is inserted at the target site and screening of mutants helps in editing process. Modern study reveals certain innovations regarding genome editing of plants such as incorporation of fluorescent gene in the expression site of CRISPR/Cas9. In plant which shows the existence of transgene of generation T0, CMS as well as BARNASE were isolated as genes that are suicidal in nature and kill pollens and embryos (Endo *et al.,* 2018).

CRISPR for transient expression

Another delivery approach such as CRISP that is a expression system which is transient is used for plants to be edited as transgene free. Mutants that were screened on the basis of markers such as antiboitic as well as herbicide is eliminated so that edited generation can be generated without any foreign gene's incorporation. First transgene free wheat plant was produced by using this strategy ny Zhang in the year 2016. Base substitution that is DNA free involves the use of adenine and cytidine. In tobacco plant transient expression that is transformation based has developed. In potato and tobacco protoplast expression that is transgene free has been used (Nishimasu *et al.,* 2018).

Ribonucleoproteins (RNPs)

In CRISPR/Cac system, another approach is being used to produce plants that are transgene free. In past during genome editing DNA having edited portions was incorporated in the cell of host plant. This kind of random addition of DNA gives rise to results that are surprising (Ding *et al.,* 2018). Even by removing the expression cassettes, portion of external DNA can still incorporate mutations in host DNA (Huang *et al.,* 2019). Certain useful techniques such as bombardment of particle and transformation of the host protoplast are in high demand to make plants that are transgene free (Cho *et al*., 2014; Tahir *et al*., 2020). Integeration of transgene can be reduced by using DNA based transient expression system with certain limitations like there is the possibility of incorporation of removed parts of DNA in the host genome. RNPs sgRNA/Cas9 is designed as a strategy that gives transgene free editing (Hu *et al.,* 2016; Hu *et al.,* 2017; Hu *et al.,* 2018).

References

1. Ahmad, S., Hera, Z. Hanif MS., Syed AH (2020). Effects of carbosulfan on the biology of bird cherry oat aphid. Biol. Clin. Sci. Res. J., 2020: e014.
2. Ali, Q., Khalil, R., Nadeem M., Azhar, M.M., Hafeez, M.M., Malik, A. (2020). Antibacterial, antioxidant activities and association among plant growth related traits of Lepidium draba. Biol. Clin. Sci. Res. J., 2020: e011
3. Anders C, Niewoehner O, Duerst A, Jinek M. (2014). Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 513:569–73.

4. Asif, S., Ali, Q. and Malik, A., Evaluation of salt and heavy metal stress for seedling traits in wheat. Biol. Clin. Sci. Res. J, 2020, Vol. 2020, p.e005.
5. Bashir, T., Ali, Q., Rashid, M.S., and Malik A. (2020). CRISPR/Cas9 in Genomics Editing: A Nature gifted molecular tool. Biol. Clin. Sci. Res. J., 2020, Vol, 2020, p. e018.
6. Belhaj K, Chaparro-Garcia A, Kamoun S, Nekrasov V (2013) Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods 9:39
7. Bibikova M, Beumer K, Trautman JK, Carroll D. (2003). Enhancing gene targeting with designed zinc finger nucleases. Science 300:764.
8. Bortesi, L. & Fischer, R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol. Adv. 33, 41–52 (2015).
9. Carroll, D. 2011. Genome engineering with zinc finger nucleases. Genetics, 188, 773-782.
10. Cermak T., Baltes N. J., Cegan R., Zhang Y., Voytas D. F. (2015). High-frequency, precise modification of the tomato genome. Genome Biol. 16:32.
11. Cho SW, Kim S, Kim Y, *et al.* (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. Genome Res 24:132–41.
12. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339:819–823.
13. Danish, P., Ali, Q., Hafeez, M.M., Malik, A. Antifungal and antibacterial activity of aloe vera plant extract. Biol. Clin. Sci. Res. J., 2020, Vol, 2020, p. e003.
14. Ding D., Chen K., Chen Y., Li H., Xie K. (2018). Engineering introns to express rna guides for Cas9- and Cpf1-mediated multiplex genome editing. Mol. Plant 11 542–552.
15. Endo, M., Mikami, M., Endo, A., Kaya, H., Itoh, T., Nishimasu, H., Nureki, O. and Toki, S., 2019. Genome editing in plants by engineered CRISPR–Cas9 recognizing NG PAM. Nature plants, 5(1), pp.14-17.
16. Fantini, E., Falcone, G., Frusciante, S., Giliberto, L., and Giuliano, G. (2013). Dissection of tomato lycopene biosynthesis through virus-induced gene silencing. Plant Physiol. 163, 986–998.
17. Fauser, F., Schiml, S. & Puchta, H. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. Plant J. 79, 348–359 (2014).
18. Hoang T, Tran T, Nguyen T, Williams B, Wurm P, Bellairs S, Mundree S (2016) Improvement of salinity stress tolerance in rice: challenges and opportunities. Agronomy 6:54.
19. Howells, R. M., Craze, M., Bowden, S., & Wallington, E. J. (2018). Efficient generation of stable, heritable gene edits in wheat using CRISPR/Cas9. BMC plant biology, 18(1), 215.
20. Hu J. H., Miller S. M., Geurts M. H., Tang W., Chen L., Sun N., *et al*. (2018). Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature 556 57–63.
21. Hu, X., Meng, X., Liu, Q., Li, J. & Wang, K. 2017, Increasing the efficiency of CRISPR-Cas9-VQR precise genome editing in rice. Plant Biotechnol. J. 16, 292–297.
22. Hu, X., Wang, C., Fu, Y., Liu, Q., Jiao, X. and Wang, K., 2016. Expanding the range of CRISPR/Cas9 genome editing in rice. Molecular plant, 9(6), pp.943-945.
23. Huang, T. K. & Puchta, H.2019, CRISPR/Cas-mediated gene targeting in plants: finally a turn for the better for homologous recombination. Plant Cell Rep. 38, 443–453.
24. Iqra L, Rashid M, Ali Q, Latif I, Malik A. Genetic variability for salt tolerance in wheat. Biol Clin Sci Res J, (2020); 2020(e016).
25. Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP.2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res.;41(20).
26. Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B., and Weeks, D. P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res. 41:e188.
27. Khalil, R., Ali, Q., Hafeez, M.M., and Malik, A. (2020). Phytochemical activities of Conocarpus erectus: An overview. Biol. Clin. Sci. Res. J., 2020: e008.
28. Khalil, R., Ali, Q., Hafeez, M.M., Malik, A. (2020). Phenolic acid profiling by RP-HPLC: evaluation of antibacterial and anticancer activities of Conocarpus erectus plant extracts. Biol. Clin. Sci. Res. J., 2020: e010.
29. Khan, H.S., Chaudhry, K.M., Ashraf, I., Usman, M., Ejaz, R., Khadaim, M.M. (2020). An investigation of the problems faced by vegetable growers regarding post-harvest practices in district Faisalabad. Biol. Clin. Sci. Res. J., 2020: e015
30. Li, J., Luo, J., Xu, M., Li, S., Zhang, J., Li, H., Yan, L., Zhao, Y. and Xia, L., 2019. Plant genome editing using xCas9 with expanded PAM

compatibility. J. Genet. Genomics, 46, pp.277-280.
31. Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., Liu, J., Zhang, H., Liu, C., Ran, Y. and Gao, C., 2017. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nature communications, 8(1), pp.1-5.
32. Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders, S.J., Barrangou, R., Brouns, S.J., Charpentier, E., Haft, D.H. and Horvath, P., 2015. An updated evolutionary classification of CRISPR–Cas systems. Nature Reviews Microbiology, 13(11), pp.722-736.
33. Masood, M., Ahsan, M., Sadaqat, H.A., Awan F., Screening of maize (Zea mays L.) inbred lines under water deficit conditions. Biol. Clin. Sci. Res. J, 2020, Vol. 2020, p.e007.
34. Mushtaq, U., Mushtaq, S., Afzal, M., Ali, Q., Malik, A. Role of modern technology for treatment of HCV. Biol. Clin. Sci. Res. J., 2020, Vol, 2020, p.e001.
35. Nazir MI, Idrees I, Danish P, Ahmad S, Ali Q, Malik A. Potential of water hyacinth (Eichhornia crassipes L.) for phytoremediation of heavy metals from waste water. Biol. Clin. Sci. Res. J, 2020, Vol. 2020, p.e006.
36. Nishimasu, H., Shi, X., Ishiguro, S., Gao, L., Hirano, S., Okazaki, S., Noda, T., Abudayyeh, O.O., Gootenberg, J.S., Mori, H. and Oura, S., 2018. Engineered CRISPR-Cas9 nuclease with expanded targeting space. Science, 361(6408), pp.1259-1262.
37. Nishizawa-Yokoi, A., Endo, M., Mikami, M., and Toki, S. (2015). CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. Biochem. Biophys. Res. Commun. 467, 76–82.
38. O'Connell, M. R., Oakes, B. L., Sternberg, S. H., East-Seletsky, A., Kaplan, M., and Doudna, J. A. (2014). Programmable RNA recognition and cleavage by CRISPR/Cas9. Nature 516, 263–266.
39. Pyott, D. E., Sheehan, E., and Molnar, A. (2016). Engineering of CRISPR/Cas9-mediated potyvirus resistance in transgene-free Arabidopsis plants. Mol. Plant Pathol. 17, 1276–1288.
40. Ran, F.A., Hsu, P.D., Lin, C.Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y. and Zhang, F., 2013. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell, 154(6), pp.1380-1389.
41. Rana H.A.A, Iftikhar, M., Chaudhry, K.M., Usman, M., Mazhar, F. (2020). Role of Information and Communication Technologies in Agricultural Extension; Comparative Study of Present and Future Aspects in District Khanewal. Biol. Clin. Sci. Res. J., 2020: e012.
42. Razzaq, A., Saleem, F., Kanwal, M., Mustafa, G., Yousaf, S., Imran Arshad, H.M., Hameed, M.K., Khan, M.S. and Joyia, F.A., 2019. Modern trends in plant genome editing: an inclusive review of the CRISPR/Cas9 toolbox. International Journal of Molecular Sciences, 20(16), p.4045.
43. Svitashev Sergei, Young Joshua K., Schwartz Christine, Gao Huirong, Falco S. Carl, Cigan A. Mark. Targeted Mutagenesis, Precise Gene Editing, and Site-Specific Gene Insertion in Maize Using Cas9 and Guide RNA. Plant Physiology. 2015;169(2):931–945.
44. Tahir, T., Ali, Q., Rashid, M.S., and Malik A. (2020). The journey of CRISPR- Cas9 from bacterial defense mechanism to a gene editing tool in both animals and plants. Biol. Clin. Sci. Res. J., 2020, Vol, 2020, p. e017.
45. Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, Qiu JL. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol. 2014;32(9):947.
46. Wang, J., Meng, X., Hu, X., Sun, T., Li, J., Wang, K. and Yu, H., 2019. xCas9 expands the scope of genome editing with reduced efficiency in rice. Plant biotechnology journal, 17(4), p.709.
47. Wei Wang, Qilani Pan, Fei He, Alina, 2018. The CRISPR journal. 65-74.
48. Woo, J.W., Kim, J., Kwon, S.I., Corvalán, C., Cho, S.W., Kim, H., Kim, S.G., Kim, S.T., Choe, S. and Kim, J.S., 2015. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nature biotechnology, 33(11), pp.1162-1164.
49. Xiao, A., Cheng, Z., Kong, L., Zhu, Z., Lin, S., Gao, G. and Zhang, B., 2014. CasOT: a genome-wide Cas9/gRNA off-target searching tool. Bioinformatics, 30(8), pp.1180-1182.
50. Yaqoob S, Fatima N, Khan S, Ali Q, Hafeez MM, Malik A., Begomoviruses and betasatellites associated with CLCuD. Biol. Clin. Sci. Res. J, 2020, Vol. 2020, p.e002.
51. Yin, K., Gao, C. and Qiu, J.L., 2017. Progress and prospects in plant genome editing. Nature plants, 3(8), pp.1-6.
52. Zhang, A., Liu, Y., Wang, F., Li, T., Chen, Z., Kong, D., Bi, J., Zhang, F., Luo, X., Wang, J. and Tang, J., 2019. Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the OsRR22 gene. Molecular Breeding, 39(3), p.47.
53. Zhang, S., Zhang, R., Song, G., Gao, J., Li, W., Han, X., Chen, M., Li, Y. and Li, G., 2018. Targeted mutagenesis using the Agrobacterium tumefaciens-mediated CRISPR-Cas9 system in

common wheat. BMC plant biology, 18(1), pp.1-12.
54. Zhang, Y., Liang, Z., Zong, Y., Wang, Y., Liu, J., Chen, K., Qiu, J.L. and Gao, C., 2016. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nature communications, 7(1), pp.1-8.

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