DOI: 10.5281/zenodo.6585382

Canadian Journal of Agricultural and Applied Sciences
CJAAS (2022) 2(1): 17-32 ISSN: 2816-2358
(January-March, 2022)

CRISPR Technology: Unlocking the Neglected Potential for Crop Improvement

Satyaranjan Padhiary[1], Priyanka Kumari[1], Rajan Kumar[1] and Nitu Rani[1]

[1]University Institute of Agricultural Sciences, Chandigarh University, Gharuan, Mohali-140413, Punjab, INDIA

Corresponding email - nitu.agri@cumail.in

Abstract

Gene editing techniques have proven to be a boon in the field of science for the insertion, deletion and repairing a desirable gene locus in the genome of a particular organism. Earlier genome editing tools such as TALENs (Transcription Activator-Like Effector Nucleases) and ZFNs (Zinc Finger Nucleases) were used. With the discovery of CRISPR/CAS9 (Clustered Regularly Interspaced Short Palindromic Repeats) technology while sequencing the gene of *E.Coli* during the late 80s, which is actually a defensive mechanism found in bacteria against viruses, the field of gene editing has witnessed a significant boost. With due course of time, scientific utility of CRISPR/CAS9 was identified and utilized mostly in gene editing techniques that are done in various animals and in about 20 plant species like reducing the gluten content in wheat, limiting Cadmium content in paddy, controlling the Phytic acid content maize, providing resistance against certain diseases and many more. Due to varsity in the application and utilization of this technology, it possess a lot of future prospect. Although, there are certain concerns of its utilization in some parts of the world.In this review work, the history, utility and application in various crops, demerits and regulatory concerns of CRISPR/CAS9 technology has been enunciated.

Keywords: CRISPR/CAS9, Genome, Gene Editing, Mutant, Coelic Diseases, Homoalleles, GMOs

Introduction

In the current scenario, the urgent need is to meet the food grains demand of the increasing worldwide population in terms of quality and quantity. As the statistics itself reflect the plight

Received on - February 09, 2022 Accepted on - March 14, 2022 Published on - March 30, 2022

of about 811 million people facing hunger every day. To worsen the situation, COVID 19 pandemic has escalated the rate of undernourishment from 8.4 percent in 2019 to 9.9 percent in 2020 as per the FAO report, 2021. By the year 2050, there is a need to increase the food production by 60-100%, as the world population will attain the figure of 10 billion [1]. Apart from the constant population growth rate, other factors such as unconducive weather and climatic conditions, decreasing availability of cultivable land, rise in biotic and abiotic stresses are the prime deterrent to farming and food production [1]. In order to overcome the mentioned challenges, our focus should be on development and utilization of technologies that can contribute to crop improvement to increase the food grain production [1].Though we have conventional plant breeding techniques such as mass selection, back crossing, interbreeding, pedigree method etc, but they are not that much effective, quiet time taking, arbitrary and desirable traits might not be obtained [2]. In the past decade, precise gene editing technologies had been illustrated in bothplantsandanimals using genome editing technologies with site specific nucleases (SSNs) [1].

Genome editing is a technique which helps to alter a specific gene,changing the DNA sequence in the genome of a living organism to obtain a desirable trait [2]. Genome refers to the complete set of genes or genetic material present in an organism. Methods by which genome editing is done includes site specific, mutation,insertion,deletion, substitution etc [3] [3]. For example, Flavr Savrtomato is the first commercially grown, genetically engineered crop developed in 1994. It was done through the introduction of antisense gene which suppresses the effect of Poly galacturonase (PG) gene. This PG gene is responsible for the degradation of pectin present in the cell wall of the tomato which causes softening and deterioration by diseases. But due to the interference in the PG gene, the tomato has become resistant to rotting, thus increasing its shelf life [5]. Previously, the first generation genome editing tools such as TALENs (Transcription Activator-Like Effector Nucleases) and ZFNs (Zinc Finger Nucleases) were used. There were several impediments while utilizing them such as complexity and costlier cost of production and the possibility of off target DNA irregular interaction [3]. But nowadays, the commonly usedgenome editing tool is CRISPR/CAS9 (Clustered Regularly Interspaced Short Palindromic Repeats), which is an adaptive viral immunity system found in bacteria [2].

The discovery of CRISPR dates back to 1987, when it was discovered accidentally in *E.Coli,* while determining the gene sequence that was coding for alakaline phosphatase isozyme that converts aminopeptidase [6]. CRISPR became more prevalaent in the initial phase of genome sequencing in archaeland bacterial genomes in late 1990s and early 2000s [6]Since the emergence of CRISPR/CAS9,it is getting popular in the modern times due to its simplicity, multiplexed mutations and efficiency over first generation genome editing tools [3]. It has superseded the not only utility of TALENs and ZFNs, but also other gene editing tools such as RNAi (RNA Interference), as it has ability to target endogenous genes, that are not possible to target specifically with precision and simplicity by RNAi technology [2].The most commonly utilized CAS9 is obtained from *Streptococcus pyrogenes* (SpCAS9)[3]. With the advancement in CRISPR technology throughout the years, various derivatives of this has been developed such as CAS9, CAS9n, dCAS9andtheir utility may vary as per the application having improved accuracy for high throughput in gene silencing, gene knockout, silencing etc. in various plants and bacteria [3].So far, CRISPR/CAS9 Technology has been applied on almost 20 crop in order to develop characteristics like improvement in yield, resistance against biotic and abiotic stresses species [1]. The Biotic stress caused by pathogenic microbes pose a serious threat in the advancement of disease resistant crops that results in 42% yield loss, which in turn results in downfall of food production by 15% [1]. But by the utilization of CRISPR/ CAS9 technology, crop resistance and resistance from abiotic factors like drought and salinity has been achieved [1]. In this review, we will elucidate the application in crop improvement of various agricultural crop, its pros and cons and its regulatory concerns.

Utility of CRISPR/CAS9 in Monocot Crops

WHEAT*(Triticumaestivum)*

Wheat is consumed as staple food throughout the world, the genetic gains in wheat is less than 1% globally, which is not sufficient to meet the future requirement [3]. But by the utilization of CRISPR/CAS9 technology, success has been achieved in developing the traits of wheat. By using the CRISPR/CAS9 technology, the International Wheat Genome Sequencing Consortium (IWGSC) published complete elucidatedtop notch reference genome of bread wheat of Chinese Spring variant, which provides more new target genes which respond to myriad of biotic, abiotic, quality and agronomical trait improvement [3].

For the first time, CRISPR/CAS9 was used to eliminate TaMLO, TaPDSandTaINOX gene locus from the wheat genome and in the next research, simultaneous elimination of three TaMLOhomoalleles to make the wheat resistant to powdery mildew [3]. CRISPR/CAS9 has also been used to edits two genes in the wheat genome, which are related to abiotic stress i.e. TaERF3 that codes for wheat dehydration responsive element binding protein 2 andTaERF3 which codes for heat ethylene responsive factor 3, which shown 70% success in expression of the traits of the edited genes [1].

Wheat being a cereal crop contains gluten protein which determines the elasticity of the dough that will be made from the flour of wheat. Glutens present in wheat can be further classified into twomajor groups, i.e. glutenins and gliading [7]. Glutenin is responsible for the viscosity of the dough whereas gliadings is responsible to form a protein network that provides elasticity [7]. Though gluten makes the food more luring, in terms of health it has an adverse effect, as its presence makes its complete digestion difficult and might also lead to coelic [49]. Coelic diseases refers to the autoimmune disorders which occur in genetically susceptible individuals by the consumption of gluten [49]. In order to avoid this, CRISPR/CAS9 gene has been utilized to knockout and delete the genes responsible for the formation of gliadings, thereby reducing the gluten content in wheat [7], further reducing the risk of coelic diseases in gluten sensitive individuals. These are some examples mentioned which explains how CRISPR/CAS9 system can be applicable in wheat.

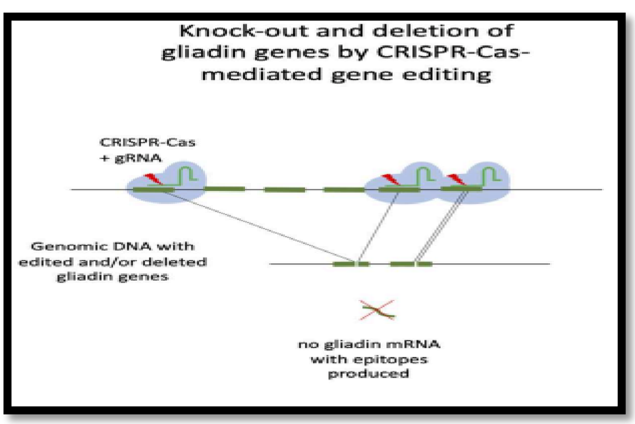

Fig 1. CRISPR/CAS9 with gliadin-preciseg RNAs (adapted from: Jouaninet al. 2020).

Paddy *(Oryza sativa)*

Paddy is the staple of more than 50% world population, but its production as well as its quality is severely affected by pathogens such as *Magnaportheoryzae (M.oryzae)* and *Xanthomonasoryzaepv. Oryzae (X. oryzaeoryzae),* which causes rice blast andbacterial blight respectively [8]. In order to protect itself, every plant has double layered innate immune system [8]. When the plant receives pathogen-associated molecular patterns (PAMPs) through cell surface pattern recognition receptors (PRRs), the first innate immune layer gets activated, which leads to functioning of PAMP Triggered Immunity (PTI)[8]. Although, this first layer of innate immunity could be overpowered by the pathogens by means of secreting PTI inhibitors effectors, which in turn causes Effector Triggered Susceptibility (ETS). To tackle this challenge, the second innate immune layer gets activated through plant a resistance gene (R genes) that particularly identifies pathogen effectors to activate Effector Triggered Immunity [8].In order to make paddy more resistant to its major diseases i.e. blast and blight, three broad spectrum R genes namely Bsr-d1, Pi21 and ERF922 were identified, and by utilizing CRISPR/CAS9 technology, target specific gene editing was done in these genes in thermosensitive genic male sterile (TGMS) rice line Longke638S (LK638S) [8]. The mutant with ERF922 modification displayed maximum resistance to rice blast, although triple mutants (mutants in which modification in all three R genes had been done) also displayed superiority in resistance against blast over wild type, whereas single mutants of Pi21 and ERF922 also displayed superiority in resistance against blight also[8].

Cadmium (Cd), a heavy metal whose intake can pose grave health risks and rice acts as a major source of cadmium in human diet [9]. The source of it in rice is the Cd present in soil [10]. The Cd from soil is mainly absorbed by the roots through the gene namely OsNRAMP5, which codes for a family of natural resistance-associated macrophage proteins [10].CRISPR/CAS9 technology has been utilized for knocking out ofOsNRAMP5, by producing three mutant alleles of OsNRAMP5 i.e. *osnramp5-4*with 1 base pair (bp) deletion, *osnramp5-5* with 17 bp deletion and*osnramp5-6* with 11 bp deletion, which resulted in frame shift mutation, thereby knocking out the OsNRAMP5[Fig 2.][10].This resulted in reduction of Cd accumulation from 0.29-033 mg/Kg to 0.05 mg/kg in *indica* and *japonica* lines [10, 48] thereby reducing the health risks posed by the consumption of rice having higher Cd accumulation.

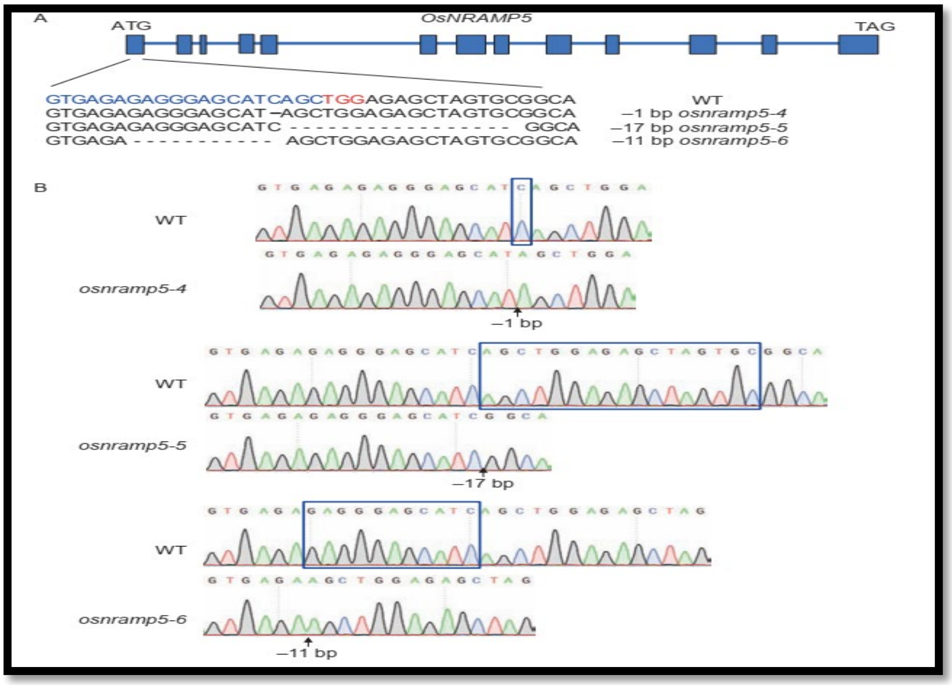

Fig 2: Mutations induced using CRISPR/CAS9 technology in *OsNRAMP5*(adapted from: Yanget al. 2019).

Maize (*Zea mays)*

Maize is a prominent cereal crop grown throughout the world. It has phytic acid that is a major constituent (70%), which is not beneficial for the digestive system of monogastric animals [1]. Phytic Acid is synthesized by the genes ZmIPK1A, ZmIPK, and ZmMRP4. By the utilization of CRISPR/CAS 9 Technology, these genes were knocked out with 31% mutationefficiency, thereby reducing the content of phytic acid in maize [1].

In maize, ARGOS8 acts as a counterbalance to ethylene and it had been found that its overexpression reduced sensitivity to ethylene, as well as the quality of grain has improved under drought condition [11]. But the endogenous of ARGOS8 mRNA is comparatively lesser, also not uniform [11].In order to tackle this problem, CRISPR/CAS 9 technology had been utilized to edit ARGOS8, which resulted in increased in grain yield by five bushels/acre under flowering stress condition, also no loss in yield under well-watered conditions [11].

Utility of CRISPR/CAS9 in Dicot Crops

Papaya (*Carica papaya L.)*

'Papaya' a multi nutrient fruit which also used for medicinal purpose. It is mainly affected by viruses. "Sunup" and "Rainbow" new varieties of papaya that have resistance against papaya ringspot virus (PRSV). These varieties affect papaya food industries in Hawaii but these variety cultivars are not exported to other countries because of many biological and non-biological factors like rapid emergence of strains, PTGS virus encoded suppressor, number of transgene copy and expose of new viruses [12.] These problems in papaya are answered by CRISPR/CAS9 technology. S-gene, a CRISPR/CAS9 mediated mutation, suppress the effect of PRSV disease in papaya [13].

Papaya also protect itself from diseases and insects through papain, cysteine, protease that are naturally present in it. However, a fungus, *Phytopthorapalmivora* affect the parts of papaya, specifically that are having more papain. Accordingly*P. palmivora* cannot able to control papain mediated resistance because it carries cysteine inhibitors. Later, CRISPR/CAS9demonstratd cysteine protease suppressor Cystatin (PpalEPIC8) that majorly inhibit the papain defense mediation. In *P. palmivora*, PpalEPIC8 mutation enhances sensitivity to papain show that it has major effect on *P. palmivora*virulence by suppressing papain [14]. Thus, through the CRISPR/CAS9 technology, the effect of pathogens (Oomycetes) is inhibited. They are highly harmful pathogen of phytopathogen class that is not possible to inhibit by conventional methods [15].

Cotton (*Gossypium sp.)*

Cotton is the major fibre crop grown throughout the world, also the oil obtained from its seeds has certain utility [1]. It thrives well in black cotton soil which is of volcanic origin. As it is the prime source of natural fibre that is generally used in textile industries, it bears a great economic importance. Cotton Curl Leaf Disease (CLCuD) is the major disease that is observed in cotton plants which is caused by *Begomoviruses* of family *Geminiviridae,* which poses a major threat to production and economic value in South East Asian region [16]. The DNA of the virus is single stranded circular which consists of about 2800 nucleotides and is associated with satellite molecules namely alpha and beta satellites [16]. This genetic material of the virus is encapsulated in a twinned icosahederal structured protein and is transmitted by whitefly [16].Using CRISPR/CAS9 technology, sgRNAs (Single Guided) were designed and incorporated in plant genome that interferes with the DNA satellite molecules of *Begomoviruses*, thereby affecting its replication, ultimately reducing onset of CLCuD[16].

CRISPR/CAS9 technology has also been utilized to bring targeted mutagenesis in Upland Cotton (*Gossypiumhirsutum L.*), particularly in two distinct sites i.e. Cloroplastosalterados 1 (GhCLA1) and vacuolar H+-pyrophosphatase(GhVP) genes, which are found in cotton protoplast [17].The mutations brought were nucleotide substitution and nucleotide deletion and upon analysis using Enzyme Restricted PCR assay and sequence analysis, it exhibited no off site mutation with an efficiency of 47.6% to 81.8% mutation in transgenic cotton plant thereby proving the efficiency and accuracy of CRISPR/CAS9 technology [17].

Soyabean (*Glycine max*)

In leguminous crops, soyabean is one of the important one it has high protein & oil content [18]. It is also a key source of secondary metabolites like iso flavonoids, which is about hundred times more than any other leguminous crop, thus having a significant nutritional and economic importance. About 12 isoflavones have been found in soyabean, which plays a vital role in reducing cardiovascular diseases and incidence of cancer. Soyabean Mosaic Virus (SMV) is one of the major disease found in soyabean that reduces its quality as well the iso flavonoid content in it [19]. CRISPR/CAS9 technology has been incorporated to target GmF3H1, GmF3H2 and GmFNSII-1 genes in hairy roots and plants of soyabean, which exhibited a mutation efficiency of 44.44% that were inherited in progeny in a stable manner, which exhibited 1/3rd less protein coat of SMV after infection, due to increase in leaf iso falvone content by two times, which concluded that increased isoflavone content enhanced the resistance against SMV [19].

CRISPR/CAS9 technology has also been utilized to reduce 'damping off' in soyabean that is caused by *Phytopthorasojae*[1]. The virulent gene of *Phytopthorasojae*i.e. Avr4/6 was replaced by a marker gene NPT II using CRISPR/CAS9 technology, which emphasized its recognition in the R gene loci of soyabean, thereby enabling it to identify & neutralize the virulence of the pathogen [1].

Potato (*Solanumtuberosum*)

After paddy and rice, potato is third most important crop that acts a major source of carbohydrates throughout the world [20]. In potato, browning is a significant observed problem, which is caused due to enzymatic browning [21]. The enzymes involved in it are polyphenol oxidases (PPOs), which catalyzes the oxiation of phenolic substrates into quinone, which leads to formation of dark brown coloured precipitates [21]. As phenolic compounds act as antioxidants in human body that reduces the incidence of cancer and cardiovascular diseases, consumption of its oxidized form is highly indesirable [21]. PPOs are associated with multi gene families, originally five such genes were reported which were categorized as StPPO and each one having a distinguished pattern of expression and tissue induction Using CRISPR/CAS9 technology, target specific mutations were induced in four alleles of StPPO, thereby reduction in PPO activity to 69%, thereby reduction in enzymatic browning by 73% hence reducing the spoilage and improving the quality of potato [21]

In potato, steroidal glycoalakloids (SGAs) α-solanine and α-chaconine are naturally produced that are bitter in taste and toxic in nature [22], thus reducing SGAs level is necessary. In genome of potato, St16DOX gene is responsible for the encoding of a steroid 16α-hydroxylase that is involved in biosynthesis of SGAs [22]. The St16DOX gene, that exists as a mono gene in the entire potato genome and thus CRISPR/CAS9 technology had been incorporated to bring target specific knockout of this gene, which completely eliminated the accumulation of SGAs from the hairs of potato leading to development of SGAs free potato tubers [22].

Cassava (*Manihatesculenta*)

Cassava (*Manihatesculenta*) is a starchy root crop that is a major food for over a billion people in tropical and subtropical regions. Almost 40% only used as food in Africa. [23, 24]. It is also known as tapioca, yuca, manioc etc. It has both drought and marginal soil tolerance [25].There is presence of toxic cyanogenic compounds (cyanogenicglucosides) in cassava, that is removed by post harvesting processes but, processing of cassava root is laborious and in Africa falls proportionately on women and girls [26]. There is decrease in nutrients after processing, also reducing the food value of processed cassava products [27,28,29,30].

Cyanogenicglucosides synthesizes with the help of cytochrome P450(CYP) enzymes. This enzyme belongs to CYP79 family [31]. CYP79D proteins catalyze the first limiting step of synthesis of cassava cyanogens, linamarin and lotaustralin [32]. They transport into storage root after synthesis into leaves and shoots [33]. CYP79D1 and CYP79D2 are the paraloges genes [31], which arose from the whole genome duplication, with the help of these genes, CYP79D is encoded in cassava [34]. Thus, cassava cyanogenicglucosides are excluded by knocking out the CYP79D genes. CRISPRC9 is used to CYP79 genes, singly and in combination, in the 60444 and West African famous TME 419 varieties. Agrobacterium-mediated CRISPR Cas9 technology is efficient in cassava, and it is also used in several cultivars [35,36,37,38]

Prospects of CRISPR/CAS9 Technology

CRISPR/CAS9 technology was originally studied by the scientists in order to understand the bacterial immunity system [39], but later on its study extended for its applicability as a gene editing tool [39]. Over a period of time, this technology shown an advancement and allowed researchers to do various functions like tackling various diseases, identification of novel diseases protective mutations which includes protection against HIV infections and hypercholesterolemia [39]. CRISPR/CAS9 technology further being explored to treat genetic disorders through direct treatment of genome of somatic cells [39]. More compact CRISPR/CAS9 Systems are also being identified to bring about more efficient mutations, even in a single unit, that can prove to be useful treat useful in auditory & visual disorders in humans too [39]. The discovery of an enzyme C2c2, also acknowledged as Cas13a enables to edit ssRNAsequence and further the CRISPR/C2c2 system can act as a helpful tool to cure several virus related diseases [40]. In recent past, another Cas enzyme i.e. Cas13b has been discovered which provides better option for fine tuning of gene functions [40]. The continuous and significant development in CRISPR technology provides a broad spectrum of opportunity the field of gene editing.

Demerits of CRISPR/CAS9 Technology and regulatory concerns

Although CRISPR/CAS9 technology has been able to show its superiority over other gene editing tools, there are certain demerits with it that are needed to be addressed. Despite CRISPR/CAS9 technology is relatively feasible and cheaper, its experience is still lacking in developing nations [41].Although CRISPR technology being relatively accurate, one of the major concerns is the appearance of off target mutations, particularly in the development of transgene free crops [42], which in some cases have been observed at frequency of ≥50% [45]. To overcome this problem, Cas9nickase(Cas9n), a variant that induces single-stranded breaks (SSBs),in combination with an sgRNA pair targeting both strandsof the DNA at the intended location to produce the double stranded break (DSB) [45] Issue like mosaicism are quite frequent in CRISPR technology as it requires actively proliferating cells in order to enable genome modification [43]. Even though CRISPR/CAS9 technology has been used to develop virus resistance against DNA and RNA based viruses, its effectiveness in eukaryotes in still being questioned due to the failure observed in providing resistance against retroviruses as well as adenoviruses [44].Therefore, emphasis in development of CRISPR/Cas13a is being given as it can prove to be useful to overcome this issue [44]. Just like other traditional gene editing techniques, issue of immunotoxicity has been a matter of concern [45] Also, the concern of pleiotropic effects (actions other than those for which it was specifically developed) of CRISPR/CAS9 technology has not been analyzed [46].

Due to such concerns, the debate on gene editing is still perpetual throughout the world and the same is applicable on CRISPR/CAS9 technology too [47] The presence of biohackers that has been detected by the United States Food and Drug Administration (FDA), who in some cases promised to deliver treatment of a disease, in which they have used the CRISPR/Cas technology, it is still not considered for medical treatment in North American laws [47] Also, due to concerns the European Union has considered the genetically edited crops under the category of GMOs (Genetically Modified Organisms), where there is a strict regulation for their ethical use [44]. One of the reasons for the non-uniformity of regulations for CRISPR/CAS9 technology is the non-participation of nations throughout the world which not only includes developed nations like the US and the EU, but also the developing nations [41]. Thus, discussions among the nations should be made in order to build an understanding of this technology and ethical utilization.

CONCLUSION

Since the time of discovery of the CRISPR/CAS9 technology, its utilization has been useful for the scientific community as well as for the humanity. Its numerous applications in medicine, agriculture, research and studies proved it to be a boon. Also, its accuracy, cost effectiveness and feasibility made it a more desirable gene editing tool over the pre-existing ones. Continuous developments and endeavors are being done in order to develop this more suitably. In today's time where a pandemic like COVID exists, it has a great significance in terms of researching on development its cure. Not only that, but also pre-existing diseases like HIV, Zika, dengue, malaria etc. Although there are certain issues regarding its regulation and utilization, which due course of time, discussions by the concerned members and authorities and development in this technology by the scientific community, the concerns aroused in this technology could also be addressed and its utility for the betterment of the human race could keep going on.

References

1. Jaganathan D, Ramasamy K, Sellamuthu G, Jayabalan S, Venkataraman G 2018. CRISPR for crop improvement: an update review. *Frontiers in plant science*, 9:985.
2. Arora L, Narula A 2017. Gene editing and crop improvementusing CRISPR-Cas9 system. Frontiers in plant science, 8:1932.
3. Kumar R, Kaur A, Pandey A, Mamrutha HM, Singh GP 2019. CRISPR-based genome editing in wheat: a comprehensive review and future prospects. Molecular biology reports, 46(3):3557-69.
4. Ahmad S, Wei X, Sheng Z, Hu P, Tang S 2020. CRISPR/CAS9 for development of disease resistance in plants: recent progress, limitations and future prospects. Briefings in Functional Genomics, 19(1): 26-39.
5. Krieger EK, Allen E, Gilbertson LA, Roberts JK, Hiatt W, Sanders RA 2008. The FlavrSavr tomato, an early example of RNAi technology. HortScience, 43(3): 962-4.
6. Han W, She Q 2017. CRISPR history: discovery, characterization, and prosperity. Progress in Molecular Biology and Translational Science, 152: 1-21
7. Jouanin A, Gilissen LJ, Schaart JG, Leigh FJ, Cockram J, Wallington EJ, Boyd LA, van den Broeck HC, van der Meer IM, America AH, Visser RG 2020. CRISPR/CAS9 gene editing of gluten in wheat to reduce gluten content and exposure—reviewing methods to screen for coeliac safety. Frontiers in Nutrition, 7: 51.
8. Zhou Y, Xu S, Jiang N, Zhao X, Bai Z, Liu J, Yao W, Tang Q, Xiao G, Lv C, Wang K 2021. Engineering of rice varieties with enhanced resistances to both blast and bacterial blight diseases via CRISPR/CAS9. Plant biotechnology journal.
9. Songmei L, Jie J, Yang L, Jun M, Shouling X, Yuanyuan T, Youfa L, Qingyao S, JianzYang H 2019. Characterization and evaluation of OsLCT1 and OsNramp5 mutants generated through CRISPR/CAS9-mediated mutagenesis for breeding low Cd rice. Rice Science, 26(2): 88
10. Yang CH, ZHANG Y, HUANG CF 2019. Reduction in cadmium accumulation in japonica rice grains by CRISPR/CAS9-mediated editing of OsNRAMP5. Journal of Integrative Agriculture, 18(3): 688-97.

11. 11.Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, Yang M, Hakimi SM, Mo H, Habben JE 2017. ARGOS 8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant biotechnology journal, 15(2): 207-16.
12. Hamim I, Borth WB, Marquez J, Green JC, Melzer MJ, Hu JS 2018. Transgene-mediated resistance to Papaya ringspot virus: challenges and solutions. Phytoparasitica, 46(1): 1-8.
13. Green JC, Hu JS 2017 Editing plants for virus resistance using CRISPR-Cas. Actavirologica, 61(2):138-42.
14. Gumtow R, Wu D, Uchida J, Tian M. 2018 A Phytophthorapalmivora extracellular cystatin-like protease inhibitor targets papain to contribute to virulence on papaya. Molecular Plant-Microbe Interactions, 31(3): 363-73.
15. Haque E, Taniguchi H, Hassan MM, Bhowmik P, Karim MR, ´Smiech M, Zhao K, Rahman M and Islam T 2018, Application of CRISPR/CAS9 Genome Editing Technology for the Improvement of Crops Cultivated inTropical Climates: Recent Progress, Prospects, and Challenges. Front. Plant Sci. 9:617. doi: 10.3389/fpls.2018.00617
16. Iqbal Z, Sattar MN, Shafiq M 2016. CRISPR/CAS9: a tool to circumscribe cotton leaf curl disease. Frontiers in plant science, 12,7: 475.
17. Chen X, Lu X, Shu N, Wang S, Wang J, Wang D, Guo L, Ye W 2017. Targeted mutagenesis in cotton (Gossypiumhirsutum L.) using the CRISPR/CAS9 system. Scientific reports, 13;7(1):1-7.
18. Cai Y, Chen L, Liu X, Guo C, Sun S, Wu C, Jiang B, Han T, Hou W 2018. CRISPR/CAS9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. Plant biotechnology journal, 16(1): 176-85.
19. Zhang P, Du H, Wang J, Pu Y, Yang C, Yan R, Yang H, Cheng H, Yu D 2020. Multiplex CRISPR/CAS9-mediated metabolic engineering increases soya bean isoflavone content and resistance to soya bean mosaic virus. Plant biotechnology journal., 18(6):1384-95.
20. Enciso-Rodriguez F, Manrique-Carpintero NC, Nadakuduti SS, Buell CR, Zarka D, Douches D 2020. Overcoming self-incompatibility in diploid potato using CRISPR-Cas9. Frontiers in plant science, 2;10:376.
21. González MN, Massa GA, Andersson M, Turesson H, Olsson N, Fält AS, Storani L, DécimaOneto CA, Hofvander P, Feingold SE 2020. Reduced enzymatic browning in potato

tubers by specific editing of a polyphenol oxidase gene via ribonucleoprotein complexes delivery of the CRISPR/CAS9 system. Frontiers in plant science, Jan 9; 10:1649.

22. Nakayasu M, Akiyama R, Lee HJ, Osakabe K, Osakabe Y, Watanabe B, Sugimoto Y, Umemoto N, Saito K, Muranaka T, Mizutani M 2018. Generation of α-solanine-free hairy roots of potato by CRISPR/CAS9 mediated genome editing of the St16DOX gene. Plant Physiology and Biochemistry.1; 131:70-7.
23. Lebot, V, Tropical Root and Tuber Crops, 2019, 2nd Edition. CABI.
24. Nweke FI. New challenges in the cassava transformation in Nigeria and Ghana. Intl Food Policy Res Inst; 2004.
25. Howeler, R., Lutaladio, N., and Thomas, G. Save and Grow: Cassava: A Guide to Sustainable Production Intensification. Rome: Food and Agriculture Organization of the United Nations (2013)
26. Ogunwande IO, Akinrinola OO, Awoniyi SO 2016. Determinants of Rural Children's Participation in Cassava Processing in Egbeda Local Government Area of Oyo State. America,61: 7-90.
27. BoakyePeprah, B., Parkes, E.Y., Harrison, O.A., van Biljon, A., Steiner-Asiedu, M., and Labuschagne, M.T. 2020 Proximate Composition, Cyanide Content, and Carotenoid Retention after Boiling of Provitamin A-Rich Cassava Grown in Ghana. Foods, 9.
28. Hawashi, M., Sitania, C., Caesy, C., Aparamarta, H.W., Widjaja, T., and Gunawan, S 2019. Kinetic data of extraction of cyanide during the soaking process of cassava leaves. Data Brief, 25: 104279.
29. Maziya-Dixon B, Dixon AGO, and Ssemakula, G 2009. Changes in total carotenoid content at different stages of traditional processing of yellow-fleshed cassava genotypes. Int. J. Food Sci. Technol, 44: 2350–2357.
30. Montagnac JA, Davis CR, and Tanumihardjo SA 2009. Nutritional Value of Cassava for Use as a Staple Food and Recent Advances for Improvement. Compr. Rev. Food Sci. Food Saf., 181–194.
31. Luck K, Jirschitzka J, Irmisch S., Huber M., Gershenzon J, and Köllner, T.G. 2016. CYP79D enzymes contribute to jasmonic acid-induced formation of aldoximes and other nitrogenous volatiles in two Erythroxylum species. BMC Plant Biol, 16: 215.
32. Andersen MD, Busk PK, Svendsen I, Møller BL 2000. Cytochromes P-450 from Cassava (ManihotesculentaCrantz) Catalyzing the First Steps in the Biosynthesis of the

CyanogenicGlucosidesLinamarin and Lotaustralin: CLONING, FUNCTIONAL EXPRESSION IN PICHIA PASTORIS, AND SUBSTRATE SPECIFICITY OF THE ISOLATED RECOMBINANT ENZYMES. Journal of Biological Chemistry, 275(3): 1966-75.

33. Jørgensen K, Bak S, Busk PK, Sørensen, C, Olsen CE, Puonti-Kaerlas J, and Møller, BL 2005. Cassava plants with a depleted cyanogenicglucoside content in leaves and tubers. Distribution of cyanogenicglucosides, their site of synthesis and transport, and blockage of the biosynthesis by RNA interference technology. Plant Physiol, 139: 363–374

34. Bredeson, JV, Lyons JB, Prochnik SE, Wu GA, Ha CM 2006. Edsinger-Gonzales E, Sequencing wild and cultivated cassava and related species reveals extensive interspecific hybridization and genetic diversity. Nat. Biotechnol, 34: 562–570.

35. Bull, S.E., Seung, D., Chanez, C., Mehta, D., Kuon, J.-E., Truernit, E., et al 2018Accelerated ex situ breeding of GBSS- and PTST1-edited cassava for modified starch. SciAdv,4: eaat6086.

36. Gomez MA, Lin ZD, Moll T, Chauhan RD, Hayden L, Renninger K 2019. Simultaneous CRISPR/CAS9-mediated editing of cassava eIF4E isoforms nCBP-1 and nCBP-2 reduces cassava brown streak disease symptom severity and incidence. Plant Biotechnol. J,17: 421–434.

37. Odipio, J., Alicai, T., Ingelbrecht, I., Nusinow, D.A., Bart, R., and Taylor, NJ. 2017 Efficient CRISPR/CAS9 Genome Editing of Phytoenedesaturase in Cassava. Front. Plant Sci, 8: 1780.

38. Veley KM, Okwuonu I, Jensen G, Yoder M, Taylor NJ, Meyers BC, Bart RS 2021. Gene tagging via CRISPR-mediated homology-directed repair in cassava. G3, 11(4): jkab028.

39. Sun D, Guo Z, Liu Y, Zhang Y 2017. Progress and prospects of CRISPR/Cas systems in insects and other arthropods. Frontiers in physiology, 8: 608.

40. Globus R, Qimron U 2018. A technological and regulatory outlook on CRISPR crop editing. Journal of cellular biochemistry, 119(2): 1291-8.

41. Razzaq A, Kaur P, Akhter N, Wani SH, Saleem F 2021. Next-generation breeding strategies for climate-ready crops. Frontiers in Plant Science, 12.

42. Plaza Reyes A, Lanner F 2017. Towards a CRISPR view of early human development: applications, limitations and ethical concerns

of genome editing in human embryos. Development, 144(1): 3-7.

43. Gonzalez-Avila LU, Vega-López JM, Pelcastre-Rodríguez LI, Cabrero-Martínez OA, Hernández-Cortez C, Castro-Escarpulli G 2021. The Challenge of CRISPR-Cas toward bioethics. Frontiers in Microbiology, 12: 1344.
44. Uddin F, Rudin CM, Sen T 2020. CRISPR gene therapy: applications, limitations, and implications for the future. Frontiers in Oncology, 10: 1387.
45. Piergentili R, Del Rio A, Signore F, UmaniRonchi F, Marinelli E, Zaami S 2021. CRISPR-Cas and its wide-ranging applications: From human genome editing to environmental implications, technical limitations, hazards and bioethical issues. Cells, 10(5): 969.
46. Tang L, Mao B, Li Y, Lv Q, Zhang L, Chen C, He H, Wang W, Zeng X, Shao Y, Pan Y 2017. Knockout of OsNramp5 using the CRISPR/CAS9 system produces low Cd-accumulating indica rice without compromising yield. Scientific reports, 7(1): 1-2.