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Application of Genome editing-CRISPR/Cas9 for the Crop Improvement

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Abstract

The application of genome editing, CRISPR/Cas9 has revolutionized plant breeding by enabling precise, efficient, and targeted modification of native genes, significantly accelerating the development of improved agronomic traits of crops. Therefore, CRISPR/Cas9 technology currently the most extensively used genome editing technique worldwide because of its simple design, cost-effectiveness, high efficiency, good reproducibility, high engineering feasibility, ability to create gene knockout, RNA editing, and quick cycle. It is used to knock in or knock out genes of interest and for generating models for genetic studies. The main components of the CRISPR/Cas9 system are an RNA-guided Cas9 endonuclease and a single-guide RNA (sgRNA). The workflow of CRISPR/Cas9 gene editing comprises selecting target sites, designing and synthesizing sgRNA, introducing transformation constructs or ribonucleoprotein (RNP) in plant cells, followed by transformation and identification of edited lines. This approach bypasses the formal regulations on GMOs, thus encouraging the widespread adoption RNA-guided gene editing in agricultural sciences and biotechnology. The system is now being utilized in the biofortification of cereal crops such as rice, wheat, barley, and maize, including vegetable crops such as potato and tomato. The world's first genome-edited rice varieties are DRR Dhan 100 (Kamala) **and** Pusa DST Rice 1 developed by the Indian Council of Agricultural Research (ICAR), New Delhi, India in 2025 with the objective of bringing about revolutionary changes in terms of higher production, climate adaptability, and water conservation. The CRISPR/Cas9-based crop genome editing has been utilized in imparting/producing qualitative enhancement in aroma, shelf life, sweetness, and quantitative improvement in starch, protein, gamma-aminobutyric acid (GABA), oleic acid, anthocyanin, phytic acid, gluten, and steroidal glycoalkaloid contents. Some varieties have even been modified to become disease and stress-resistant. Therefore, CRISPR/Cas9 is aiding in developing climate-ready crops and improving crop quality parameters such as appearance, palatability, nutritional components, and other preferred traits. Gene editing tools are used to generate changes to the native genetic material. Unlike GMOs, which introduce novel configurations of genetic materials typically derived from other organisms, gene editing methods modify existing genetic material in ways that can yield beneficial outcomes.

Keywords: Biofortification; CRISPR/Cas9; Cas9 endonuclease; Genome editing; Indian Council of Agriculture Research (ICAR); single-guide RNA (sgRNA)

1. Introduction

Human population is expected to reach to about 10 billion by 2050. Climate change affects crop production, thus posing food security challenges [1-30]. However, crop improvement by classical breeding methods is very slow and time consuming. Bhattacharya et al., (2021) [6] and Yıldırım and Kavas (2026) [73] reviewed that conventional breeding alone will not bridge the gap between current level of crop production and expected levels in the decades to come in the food production systems [1-73-171]. Traditional plant breeding techniques such as crossbreeding, mutation breeding, and marker-assisted selection have significantly contributed to crop improvement over the past century [1-24-35-67-73-171]. However, these methods are often limited by long breeding cycles, low precision, and the unintended

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transfer of undesirable traits. To address these challenges, transgenic breeding emerged as a powerful tool, enabling the introduction of specific foreign genes to confer desirable traits such as pest resistance or herbicide tolerance [1-34-56-69-77-171]. While highly effective and precise, transgenic approaches face considerable regulatory and public acceptance barriers, particularly in regions with strict GMO legislation. Yıldırım K, Kavas (2026) [73] reviewed that the use of CRISPR/Cas9 genome editing has revolutionized plant breeding by enabling precise, efficient, and targeted modification of native genes, significantly accelerating the development of improved crops. Among CRISPR-based methods, transgene-free genome editing has gained prominence for producing enhanced plant varieties without integrating foreign DNA, thus avoiding many regulatory constraints associated with GMOs [1-79]. Molecular breeding includes gene editing and marker-assisted selection [1-45-67-73-171]. The genome-editing tool, CRISPR/Cas9 has been extensively used in crop improvement programs due to its more straightforward design, low methodology cost, high efficiency, good reproducibility, and quick cycle. Sharma et al., (2025) [5] reviewed that biotechnology-enabled crops developed through genome editing will have a part to play in improving crop productivity, meeting food, nutrition security besides catering to regional preferences and fetching valuable foreign exchange [1-5-90]. Political, social, economical proposition, scientific will, retailer and consumer acceptance are a must for genome editing (GE) to succeed and add value in the food value chain [1-83-171].

CRISPR – clustered regularly interspaced short palindromic repeats were first discovered in the sequences of DNA from *Escherichia coli* bacteria and described in 1987 by Ishino et al., from Osaka University (Japan) [2]. Most bacteria and *Archaea* have an adaptive defense system called CRISPR-Cas that protects them from phages, viruses, and other foreign genetic material [2-86]. At that time sequencing of these difficult-to-study DNA fragments took several months, but neither their origin nor their significance in the bacterial cell were understood by their discoverers [2]. Although in the early work in this field, the biological function of the CRISPR system had not yet been elucidated, scientists had already proposed a way to use the information encoded in CRISPR [2-100]. CRISPR/Cas9 was adapted into a programmable gene-editing tool in 2012 by biochemist **Jennifer Doudna** (University of California, Berkeley) and microbiologist **Emmanuelle Charpentier** (then at Umeå University), a breakthrough that earned them the 2020 Nobel Prize in Chemistry [2]. For the first time in history, a Nobel prize was awarded to two women, Emmanuelle Charpentier and Jennifer Doudna, who made key discoveries in the field of DNA manipulation with the CRISPR/Cas9 system, so-called "**genetic scissors**" [2-118]. It is difficult to overestimate the importance of the technique as it enables one not only to manipulate genomes of model organisms in scientific experiments, and modify characteristics of important crops and animals, but also has the potential of introducing revolutionary changes in medicine, especially in treatment of genetic diseases [2]. The original biological function of CRISPR/Cas9 system is the protection of prokaryotes from mobile genetic elements, in particular viruses [2-113]. Currently, CRISPR/Cas9 and related technologies have been successfully used to cure life-threatening diseases, make coronavirus detection tests, and even to modify human embryo cells with the consequent birth of babies carrying the introduced modifications [2-120]. This intervention with human germplasm cells resulted in wide disapproval in the scientific community due to ethical concerns, and calls for a moratorium on inheritable genomic manipulations [2].

In the last 20 years since its discovery, clustered regularly interspaced short palindromic repeats (CRISPR) has evolved from a mere bacterial immune system to a tool that can be programmed to achieve directed, efficient and sequence-specific modifications to the host genome [1-2-119]. In research labs, CRISPR is now frequently used to achieve fast adjustments to host cell lines [1-5-120]. It is used to knock in or knock out genes of interest and for generating models for genetic studies [1-129]. The system is now being utilized in the biofortification of cereal crops such as rice, wheat, barley, and maize, including vegetable crops such as potato and tomato [1-129]. The CRISPR-Cas-based crop genome editing has been utilized in imparting/producing qualitative enhancement in aroma, shelf life, sweetness, and quantitative improvement in starch, protein, gamma-aminobutyric acid (GABA), oleic acid, anthocyanin, phytic acid, gluten, and steroidal glycoalkaloid contents [2-127]. Some varieties have even been modified to become disease and stress-resistant [4,5, 6, 19-26]. Sharma et al., (2025) [5] reported that ICAR has developed India's first genome-edited(GE) rice varieties—DRR Rice 100 (Kamla) and Pusa DST Rice 1 in 2025 with the objective of bringing about revolutionary changes in terms of higher production, climate adaptability, and water conservation [5].

2. CRISPR/Cas9 system: Genome modification

Multiple tools exist to facilitate gene editing but **CRISPR/Cas9** is by far the most commonly used [1-15-34-90-120]. As a result, the term CRISPR is sometimes used interchangeably with gene editing [1-5-120-171]. To date, gene editing has been applied to a large variety of plants and animals, including fruits, vegetables, wine grapes, fish, rice and other popular foods. Some of these gene-edited products are now on the market [1-5-129]. The main components of the CRISPR/Cas9 system are an RNA-guided Cas9 endonuclease and a single-guide RNA (sgRNA) [1-5-126-171]. Type II CRISPR/Cas9 is one of the best-defined and most commonly utilized categories in multiple CRISPR/Cas systems [1-5-128]. Cas9 endonucleases HNH domain cuts one strand of sgRNA, while the RuvC-like domain cuts the opposite strand

of dsDNA, resulting in double-strand breaks (DSBs) [1-5-126]. As a result, the plant endogenous repair system automatically repairs DSBs in vivo, utilizing error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR), resulting in massive insertion or fragment replacement [1-5-128-171].

The CRISPR/Cas system allows rapid, site-specific genome modification in a single cell or a whole organism [1-5-120-171]. It regulates transcription, changes epigenomes, runs genome-wide screens, and imaging chromosomes [1-5-128-171]. The CRISPR/Cas system is now increasingly used for developing edited crops due to its diverse applications in genome editing [1-45-120-171]. Using the CRISPR/Cas, research groups have engineered several crop systems for disease resistance, drought, salinity, and thermotolerance [1-35-58-120-171]. CRISPR/Cas is aiding in developing climate-ready crops and improving crop quality parameters such as appearance, palatability, nutritional components, and other preferred traits [1-5-120-171]. The CRISPR/Cas system is an efficient, convenient, and cost-effective genome editing tool through which major crops can be biofortified for deficient vitamins or minerals [1-45-120]. Furthermore, due to the non-insertion of foreign DNA and lesser regulatory restrictions, the products of this technology are easily acceptable by people in society [1-30-120-171]. This genome editing tool has immense potential to eliminate the nutrient deficiency of crops and provide food security for the ever-increasing population [1-5-129-171]. So far, CRISPR technology has been specifically utilized to modify a single gene for crop improvement [1-59-120]. However, it holds the potential to manipulate several genes simultaneously, either by assembling multiple sgRNA expression cassettes in a single CRISPR vector or by producing more RNA through an endogenous RNA processing system [1-5-128-171].

CRISPR/Cas9 technique is a promising tool for crop improvement by delivering unprecedented accuracy and prompt development of crops capable of surviving food security and environmental stresses [1-55-56-79-120-171]. Through its targeted modifications for improving crop productivity, nutritional quality, and resilience, it serves as an essential method for attaining global food security and climate-smart agriculture [1-5-120]. It is imperative to encourage interdisciplinary collaboration, ethical rules, and fair accessibility for ensuring its responsible adoption by scientists, stakeholders, and policy makers [1-15-34-129-171].

3. Genome edited (GE) and Genetically modified (GMOs)

The core difference is that GMOs contain foreign DNA inserted from another species, while genome-edited crops simply tweak, delete, or repair the plant's existing DNA without adding outside genetic material [1-89-127]. Therefore, GMOs as adding a new tool to a toolbox, and genome editing as sharpening the tools already inside. Disadvantages of genetically modified (GMOs) crops include allergic reactions in humans and reduced nutrition [1-55-129]. Also, they cause environmental impact by releasing toxins in the soil, induce pest resistance, and disruption of crop biodiversity. Several ethical concerns are associated with GMO crops [1-5-120-171]. In view of the disadvantages of GMO crops, genome editing (GE) technology offers distinct genetically modified (GMO) crops [1-5-120]. The best example is a widely grown GMO is **Bt cotton** — a cotton variety that has been designed to contain a gene from a soil organism that provides inherent resistance to the bollworm and other insect pests [1-67-120]. This genetic pest control mechanism can reduce pesticide applications and improve yields. Though just 10 GMO crops are grown commercially, GMOs are found in much of the Western food supply due to ingredients derived from GM corn, soybeans and sugar beets [1-5-120]. Other GMO crops approved for sale include potato, summer squash, papaya, apples, canola, cotton and alfalfa [1-5-129-171]. Genetically modified organisms can leverage the genetics of other organisms to improve desired traits. In the case of Golden Rice, a gene from daffodil and another gene from a soil bacterium were inserted into the rice genome to produce beta carotene, which is converted into the essential nutrient vitamin A [1-5-120-171]. Golden Rice is intended to reduce vitamin A deficiency among nations like the Philippines, where rice is a staple food [1-5-120]. In research contexts there are many examples of GMOs representing a diversity of plants and traits [1-57-87-120]. However, the translation of this technology to commercial or applied settings has reached a bottleneck due to a stringent and extremely expensive regulatory process [1-5-120].

This bottleneck could be eased by gene editing, which has recently come on the scene as an important mechanism for crop improvement [1-5-129-171]. The discovery and application of CRISPR/Cas9 for agricultural improvement has been effective at generating novel varieties in myriad crops across many traits [1-46-57-79-120-171]. Gene editing tools are used to generate changes to the native genetic material [1-66-120]. Unlike GMOs, which introduce novel configurations of genetic materials typically derived from other organisms, gene editing methods modify existing genetic material in ways that can yield beneficial outcomes [1-5-120-171]. Regulatory frameworks governing gene editing are nascent and generally much less prohibitive relative to regulation of GMOs [1-5-120-171]. Legislation regarding gene editing is emerging globally and trending towards allowing gene-edited products to pass from research to production with relative ease, compared to GMOs [1-52-67--129]. A favorable regulatory environment may facilitate the emergence of gene-edited products generated by public sector institutions [1-5-110]. Both GMOs and gene editing offer powerful tools for increasing crop yields, adapting to climate change, enhancing the nutritional qualities of foods,

improving animal welfare and developing products with many other important traits [1-13-46-79-120-171]. Where GMOs are able to draw on the diversity of all living organisms to extract beneficial genetic material, varieties produced using gene editing are limited to changing the standing genetic material in ways that could be beneficial [1-55-129]. It is noteworthy that gene editing tools can be used to produce GMOs. In this case, novel configurations of genetic material can be precisely inserted into the genomes of organisms by using gene editing machinery [1-35-46-57-170]. These gene-edited GMO products typically would be regulated as a GMO [1-5-129]. Though GMOs and gene-edited organisms are imbued with technological benefits and drawbacks, they do provide potent mechanisms to develop next-generation crop and animal varieties to address current and future demands on global food systems [1-5-120-171].

4. Genome editing (GE) for crop improvement in India

Genome editing has changed plant biology and accelerated crop improvement. CRISPR/Cas9 allows precise and efficient genetic changes in many species [1-35-116-117-120]. Furthermore, Cas9 has limits like PAM restrictions, off-target effects, and varying editing success [1-5-120]. This led to new systems. Editors like Cas12a, CasΦ, CasMINI, and CasX offer more targeting options, can edit RNA, and work better with hard to edit plant genomes [1-5-120-171]. Precision tools such as base editors and prime editors make precise changes by swapping nucleotides or adding small pieces without cutting both DNA strands. This improves accuracy [1-120]. Beyond single tools, combined and step by step editing methods can be used for handling complex traits controlled by many genes [1-55-120]. Using several methods like CRISPR knockouts, base and prime editing, epigenome editing and recombinase systems-breeders can improve traits while reducing unwanted side effects [1-5-120]. Stepwise editing helps to test changes, confirm their effects, and improve entire biological pathways [1-58-90-120]. Combining these approaches with AI-driven analysis, target prediction, and design optimization makes it easier to pick the best genes and edits for desired traits [1-58-90-120]. These advanced editing methods are used to boost stress tolerance, fight diseases, improve nutrition, increase yields, and enhance quality after harvest [1-58-90-120-171]. Despite progress, problems remain with how efficient edits can be made, delivering tools into plants, reliance on specific genotypes, unclear regulations, and acceptance by society [1-58-90-120]. The genome editing with AI, a fast breeding techniques helps to develop stronger, high yielding crops and support global food security [1-58-90-129-171].

The adoption of genome editing(GE) for crop improvement has the potential to bring India into the epicentre of international trade in agricultural products [4-6, 19,20, 23-27, 35, 37, 39, 40, 48, 49, 52, 58, 60, 62, 64, 66, 82, 83, 85, 105, 109, 110, 122, 127, 128-171]. Genome editing(GE) has revolutionized the agricultural sector because of its ability to create precise, stable and predictable modifications in the genome and therefore, offers great opportunities for crop improvement in India [1-58-90-120]. However, for harvesting the real benefits of this technology in agriculture sector, there is a strong need of creating awareness among the end users and development of suitable policies for regularization of genome edited products [1-58-90-120-171]. Many regulatory agencies around the world have been modernizing their regulatory approaches to be more risk proportionate and to reflect a more science-based approach [1-58-90-129]. It will also provide great opportunities for the government to push toward making India a global seed hub. Small stakeholder farmers will benefit immensely from high-quality seed development, resulting in higher agricultural returns [1-58-90-120-171].

Genome editing(GE) is a popular method used to make changes to an organism's DNA [1-58-90-120-171]. In agriculture, it has been used to design and develop crop varieties with improved disease resistance, climate resilience, and nutritional quality. Popular tools for genome editing(GE) include the CRISPR/Cas9 system, TALENs, and Zinc finger nucleases [1-58-90-129-171]. These tools have been shown to be successful in the development of improved crop varieties. Several genome-edited (GE) commodity and specialty crops have been developed and deployed globally, including herbicide-resistant soybean and corn, non-browning mushrooms, and high-yielding tomatoes [1-58-90-120]. High-nutrition rice, wheat, mustard, and millets have also been developed. From an economic standpoint, the cost of developing genome-edited crops is comparable to that of traditional breeding and the process could even be faster [1-58-90-120]. The process allows for the improvement of crop varieties that can be designed to adapt to an array of growing conditions, taking a big step forward in terms of food security, especially with respect to generating food in areas where demand is the highest [1-58-90-120-171].Unfortunately, gaining public acceptance of genome edited (GE) products has been challenging because they are often categorized as genetically modified organisms (GMOs), a designation that includes transgenic crops created through the insertion of foreign DNA [1-58-90-120]. Additionally, due to a lack of scientific awareness, there have been concerns about carcinogenicity, antibiotic resistance, and nutritional defects resulting from GMOs. While genome edited (GE) crops do not feature foreign DNA, confusion between genome edited (GEs) and GMOs has led to a dip in popularity among consumers and hampered adoption in India [1-58-90-129]. If the categorization confusion between genome edited (GE) crops and transgenic crops can be overcome through increased awareness, genome edited (GE) products have the potential to be better accepted by consumers [1-58-90-120]. However, this is only possible as the government now revises its regulation of genome edited

(GE) crops and provides incentives to better promote genome-editing research in India [1-58-90-120]. It is also imperative that authorities work to raise awareness of the scientific principles behind genome edited (GE) technology so that genome edited (GE) crops are accepted by farmers and consumers alike [1-58-90-120-171].

Sharma et al., (2025) [5] reviewed that the Indian Council of Agricultural Research (ICAR) (Figure-1) has developed India's first genome-edited rice varieties—DRR Rice 100 (Kamla) and Pusa DST Rice 1—with the objective of bringing about revolutionary changes in terms of higher production, climate adaptability, and water conservation [4-6, 19,20, 23-27, 35, 37, 39, 40, 48, 49, 52, 58, 60, 62, 64, 66, 82, 83, 85, 105, 109, 110, 122, 127, 128, 130-171]. These new varieties were developed using genome-editing(GE) technology based on CRISPR/Cas9. Sharma et al., (2025) [5] reported that the DRR Rice 100 (Kamala) variety was developed by the ICAR-Indian Institute of Rice Research (IIRR) in Hyderabad, based on Samba Mahsuri (BPT 5204). Researchers edited a cytokinin oxidase gene (OsCKX2), developing a novel allele. The result was a 19% boost in grain yield, earlier maturity by up to 20 days (~130 days), and better performance under low-fertilizer and drought conditions. Due to its shorter duration, it helps to save water and fertilizers and reduces methane gas emissions [4-6, 19,20, 23-27, 35, 37, 39, 40, 48, 49, 52, 58, 60, 62, 64, 66, 82, 83, 85, 105, 109, 110, 122, 127, 128]. Its stalk is strong and does not fall. Sharma et al., (2025) [5] reported that the rice quality is similar to the original variety, Samba Mahsuri. The second variety, Pusa DST Rice 1, was developed by the ICAR-Indian Agriculture Research Institute (IARI) in New Delhi. It is based on MTU 1010 for improved drought and salt tolerance. By knocking out a gene responsible for suppressing stress resistance, the Indian scientists achieved plants with reduced stomatal density and water use, alongside improved tillering, grain yield, and salt resilience. Field tests showed significantly higher yields under drought and saline stress compared to the parent MTU1010 variety. Sharma et al., (2025) [5] indicated that this variety can increase yields by 9.66% to 30.4% in saline and alkaline soils, with the potential for up to a 20% increase in production [5]. These genome-edited rice varieties were developed for states such as Andhra Pradesh, Telangana, Karnataka, Tamil Nadu, Puducherry, Kerala (Zone VII), Chhattisgarh, Maharashtra, Madhya Pradesh (Zone V), Odisha, Jharkhand, Bihar, Uttar Pradesh, and West Bengal (Zone III). Field evaluations have been conducted across multiple agroecological zones [4-6, 19,20, 23-27, 35, 37, 39, 40, 48, 49, 52, 58, 60, 62, 64, 66, 82, 83, 85, 105, 109, 110, 122, 127, 128, 130].

The launch of DRR Dhan 100 (Kamala) and Pusa DST Rice 1 marks a major milestone in Indian agriculture, demonstrating the transformative potential of genome editing in addressing key challenges in food production [5, 130]. With their enhanced yields, environmental resilience, and suitability for diverse growing conditions, these innovative rice varieties offer practical solutions to climate change, soil degradation, and food insecurity [5, 130]. By embracing genome-edited crops, India sets a global precedent, paving the way for broader adoption of agricultural biotechnology and delivering lasting benefits to farmers, consumers, and the nation's food security landscape [5, 130].

Genome editing, a New Breeding Technique (NBT), has fascinated plant biologists worldwide [1-5-129]. This technique for crop improvement has allowed the development of biotic stress-resistant traits, climate-resilient crop varieties, and products with improved quality. The recent development of a new biosafety regulatory policy for genome edited crops(GE) by the government of India, wherein SDN-1 and SDN-2 categories of genome edited events have been exempted from stringent biosafety regulations, has provided an impetus for genome editing research initiatives in India [4,5-129]. Various government organizations, including the Indian Council of Agricultural Research (ICAR), Council of Scientific and Industrial Research (CSIR), Department of Biotechnology (DBT), and Department of Science and Technology (DST), have started funding genome editing research for various crops [4-6, 19,20, 23-27, 35, 37, 39, 40, 48, 49, 52, 58, 60, 62, 64, 66, 82, 83, 85, 105, 109, 110, 122, 127, 128, 130]. Recently, ICAR launched a mega project on "Enabling Climate Resilience and Ensuring Food & Nutritional Security Through Genome Editing in Agricultural and Horticultural Crops," covering major agricultural and horticultural crops in crop-based research institutes of ICAR [1-31-129]. Furthermore, ICAR has also launched the "All India Network Project on Biotech Crops" to support translation work on transgenic and gene edited crops [1-31-129]. DBT is funding a number of consortium-based network projects in various crops, including rice, maize, Indian mustard, potato, and tomato, under the platform "Genome Editing of Crops for Enhanced Attributes." DBT is also supporting research grants to enhance the capacity of scientists working on genome editing and is collaborating with international organizations, such as the Indo-U.S. Science and Technology Forum, on initiatives related to genome engineering and gene editing [4-6, 19,20, 23-27, 35, 37, 39, 40, 48, 49, 52, 58, 60, 62, 64, 66, 82, 83, 85, 105, 109, 110, 122, 127, 128-171]. There is a need to create awareness among stakeholders about the positive aspects of this technology to reap the real benefits of genome edited crops [4-6, 19,20, 23-27, 35, 37, 39, 40, 48, 49, 52, 58, 60, 62, 64, 66, 82, 83, 85, 105, 109, 110, 122, 127, 128]. The recent release of two genome edited rice varieties by the government of India has opened the gateway for many genome edited products to get to the market in the coming years [1-31-129-171].

Figure 1 Worlds first genome edited rice varieties developed by Indian Council of Agricultural Research (ICAR), India

The successful implications of CRISPR/Cas9 system have been stated in different plants, including model plants like *Arabidopsis*, to major crops, for example wheat, rice, apple, citrus, banana, tomato etc [1-31-129-171]. Using various plant materials such as leaf discs, flowers, calli and protoplasts, experiments have been conducted for improving biotic/abiotic stress resistance and enhancing crop productivity. The resulting mutations are heritable, highlighting the usefulness of CRISPR Cas/9 in plant molecular biology and crop improvement programs [1-31-129]. One of the strengthening features of CRISPR/Cas9 system is its efficiency in modifications of multiple target genes. Through multiplex editing, the natural domestication of plants can effectively be produced within a significantly short span of time, allowing rapid development of improved plants with desirable traits [1-31-129]. It also enables the induction of specific deletions among sites, thus offering a useful means to interrupt DNA sequences and generating knockouts via eliminating specific region instead of frame-disruptive mutations [1-31-129-171].

The induction of gene knockouts through non-homologous end joining (NHEJ) pathway is the primary function of CRISPR/Cas9 system, while other gene editing approaches such as homology-directed pair (HDR) and epigenetic modulation also play a significant role in improving desirable agricultural characteristics [1-41-129-171]. The effectiveness of gene targeting through homology-directed pair (HDR) yet remains lower in plant cells. Various innovative approaches have been established to improve the extent of gene targeting in plants by HDR pathway, such as donor DNAs amplification by using virus replicons [1-34], inhibiting NHEJ pathway [1-35,36], and using Cas9 expression by egg-specific promoters [1-37-129-171].The workflow of CRISPR/Cas9 gene editing comprises selecting target sites, designing and synthesizing sgRNA, introducing transformation constructs or ribonucleoprotein (RNP) in plant cells, followed by transformation and identification of edited lines [1-31-129]. Currently, this technology and its novel derivatives have unveiled broad functions including gene disruption, gene insertions, transcriptional repression and activation [1-31-129]. Besides, multiplex gene editing have played a significant role in an advanced level research and regulatory pathways [1-31-129-171]. Earlier setbacks faced in developing gene targeting technology [1-38, 39-125], have now been fixed by the arrival of CRISPR/Cas9 system, which basically target single genes instead of entire genome [1-39,40-129]. Furthermore, unconventional strategies like the delivery of Cas9 protein-sgRNA ribonucleoproteins through CRISPR/Cas9 enables gene insertion into plants without transgenic traces [1-51-68-86-90-129]. This approach bypasses the formal regulations on GMOs [1-43], thus encouraging the widespread adoption RNA-guided gene editing in agricultural sciences and biotechnology [1-31-129]. Together with other transformative nucleases, CRISPR/Cas9 has become a revolutionary technology that is shifting the paradigm of plant sciences [1-31-129-171].

5. Crops with the target trait(s) for genome editing (GE) in India

- Rice: Drought and salinity tolerance, bacterial blight and blast resistance, herbicide resistance, higher yield, de novo domestication, nitrogen use efficiency (NUE), blast and blight resistance, nematode resistance, and virus resistance.
- Wheat: Nitrogen use efficiency (NUE) and phosphorus use efficiency (PUE), heat stress, salinity and herbicide tolerance, biofortification of Fe and Zn, resistant starch.
- Maize: Development of haploid inducer lines, enhanced kernel number per plant, high amylose/low glycemic maize, BLSB resistance, root architecture improvement and herbicide tolerance.
- Chickpea: Fusarium wilt resistance, drought tolerance, improving productivity and nodulation
- Pigeon Pea: Productivity improvement.
- Grass Pea: Anti-nutritional factor removal.
- Urd Bean: Tolerance to MVYV and UCLV, and productivity enhancement.
- Lentil: Productivity enhancement.
- Indian Mustard: Early flowering, improved grain and oil yield, shattering resistance, improved plant architecture, stem rot resistance, orobanche resistance, development of haploid inducer lines, reduced seed glucosinolate content, low erucic acid.
- Groundnut: Enhanced oil content
- Soybean: Seed and oil content, insect resistance, pod shattering resistance, resistance to Yellow Mosaic Disease (YMD), high oleic acid content, photo-insensitivity, drought, water-logging and salinity tolerance.
- Castor: Bio-detoxification.
- Sunflower: Powdery mildew resistance
- Sesame: Shattering resistance, determinate habit and high oleic acid oil, more number of seeds per capsule, high oil content and seed weight.
- Linseed: More 1000-seed weight, early flowering.
- Pearl Millet: Enhanced grain yield, enhanced shelf life
- Foxtail Millet: Enhanced grain yield
- Finger Millet: Enhanced grain yield, drought stress tolerance
- Sorghum: Herbicide tolerance.
- Sugarcane: Drought tolerance, enhanced sucrose content, greater number of tillers, enhanced biomass, red rot and virus resistance, reduced lignin content.
- Cotton: Tolerance to premature square and boll shedding, compact architecture trait, determinate sympodial shoots, improved seed oil quality and content, fiber quality, enhanced yield.
- Jute: Resistance to short-day flowering, herbicide tolerance, improved fiber quality, heat stress tolerance, and development of haploid inducer lines.
- Apple: Early flowering and drought tolerance
- Papaya: Resistance to papaya ringspot virus (PRSV).
- Banana: Multiple diseases and virus resistance, biofortified banana with enriched vitamin A content, development of seedless Bhimkol banana.
- Grape: Powdery mildew resistance.
- Potato: Late blight resistance, bacterial wilt resistance, potato scab resistance, High temperature tolerance, enhancement of folic acid, vitamin D, yield, reducing glycemic index level & steroid glycoalkaloids (SGA) content, development of haploid inducer lines.
- Tomato: Resistance to ToLCD, fusarium wilt, enhancement of Total Soluble Sugars (TSS), vitamin D content, enhanced postharvest life and nutrition quality, modification of plant architecture for enhanced yield
- Chilli: Resistance to chilli leaf curl virus (chilli LCV), potyvirus, and anthracnose disease.
- Cucumber: Resistance to powdery mildew disease
- Muskmelon: For delayed fruit ripening, modifying ripening behavior, and extended shelf life
- Onion: Heat and drought tolerance, and development of male sterile lines.
- Cassava: Low amylose starch lines and resistant starch lines.
- Black Pepper: Resistance to Phytophthora disease.
- Ginger: Resistance to Ralstonia solanacearum and Pythium disease.
- Cumin: Resistance to Alternaria blight disease
- Coriander: Resistance to stem gall disease
- Marigold: Enhanced lutein and zeaxanthin content, and development of haploid inducer lines.

6. Genome Editing(GE) by CRISPR/Cas9

Genome editing produces predictable and inheritable mutations in specified regions of the genome, with minimal off-target effects and no external gene sequence integration [1-31-129-171]. Deletions, insertions, single-nucleotide substitutions, and extensive fragment substitutions are used for GE-mediated DNA alterations [1-31-129]. Systems such as homing endonucleases or meganucleases (HEs), Zinc-Finger Nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) were engaged as genome editing tools before the discovery of CRISPR-associated protein (Cas) [1-61-129]. Meganucleases, also known as homing endonucleases, are rare-cutting enzymes found in all microbial genomes [1-31-129-171]. These enzymes identify and cleave lengthy DNA sequences (usually 18–30 base pairs), resulting in double-strand DNA breaks (DSBs) [1-31-129]. Various designed meganuclease variants are available to cleave unique DNA targets for genomic changes for creating important characteristics in crop species. Homing endonucleases technology has suffered from technical problems in the manufacture of these nucleases and designing vectors for their entrance into cells and off-targeting consequences [1-31-129-171]. Zinc-finger nucleases (ZFNs) are "nucleases" consisting of engineered zinc-finger DNA-binding domains paired with a nuclease, most often the FokI nuclease. ZFN-induced double-strand breaks are exposed to cellular DNA repair processes, resulting in remarkably targeted mutagenesis and targeted gene replacement [1-31-129]. The zinc-finger domains consist of four to six 30 amino acid domains that may bind to trinucleotide sequences, limiting total DNA-binding domain specificity to 12–18 nucleotide sequences [1-61-129-171]. However, ZFN technology has disadvantages such as complex design (which requires customized protein for each DNA sequence), low engineering feasibility, low specificity, normal efficacy, and inability to gene knockout and RNA editing [1-31-129]. Transcription activator-like effector nucleases (TALENs) are a nonspecific DNA-cleaving nuclease linked to a DNA-binding domain that could be tailored to target specific sequences [1-61-129]. TALENs are made up of an engineered array of TALE repeats fused to the FokI nuclease domain and are used for editing the genome [1-31-129-171]. However, TALENs also have disadvantages such as complex design, lower engineering feasibility, lower specificity, low efficacy, and inability to gene knockout and RNA editing [1-31-129]. Thus, CRISPR/Cas technology has emerged as a promising genome editing tool overcoming many of the abovementioned disadvantages of HEs, ZFNs, and TALENs [1-31-129]. Therefore, CRISPR/Cas technology currently the most extensively used genome editing technique worldwide because of its simple design, cost-effectiveness, high efficiency, good reproducibility, high engineering feasibility, ability to create gene knockout, RNA editing, and quick cycle [1-31-129-171].

7. CRISPR/Cas9 technology: Applications in Crop improvement

Carotenoids are widely distributed isoprenoid pigments essential for photosynthetic organisms. Humans do not produce carotenoids de novo, but they require them in their food, notably as β-carotene and vitamin A precursors [1-31-129-171]. Vitamin A is necessary for biological functions that include light transduction in vision, embryonic development, immunological operation, and overall health maintenance in human beings. Vitamin A deficiency (VAD) is one of the most severe worldwide health issues, resulting in a variety of symptoms such as xerophthalmia, night blindness, pediatric blindness, and an increased risk of morbidity and mortality, particularly in young children. Given this, carotenoid-enriched staple crops (golden crops), either through traditional breeding or genetic engineering, were initiated to combat VAD [1-31-129-171]. Among all the genetic engineering techniques, the CRISPR-based genome-editing technique seems the most efficient and widespread method, making rapid, DNA/transgene-free, and targeted multiplex genetic modification of organisms: a reality for developing "golden" staple crops. Through CRISPR/Cas-based systems, various genome-edited golden crops, that is, carotenoid biofortified crops have been produced to combat VAD. For instance, the Golden rice cultivar Kitaake has been developed by Knock-in, a 5.2-kb carotenogenesis cassette consisting of CrtI and maize PSY genes. The variety contains 7.9 μg/g dry weight (DW) β-carotene in the endosperm [1-61-129-171].

Vitamin E (tocopherol) is a potent lipid-soluble antioxidant and an essential component of the human diet [1-51-68-86-90-129]. Many human diseases, such as cardiovascular disease and certain cancers, are associated with insufficient vitamin E intake. The daily requirement of vitamin E for humans lies between 15 and 30 mg [1-31-129-171]. Through CRISPR/Cas9 technology, significant increase in tocopherols and tocotrienol content was achieved by targeted overexpression of *Hordeum vulgare* homogentisate phytyltransferase (HvHPT) and *Hordeum vulgare* homogentisate geranylgeranyltransferase (HvHGGT) [1-51-68-86-90-129-171]. These genes can be utilized for enhancing vitamin E content in other crops. Iron is involved in various metabolic activities such as oxygen transport and electron transport chain. Iron metabolism disorders cause the most frequent diseases in humans, encompassing multiple conditions with various clinical presentations, ranging from anemia to neurodegenerative diseases [1-31-129]. The iron need in humans can be fulfilled through dietary and crop biofortification. CRISPR/Cas9 is responsible for the latter type of

biofortification. This system has been reported to disrupthe Inositol pentakisphosphate 2- kinase 1 (IPK1) gene causing iron biofortification in wheat [1-31-129-171].

CRISPR/Cas systems, originally identified as adaptive immune mechanisms in bacteria and archaea, have been repurposed for targeted genome editing in plants [1-31-129-171]. The CRISPR/Cas9 system, in particular, has emerged as a powerful tool for introducing site-specific double-strand breaks, enabling precise genetic modifications [1-31-129]. The three-stage process of adaptation, expression, and interference underlies the CRISPR mechanism, with guide RNAs directing Cas endonucleases to specific genomic loci [1-31-129-171]. Advances in CRISPR/Cas9 technology have expanded its applications beyond gene knockouts, encompassing base editing, prime editing, and epigenome editing [1-31-129]. These innovations have facilitated the development of crops with enhanced yield, stress tolerance, disease resistance, nutritional content, and post-harvest quality [1-31-129-171]. However, challenges related to off-target effects, regulatory hurdles, ethical concerns, and public acceptance must be addressed to fully harness the potential of CRISPR/Cas9 in agriculture [1-31-129-171]. Integration of CRISPR/Cas9 with other cutting-edge technologies, such as synthetic biology, artificial intelligence, and high-throughput phenotyping, holds immense promise for accelerating crop improvement efforts [1-31-129-171]. As research continues to refine CRISPR/Cas9 tools and expand their applicability across diverse plant species, this transformative technology is poised to play a pivotal role in shaping a sustainable, resilient, and productive global food system for future generations [1-31-129-171].

Plant diseases caused by bacteria, viruses, fungi, and oomycetes threaten global crop productivity and food security [1-31-129-171]. Conventional control method chemical treatments, crop rotation, and traditional breeding are often slow, unsustainable, or ineffective against rapidly evolving pathogens. CRISPR/Cas9 genome editing offers a transformative approach by enabling precise modifications to host and pathogen-related genes, thereby enhancing crop disease resistance [1-31-129-171]. A significant strategy involves knocking out susceptibility (S) genes that pathogens exploit. For example, editing OsSWEET11, OsSWEET13, and OsSWEET14 promoters in rice prevents binding by *Xanthomonas oryzae* TALEs, conferring resistance to bacterial blight without affecting development [1-31-129-171]. Similarly, CRISPR/Cas9-mediated disruption of TaMLO homoeologs in wheat and SlMLO1 in tomato provides durable resistance to powdery mildew with minimal agronomic cost, while editing SlPMR4 enhances basal immunity [1-51-68-86-90-129]. Beyond S genes, CRISPR targets viral genomes and host–virus interactions [1-31-129]. In sugar beet, CRISPR disrupts viral DNA to combat Beet Curly Top Virus, while editing SlTOM1 in tomato impairs tobamovirus replication. Cas13, an RNA-targeting variant, effectively suppresses RNA viruses such as PVY and TuMV in potato and Arabidopsis [1-31-129]. For oomycete resistance, StDMR6-1 knockouts in potato reduce *Phytophthora infestans* severity, and DMR6 mutants in *Arabidopsis* resist both oomycetes and bacterial infections. CRISPR/Cas9 also modulates plant immune pathways by editing regulators like EDS1, PAD4, and NPR1, boosting defense responses without triggering autoimmunity. Multiplexed CRISPR strategies allow the stacking of multiple resistance (R) genes at defined loci to ensure durable protection, overcoming the limitations of single-gene resistance [1-31-129-171].

CRISPR/Cas9 genome editing is an important tool for increasing crop yields, which is critical in global food insecurity and climate change [1-51-68-86-90-129]. By precisely targeting yield-related genes, this system enhances productivity without transgenic methods. In rice, CRISPR targets yield-suppressing genes like Gn1a, GS3, GW2, and DEP1, which control grain number, size, and panicle architecture. Multiplex editing (targeting multiple genes simultaneously) produces rice with larger grains and higher yields [1-51-68-86-90-129]. Editing IPA1, a tillering and panicle development regulator, yields plants with improved architecture and greater biomass. CRISPR/Cas9 edits TaGW2, a gene limiting grain width and weight in wheat [1-51-68-86-90-129-171]. Mutations across all three homoeologs increase grain size and yield [1-51-68-86-90-129]. This technology modifies ZmCEP1 and ZmIPT2 in maize, which boosts nitrogen use efficiency, indirectly increasing yield Beyond cereals, CRISPR enhances tomato yield by editing SELF-PRUNING (SP) and SP5G genes, creating compact, early-flowering plants with higher fruit production in shorter cycles. CRISPR optimizes soybean flowering and architecture in legumes by targeting GmFT2a and GmFT5a, boosting yields [1-51-68-86-90-129-171].

Herbicide tolerance is vital in modern agriculture, enabling effective weed control, reduced labor, and maximized yields [1-51-68-86-90-129]. Traditionally achieved via transgenic methods or chemical mutagenesis, herbicide-tolerant crops have advanced with CRISPR/Cas genome editing [1-51-68-86-90-129]. This system precisely modifies the target genes to obtain herbicide-resistant crops. In rice, CRISPR-mediated HDR was also employed to introduce precise point mutations at amino acid positions W548 and S627 of the ALS gene which enabled resistance to bispyribac-sodium, yielding novel herbicide-tolerant germplasms [1-51-68-86-90-129]. In rapeseed, glyphosate resistance was achieved by combining CRISPR/Cas9 with a geminiviral replicon-based donor delivery system. CRISPR/Cas9 has been also successfully applied in soybean and maize to develop herbicide-resistant lines by precisely modifying ALS and EPSPS through both base editing and HDR strategies [1-51-68-86-90-129].

Abiotic stresses, including drought, salinity, and extreme temperatures, significantly constrain global crop production and yields [1-51-68-86-90-129-171]. CRISPR/Cas genome editing has also been used as a powerful approach to enhance crop resilience by modifying genes involved in stress sensing, signaling, and adaptive responses [1-51-68-86-90-129]. For instance, targeted mutation of the OsDST gene in the rice cultivar MTU1010 resulted in broader leaves, reduced stomatal density, and improved water retention under drought conditions [1-51-68-86-90-129]. Similarly, OsbHLH024 knockout in rice elevated the expression of key ion transporter genes (OsHKT1;3, OsHAK7, and OsSOS1), improving salt stress tolerance. Cold and heat stress, which impair metabolic and cellular functions, have also been addressed via CRISPR [1-51-68-86-90-129-171].

The use of meristematic tissues offers a strategic approach to genome editing in woody species, especially those that are recalcitrant to conventional tissue culture, as these regions contain totipotent, highly regenerative cells capable of giving rise to whole plants. Rocha et al., (2026) [119] employed biolistic delivery of genome-editing reagents into the shoot apical meristem (SAM) of citrus and the axillary meristems (AXM) of poplar [119]. The system was first validated using a GFP expression construct and subsequently applied for targeted genome editing [119]. In citrus, edited plants were obtained at the CsNPR3 locus exclusively through the delivery of CRISPR/Cas9 ribonucleoproteins (RNPs), whereas plasmid-based vectors were unsuccessful. Similarly, genome editing in poplar was achieved using RNPs targeting the Pt4CL1 gene [119]. Although chimeric events were detected, this approach provides a feasible and innovative framework for producing transgene-free edited perennial plants [119].

7. How to design guide RNA(gRNA) for CRISPR/Cas9 technology

To design a guide RNA (gRNA) for CRISPR/Cas9, identify a 20-nucleotide target sequence immediately upstream of a PAM site (usually 5'-NGG) [1-51-68-86-90-129-171]. Always use predictive design software for the best results. **Find the PAM Site:** The Cas9 enzyme requires a Protospacer Adjacent Motif (PAM) sequence to bind and cut the DNA [1-51-68-86-90-129]. For the most commonly used *Streptococcus pyogenes* (SpCas9), this sequence is **NGG** (where N can be any nucleotide). **Select the Spacer Sequence:** The gRNA uses a ~20-nucleotide sequence that is exactly complementary to your target DNA, located immediately before (5' of) the PAM site. The PAM itself is not included in the gRNA sequence. **Optimize GC Content:** Effective gRNAs have a GC content between 40% and 80%**.** Avoid extreme poly-T or poly-A stretches, as these can cause premature transcription termination or synthesis. **Prioritize Specificity:** Check that your 20-nucleotide spacer does not match other areas in the genome to avoid "off-target" effects [1-51-68-86-90-129].

Manual design is rarely used today because sophisticated algorithms can automatically score guides for cutting efficiency and off-target risk. Input the gene sequence into one of these widely used, free platforms: **Benchling:** A popular, user-friendly platform with built-in CRISPR design tools that can search reference genomes, identify PAM sites, and score candidates. **CHOPCHOP:** An excellent web tool that lets to paste your sequence or target genomic coordinates to find optimal guides. **CRISPOR:** Highly regarded for providing detailed off-target predictions and specific efficiency scores for different Cas9 variants [1-51-68-86-90-129].

A genome editing experiment requires the following components at the least: A host cell whose genome needs to be edited. A guide RNA which is transcribed and targets a region of interest in the genome. Cas proteins that are encoded within the cell or delivered to enable target binding and subsequent cleavage [1-51-68-86-90-129]. The most commonly used protein in genome editing experiments is spCas9 found in *S. pyogenes*. spCas9 facilitates genome editing by generating DSBs. This cleavage is dependent on spCas9 binding to the 5′- NGG-3′ PAM sequence that is located downstream of the cleavage site [1-51-68-86-90-129]. Depending on the Cas protein used for genome editing and the organism it is isolated from, the PAM will vary [1-51-68-86-90-129]. Despite its popularity, spCas9 has some limitations. It has the ability to recognize other PAMs such as 5′-NAG-3′ and 5′-NGA-3′. spCas9 can also bind and cleave sequences in the genome that have some similarity to the crRNA [1-51-68-86-90-129]. These low-fidelity aspects of spCas9 result in off-target effects. spCas9 is also a rather large protein and delivery into cells can be a challenge. For this reason, scientists have discovered and engineered alternatives to spCas9 that can also be used in genome editing [1-51-68-86-90-129]. Some examples of other commonly used Cas nucleases are Cas12a, Cas13a, lbCas9, asCas9 and CasX [1-51-68-86-90-129]. Other high-fidelity engineered variants including HypaCas9, eSpCas9 and Hi-FiCas9 offer many advantages over the traditional Cas9. These variants have a weakened DNA phosphate backbone interaction with the nuclease. This results in genome-wide specificity and undetectable off-target effects. However, each specific variant has certain improved characteristics which can benefit the process, depending on the type of experiment being performed [1-51-68-86-90-129-171].

In a genome editing experiment with spCas9, a guide RNA can be designed in two ways: a two-component crRNA plus a tracrRNA system or as a single guide RNA (sgRNA). In bacteria, a tracrRNA includes a segment at its 5′ end which is complementary to the repeat-derived segment of the pre-crRNA, allowing the two to hybridize [1-51-68-86-90-129].

The guide region of the crRNA is typically 20 nucleotides long and is synthesized along with an optimized tracrRNA length and joined by a linker loop to form one continuous molecule. sgRNAs are preferred as the separate crRNA and tracrRNA molecules may not anneal efficiently. Only the guide region in the sgRNA determines the region to be targeted in the genome and this flexibility is what makes CRISPR-based genome editing useful and easy [1-51-68-86-90-129]. The DNA sequence encoding the guide RNA does not contain a PAM and this prevents it from being cut by the Cas proteins, though some applications of PAMs in homing guide RNAs have been developed. While designing guide RNAs, it is recommended to design three to four different guides to increase the chances of at least one of them binding efficiently and cleaving the target [1-51-68-86-90-129]. While designing guide RNAs for a knock-in, the cut site should be as close to the knock-in site as possible. The recommended distance is less than 10 nucleotides between the two. However, if the knock-in is on just one allele, the cut site to knock-in site distance can be increased up to 20 nucleotides to increase the frequency of monoallelic edits to biallelic edits [1-51-68-86-90-129]. It is important to keep in mind that guide designs must be optimized to reduce off-target binding and cleavage. There are many tools available for guide design that predict the on-target activity based on certain established scoring rules. Benchling, IDT (Integrated DNA Technologies), Broad Institute GPP, CasOFFinder, CRISPOR and Synthego have some useful resources that can help with this process [1-51-68-86-90-129].

In India, companies like **CrisprBits** (headquartered in Bengaluru, Karnataka with a lab at C-CAMP) offer CRISPR design, precision medicine, and clinical-research solutions. Additionally, specialized bioinformatics and genomics firms such as **EdGene BioMed** provide bespoke guide RNA (gRNA) design for CRISPR-Cas9 genome editing and sequence analysis [1-51-68-86-90-129]. Because evaluating on-target activity and off-target risks manually is complex, researchers rely on specialized bioinformatics tools. These platforms score guides automatically and provide synthesis instructions: **Synthego CRISPR Design Tool:** Provides rapid, high-scoring guide recommendations for thousands of species and genomes. IDT CRISPR-Cas9 Design Checker: Allows you to input sequences to assess on-target and off-target potential and order modifications. **CHOPCHOP**: A widely used, free academic web tool for identifying target sites and calculating off-target scores across many genomes [1-51-68-86-90-129]. **Benchling**: An end-to-end biological software suite that includes integrated gRNA design, off-target analysis, and plasmid mapping. gRNA Format: A standard single-guide RNA (sgRNA) links the targeting crRNA to the structural tracrRNA [1-51-68-86-90-129]. The basic architecture looks like this:

5'- [20-nucleotide Target Spacer]- [Tetraloop Scaffold]- [tracrRNA Scaffold]-3'

The rapid rise of CRISPR as a technology for genome engineering and related research applications has created a need for algorithms and associated online tools that facilitate design of on-target and effective guide RNAs (gRNAs) [1-51-68-86-90-129]. The state of the art in CRISPR, gRNA design for research applications of the CRISPR/Cas9 system, including knockout, activation, and inhibition. Notably, achieving good gRNA design is not solely dependent on innovations in CRISPR technology. Good design and design tools also rely on availability of high-quality genome sequence and gene annotations, as well as on availability of accumulated data regarding off-targets and effectiveness metrics [1-51-68-86-90-129].

With the CRISPR/Cas9 system, however, targeting of the nuclease to a particular locus relies on the guide RNA (gRNA), as well as the presence at the target DNA region of a protospacer adjacent motif (PAM) sequence [1-51-68-86-90-129]. To design a gRNA, the following must be defined: (a) the target region or gene; (b) the version of Cas9 protein to be used, including what PAM sequence(s) is recognized; (c) what promoter will be used for in vitro or in vivo expression of the gRNA, i.e. so that the terminator sequence for the promoter can be excluded from the gRNA sequence; and (d) the cloning strategy, which might put additional constraints on gRNA sequence and will impact the design of the specific oligonucleotides to be synthesized and either used as a template for RNA production or cloned into an expression vector [1-51-68-86-90-129]. Importantly, design of gRNAs must also be coupled with the intended application, e.g. knockout via nonhomologous end joining (NHEJ), knock-in, CRISPR activation (CRISPRa), or CRISPR interference (CRISPRi) [1-51-68-86-90-129]. For example, when generating a knockout in a genetic system, the importance of limiting off-targets on the same chromosome might outweigh the importance of limiting off-targets on other chromosomes that can be ‘cleaned up’ in subsequent back-crosses or outcrosses. Moreover, for applications such as CRISPRa or CRISPRi, the appropriate position of gRNA(s) relative to the transcription or translation [1-51-68-86-90-129].

The start site of the target gene might be different than the appropriate position of gRNA(s) intended to be used in generation of a knockout or knock-in allele. In addition, some approaches required the design of multiple gRNAs for a single target, such as design of a pair of gRNAs for use with a nickase version of the Cas9 protein; design of nearby or distal gRNAs for use with a knock-in construct; or design of two or more gRNAs upstream of the transcription start site for CRISPRa [1-51-68-86-90-129]. The CRISPR system can be used for a growing variety of applications, and each CRISPR approach requires at least two basic components: a Cas protein and guide RNA (gRNA). The most commonly

used Cas endonuclease is engineered from *Streptococcus pyogenes* (a.k.a. SpCas9 or, with codon optimization for expression in human cells, hCas9) [1-51-68-86-90-129]. It is an RNA-guided DNA nuclease which can generate double-strand breaks (DSBs) at target sites. Cas9 is localized to a target site in the host genome by the complementary gRNA, and the DSB is introduced if the gRNA was designed to target a region immediately adjacent to a Protospacer Adjacent Motif (PAM). The PAM sequence is dependent on the particular Cas enzyme being used: for SpCas9, the PAM sequence is NGG or NAG [1-51-68-86-90-129].

Once DSBs are generated by the CRISPR system, cells most commonly activate the non-homologous end joining (NHEJ) pathway to repair the DNA breaks, which usually results in small random deletions, or more rarely insertions and base substitutions. When these mutations disrupt a protein-coding region (e.g. a deletion causing a frameshift), they may lead to functional gene knockout [1-51-68-86-90-129]. Dual gRNAs can be introduced with Cas9 to create two DSBs, thereby deleting a DNA fragment located between the two target sites, or dual gRNAs can be used to target two different genes simultaneously [1-51-68-86-90-129]. Alternatively, and less efficiently, DSBs can be repaired via the homology-directed repair (HDR) pathway when a DNA template is present. When donor DNA is introduced with the CRISPR components, HDR can result in replacement of the target genomic DNA sequence with the donor sequence, generating precise changes such as point mutations or knockin [1-51-68-86-90-129]. . The donor DNA template can be a single-stranded oligo nucleotide (ssODN) or a dsDNA fragment (usually linearized DNA derived from either regular plasmid or AAV) [1-51-68-86-90-129]. ssODNs are suitable for introducing point mutations or small tag insertions, while dsDNA fragments are widely used to introduce large fragment knockins like targeted stable integration of transgenes. Another widely used Cas9 variant, Cas9 "nickase" (e.g. Cas9(D10A)), generates single-stranded cuts in DNA [1-51-68-86-90-129]. If Cas9 nickase is used in conjunction with two gRNAs targeting the two opposite strands flanking a single target region, the nickase will generate two single-stranded cuts, one on each strand, resulting in wide-spread DSBs around the target region. Because single nicks are generally repaired with high fidelity, efficient cleavage occurs only when both offset gRNAs bind correctly, creating a staggered DSB. This double-nicking strategy, therefore, dramatically reduces off-target activity while maintaining high on-target efficiency at many loci [1-51-68-86-90-129].

Cas9 endonuclease binding to the target genomic locus is mediated both by the target sequence contained within the guide RNA and a 3-base pair sequence known as the Protospacer Adjacent Motif or PAM [1-51-68-86-90-129]. In order for dsDNA to be cut by Cas9, it must contain a PAM sequence immediately downstream (3') of the site targeted by the guide RNA. In the absence of either the guide RNA or a PAM sequence, Cas9 will neither bind nor cut the target. Cas9 homologs from different organisms or Cas9 mutants developed in a variety of labs have different PAM requirements [1-51-68-86-90-129]. These different PAM requirements allow researchers to target many different genomic loci. Cas9 and its variants have two endonuclease domains: the n-terminal RuvC-like nuclease domain and the HNH-like nuclease domain near the center of the protein. Upon target binding, Cas9 undergoes a conformational change that positions the nuclease domains to cleave opposite strands of the target DNA. Thus, the end result of Cas9-mediated DNA damage is a DSB within the target DNA ~3-4 nucleotides upstream of the PAM sequence [1-51-68-86-90-129].

In the native Type II CRISPR/Cas system, Cas9 is guided to its target sites with the aid of two RNAs: the **crRNA** which defines the genomic target for Cas9, and the **tracrRNA** which acts as a scaffold linking the crRNA to Cas9 and facilitates processing of mature crRNAs from pre-crRNAs derived from CRISPR arrays [1-51-68-86-90-129]. In most systems used for CRISPR-mediated genome editing, these two small RNAs have been condensed into one RNA sequence known as the guide RNA (gRNA) or single guide RNA (sgRNA) [1-51-68-86-90-129]. The gRNA contains both the 20 nucleotide target sequence to direct Cas9 to a specific genomic locus and the scaffolding sequence necessary for Cas9 binding. When using CRISPR/Cas9 for genome editing, researchers simply need to express a gRNA designed to direct Cas9 to their target sequence of choice and their prefered Cas9 variant (with the appropriate PAM sequence) to modify the desired genomic locus [1-51-68-86-90-129].

7.1. Designing guide RNA for Gene Knockouts

Perhaps the most widely adopted use of CRISPR is to knock out (KO) specific genes in the genome of an organism to test their function. A successful genetic knockout generally results in a loss of protein function and often (but not always) manifests as a change in phenotype. The most common way to generate CRISPR-mediates knockouts is through the non-homologous end joining (NHEJ) repair pathway [1-51-68-86-90-129]. In this mechanism, the Cas nuclease is directed to the correct genomic locus by the guide RNA and creates a double-strand break (DSB). The DSB is fixed by NHEJ, which is a "quick fix" repair pathway. This pathway often results in insertion or deletion (indel) of one or more nucleotides at the point of cleavage, which leads to frame-shift mutations that completely knock out gene function [1-51-68-86-90-129]. The entire basis of a CRISPR gene knockout experiment relies on indels being introduced by NHEJ during repair. However, an indel does not guarantee the disruption of protein function, especially if the cut site is not carefully planned. When designing the guide RNA for a knockout experiment, it is important to select target sites in exons that are crucial for

protein function. For this reason, targeting regions encoding amino acids too close to the N- or C-terminus of the protein should be avoided [1-51-68-86-90-129]. In the case of the former, the cell could possibly find another start codon (ATG) downstream, while in the latter case, the target sites could code for non-essential part of the protein [1-51-68-86-90-129]. The above parameters for a knockout experiment are important, but they still offer a wide range of genomic locations for designing the gRNA. Therefore, for these experiments, researchers can select the gRNA with highest sequence complementarity within the specified location range [1-51-68-86-90-129].

7.2. Designing Guide RNA for CRISPR Knock-ins

A knock-in (KI) experiment aims to edit the target genome by inserting a new DNA fragment into the genome. In this case, researchers aim to divert the cell from using its "quick and dirty" NHEJ repair pathway to another more sophisticated repair pathway, referred to as the homology-directed repair (HDR) pathway [1-51-68-86-90-129]. In HDR, the cell copies the sequence of a donor DNA template to fill in the broken piece accurately. In knock-in experiments, researchers supply a surplus of template DNA bearing their desired genomic change, improving their chances that the cell will pick this DNA to use for the repair. As KI experiments require additional elements such as external DNA for HDR, there is less freedom in the target site location for designing a guide RNA [1-51-68-86-90-129]. Studies have shown a dramatic drop in efficiency of knock-in experiment when the cut site was not close to ends of the repair template [1-51-68-86-90-129]. This restricts the potential gRNAs that can be designed around this limited location. These locational constraints are even more stringent for the Cas9 nucleases that edit bases without cutting the DNA. Thus, for gene editing, location, rather than sequence complementarity, is the limiting design parameter [1-51-68-86-90-129].

7.3. Designing Guide RNAs for CRISPRa & CRISPRi

The critical parameters for gRNA design are also different for CRISPRa (gene expression) and CRISPRi (gene silenced) experiments. These experiments involve gene activation or inhibition by targeting the promoter of the target gene [1-51-68-86-90-129]. The location target DNA range for guide RNA is therefore quite narrow, necessitating a balance of complementary sequence and optimized location while designing [1-51-68-86-90-129]. Guide RNA design is crucial for CRISPR experiments because it can directly determine the specificity and efficiency of the editing action, which is essential for accurate genome editing [1-51-68-86-90-129]. To design our own guide RNA, select target sequences using your favorite CRISPR design platform, such as CHOPCHOP, Benchling, CRISPOR, or Cas-Designer CRISPR RGEN Tools. To order guide RNA, upload target sequences without the PAM sequence into easy-to-use ordering interface of biotechnology Inc (Synthego CRISPR Design Tool), so that company can synthesize guide RNA. For experiment result analysis via sanger sequencing, we recommend the ICE tool hosted by Editco [1-51-68-86-90-129].

7.4. Synthego CRISPR Design Tool

This is the world's most efficient CRISPR design tool for gene knockouts. The Synthego CRISPR Design Tool enables guide RNA design for over 120,000 genomes and 9,000 species, while also reducing guide design time from hours down to minutes. The tool automatically recommends the guide(s) that have the highest chances of gene knockout and the lowest off-target effects [1-51-68-86-90-129]. The tool also offers the ability to validate guide designs created by other methods, including the tools listed above, and has an integrated process for easily ordering the top scoring synthetic sgRNAs [1-51-68-86-90-129]. Within 5 minutes, one can easily design a CRISPR guide for gene knockout. Whether a new CRISPR user or an advanced researcher, the Synthego CRISPR Design Tool generates great knockout designs for all.

7.5. Ensure On-Target Activity of Guide RNA

Logically, it seems obvious that an optimum guide RNA should be specific for only the target DNA sequence and not anywhere else in the genome (i.e. sequence complementarity and target location proximity are important parameters driving the design criteria) [1-51-68-86-90-129]. But from the above sections, it is clear that the decision regarding whether to compromise on complementarity or location will be determined by the goals of each experiment [1-51-68-86-90-129]. In 2016, Doench et al. analyzed the specificity of thousands of guide RNAs while creating genome-wide mice and human libraries. Using sophisticated computational biology tools, the team established scoring rules to predict the on-target activity of gRNAs, and the "Doench rules" are now implemented in several online gRNA design tools.

7.6. Selecting the appropriate Cas protein

The most commonly used protein in genome editing experiments is *sp*Cas9 found in *S. pyogenes*. *sp*Cas9 facilitates genome editing by generating DSBs [1-51-68-86-90-129]. This cleavage is ependent on *sp*Cas9 binding to the 5′-NGG-3′ PAM sequence that is located downstream of the cleavage site [1-51-68-86-90-129]. Depending on the Cas protein used for genome editing and the organism it is isolated from, the PAM will vary. Despite its popularity, *sp*Cas9 has some limitations. It has the ability to recognize other PAMs such as 5′-NAG-3′ and 5′-NGA-3′. *sp*Cas9 can also bind and cleave

sequences in the genome that have some similarity to the crRNA. These low-fidelity aspects of *sp*Cas9 result in off-target effects. *sp*Cas9 is also a rather large protein and delivery into cells can be a challenge. For this reason, scientists have discovered and engineered alternatives to *sp*Cas9 that can also be used in genome editing [1-51-68-86-90-129]. Some examples of other commonly used Cas nucleases are Cas12a, Cas13a, *lb*Cas9, *as*Cas9 and CasX. Other high-fidelity engineered variants including HypaCas9, eSpCas9 and Hi-FiCas9 offer many advantages over the traditional Cas9. These variants have a weakened DNA phosphate backbone interaction with the nuclease. This results in genome-wide specificity and undetectable off-target effects. However, each specific variant has certain improved characteristics which can benefit the process, depending on the type of experiment being performed [1-51-68-86-90-129-171].

7.7. Guide RNA design

In a genome editing experiment with *sp*Cas9, a guide RNA can be designed in two ways: two-component crRNA plus a tracrRNA system or as a single guide RNA (sgRNA). In bacteria, a tracrRNA includes a segment at its 5′ end which is complementary to the repeat-derived segment of the pre-crRNA, allowing the two to hybridize [1-51-68-86-90-129]. The guide region of the crRNA is typically 20 nucleotides long and is synthesized along with an optimized tracrRNA length and joined by a linker loop to form one continuous molecule. sgRNAs are preferred as the separate crRNA and tracrRNA molecules may not anneal efficiently. Only the guide region in the sgRNA determines the region to be targeted in the genome and this flexibility is what makes CRISPR-based genome editing useful and easy [1-51-68-86-90-129]. The DNA sequence encoding the guide RNA does not contain a PAM and this prevents it from being cut by the Cas proteins, though some applications of PAMs in homing guide RNAs have been developed. While designing guide RNAs, it is recommended to design three to four different guides to increase the chances of at least one of them binding efficiently and cleaving the target [1-51-68-86-90-129-171]. While designing guide RNAs for a knock-in, the cut site should be as close to the knock-in site as possible. The recommended distance is less than 10 nucleotides between the two. However, if the knock-in is on just one allele, the cut site to knock-in site distance can be increased up to 20 nucleotides to increase the frequency of monoallelic edits to biallelic edits [1-51-68-86-90-129]. It is important to keep in mind that guide designs must be optimized to reduce off-target binding and cleavage [1-51-68-86-90-129-171]. There are many tools available for guide design that predict the on-target activity based on certain established scoring rules. Benchling, IDT (Integrated DNA Technologies), Broad Institute GPP, CasOFFinder, CRISPOR and Synthego have some useful resources that can help with this process.

8. Delivery of the Cas protein and guide RNA into the cells

Once the design is ready, the Cas reagents can be delivered into cells in these forms [14]. 1) **Naked DNA**: The guide RNA sequences along with the Cas protein can be cloned into a plasmid which can then be delivered into cells and be selected. Sometimes they are expressed on different plasmids but it is easier when they are on the same plasmid and controlled by two different promoters [14]. U6 is the most common promoter used for guide RNA expression and for *sp*Cas9, a stronger promoter such as T7, CMV or a cell-specific promoter is used [1-51-68-86-90-129]. Delivery in the form of an mRNA is also another option [14]. It is important to optimize the amount of DNA template based on each experiment and it can be delivered in these forms: a) Plasmid/double stranded DNA: These are suitable if the insert is long (>50 bp), especially if the goal is to replace an entire gene or insert a reporter gene [14]. The template is in the form of a plasmid or double stranded fragments can be generated using PCR [1-51-68-86-90-129-171]. The homology arms should be 50–800 bp in size to ensure efficient homology-directed repair [14]. The disadvantage is that sometimes the plasmid DNA can integrate itself into off-target sites [14]. Make sure to include a nuclear localization sequence for transcription within the cell [14]. Usually, 400 ng of the plasmid donor (~5 kb) per 2.5×10^6 cells is sufficient for transfection. b) ssDNA: This method is useful when the knock-in fragment is less than 50 bp [14]. The homology arms are recommended to be 50–80 bp long (50 bp long for single nucleotide changes). It is recommended to start with at least 60 pmols of ssDNA donor template per 1×10^6 to 2.5×10^6 host cells for high-efficiency knock-ins. These should be end-modified to be protected from cellular nucleases [1-51-68-86-90-129-171].

2**) Viral transduction methods**: Different types of viral vectors are used to transduce DNA/RNA into the host cells [14]. To do so, plasmids containing the viral genes and the CRISPR/Cas components are introduced into a packaging cell line (e.g., 293 T cells) from which the viral particles are harvested. These are later introduced to a host cell line. Examples of such vectors are [14].

a) Adeno-associated viruses: These non-pathogenic single stranded viruses can be modified by removing all their viral genes and inserting the sequence of interest containing inverted terminal repeats [14]. They have a natural ability to induce homologous recombination; however, the drawbacks are that the size of the insert is limited to 4.5 kb and this method is generally more expensive [14]. The advantage, however, is that since AAVs do not trigger an immune response and do not integrate within the genome, allowing less chances for insertional mutagenesis [14].

b) Lentiviruses: These belong to the family of retroviruses and integrate into the genome of the host cells by penetrating through the nuclear envelope [14]. This makes them highly effective in non-dividing cells but also increases their chances of disrupting essential genes [14].

3) **Riboprotein complex**: The Cas protein-guide RNA complex can be delivered into cells via various methods such as lipofection, electroporation, nucleofection and microinjection [14]. For a preformed RNP complex, the transfection can be done directly into the nucleus or via microinjection. The type of transfection method used will depend on whether there is a need for permanent expression of the CRISPR-Cas components or if a transient expression would suffice [14].

9. Data analysis with CRISPR

Once the genome is edited, it needs to be validated. Regardless of the design of the experiment or the method of repair, the first step is always to PCR-amplify the edited region [14]. This amplified region can then be subject to Sanger sequencing or high-throughput sequencing [14]. After the sequencing results arrive, remember to compare it to a control sequence to determine the presence of off-target mutations, if any, and to account for any other indels in the target region [14]. Most programs used for CRISPR data analysis provide scores that can be used as an indication of a successful genome editing experiment [14].

10. Genome editing: CRISPR/Cas Technology: Challenges

There are several challenges to the widespread CRISPR-based agriculture revolution which include varying legislation and regulatory frameworks for gene-edited crops, delivery of CRISPR/Cas payloads, and off-target activity in CRISPR/Cas systems [1-14-129]. Another limitation of CRISPR technology is off-target changes in the host genome, which is needed to be rectified through alteration in the current CRISPR/Cas methodology to minimize off-target binding for optimizing sgRNA, Cas proteins, and delivery methods [1-51-68-86-90-129]. Inclusion of highly specific sgRNAs may lower off-target rates. Other strategies, including the extension of the sgRNA sequence and 3′-terminal cleavage of the sgRNA, may reduce the off-targeting effect [1-51-68-86-90-129]. Despite the persisting challenges, several essential crops have been altered using CRISPR/Cas for nutrition, quality, and productivity enhancement [1-51-68-86-90-129]. Nonetheless, additional study is needed to investigate more crop diversity in terms of nutrition, quality, and productivity enrichment to identify effective biofortification targets and optimize CRISPR delivery methods [1-51-68-86-90-129]. The CRISPR-based agricultural genome editing is the future of crop fortification as it can design genes for increased vitamin synthesis, crop quality features, and crop production qualities. CRISPR-based genome editing has a great potential to achieve the 2030 goal of eradicating hunger, s a great potential to achieve the 2030 goal of eradicating hunger, food insecurity, and all forms of human malnutrition [1-51-68-86-90-129-171].

Although CRISPR/Cas technology holds an incredible potential for developing new crops with improved traits, however, several challenges persist, such as efficient delivery of CRISPR reagents, consumer acceptance, intellectual property rights, trait stacking and combinatorial editing, and different jurisdictions of CRISPR edited plants, to fully realize the potential of this revolutionary technology [1-51-68-86-90-129-171]. Moreover, the difficulty in detection and traceability of CRISPR edited SDN1 and SDN2 crops is an important consideration in regulation, labeling, and commercialization of these crops [1-51-68-86-90-129-171]. With the rapid rise in CRISPR technology, the old paradigms and regulatory frameworks of conventional GMOs should be re-evaluated to accommodate new developments such as transgene free CRISPR edited crops with precise and point mutations [1-51-68-86-90-129-171]. Thus, it is important to enhance international coordination among all stakeholders including scientists, policy makers, regulatory authorities, politicians, farmers, industry representative, and public to revisit the regulatory framework [1-51-68-86-90-129-171]. All stakeholders must be engaged for globally harmonized definitions and regulatory policies for precise genetic modifications and increased public awareness to address the unique challenges about regulation of gene edited crops [1-51-68-86-90-129-171]. Development of a universal, transparent, scalable, and mutually agreed-upon regulation for gene edited plants, holds a tremendous potential to address the global challenges related to food security, sustainable agriculture, and the growing world population [1-41-69-80-90-129-171]. At the same time, ethical responsibilities like self-determination of exemption of CRISPR modified crops by farmers and agricultural companies should be controlled by strict monitoring. The long-term effect of GE crops should not be ignored for a healthier and sustainable environment [1-51-68-86-90-129-171].

11. Conclusion

Global food security is escalating by population growth, climate change and depletion of basic resources, and explicitly demands the implementation of cutting-edge approaches to improve crop yield, resilience, and nutritional quality.

CRISPR/Cas9 technology has transformed modern agriculture by introducing accurate and inherently stable modifications in different plants. The advent of CRISPR-based genome editing has revolutionized crop improvement, offering unprecedented precision and efficiency in modifying key agronomic traits. CRISPR/Cas9 enables highly precise, rapid plant breeding to address global food security. State-of-the-art applications for crop improvement focus on AI-driven guide RNA design, base/prime editing for precise nucleotide substitutions, and epigenome editing for climate resilience. These breakthroughs accelerate the development of crops with higher yields, disease resistance, and enhanced nutritional profiles. CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/Cas associate protein 9), a gene editing tool to reshape the genomic landscape of not only bacteria, but also animals and plants to achieve goals in food security, therapeutics, and human health. CRISPR/Cas9, the "genetic scissors", is being presaged as a revolutionary technology, having tremendous potential to create designer crops by introducing precise and targeted modifications in the genome to achieve global food security in the face of climate change and increasing population. Therefore, this technique grabbed the attention of scientists and private companies to engineer agricultural crops with climate resilience, disease resistance, and better nutritional profile. Similarly, CRISPR/Cas technology has been adopted universally for translational applications in human health, therapeutics, and product development. Traditional genetic engineering relies on random and unpredictable insertion of isolated genes or foreign DNA elements into the plant genome. However, CRISPR/Cas based gene editing does not necessarily involve inserting a foreign DNA element into the plant genome from different species but introducing new traits by precisely altering the existing genes. These advancements include the use of base and prime editing to accurately alter metabolic pathways for nutritional enhancements, along with designing Cas variants with limited dependency on PAM, to facilitate editing in complex genome crops like wheat.

Moreover, the integration of artificial intelligence-driven target prediction and speed breeding has significantly improved varietal development by shortening breeding period and increasing resilience to various biotic and abiotic stresses. Case studies in cereal (Rice, wheat, maize, and sorghum) and horticultural crops provide evidence of CRISPR's major contribution towards limiting food security, improving nutritional value, and mitigating postharvest waste. CRISPR edited crops are touching markets, however, the world community is divided over whether these crops should be considered genetically modified (GM) or non-GM. Classification of CRISPR edited crops, especially transgene free crops as traditional GM crops, will significantly affect their future and public acceptance in some regions. Therefore, the future of the CRISPR edited crops is depending upon their regulation as GM or non-GMs, and their public perception.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

References

[1] Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR/Cas nucleases, base editors, transposases and prime editors. Nat Biotechnol. 2020; 38:824–844.

[2] Gostimskaya I. CRISPR/Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing. Biochemistry (Mosc). 2022;87(8):777-788.

[3] Ishino Y, Shinagawa H, Makino K, Amemura M, Nakatura, A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isoenzyme conversion in Escherichia coli, and identification of the gene product, J. Bacteriol. 1987; 169, 5429-5433, doi: 10.1128/ jb.169.12.5429-5433.1987

[4] Kumar D, Yadav A, Ahmad R, Dwivedi UN and Yadav K. CRISPR-Based Genome Editing for Nutrient Enrichment in Crops: A Promising Approach Toward Global Food Security. Front. Genet. 2022; 13:932859. doi: 10.3389/fgene.2022.932859.

[5] Sharma N, Thakur K, Zinta R, Mangal V, Dalamu, Tiwari JK, Sood S, Dutt S, Kumar V, Singh B, Thakur AK. Genome editing research initiatives and regulatory landscape of genome edited crops in India. Transgenic Res. 2025; 34:13.

[6] Bhattacharya A, Parkhi V, Char B. Genome editing for crop improvement: A perspective from India. In Vitro Cell Dev Biol Plant. 2021;57(4):565-573.

[7] Ahmad A, Jamil A, Munawar N. GMOs or non-GMOs? The CRISPR Conundrum. Front Plant Sci. 2023;14:1232938.

[8] Doench JG. Am I ready for CRISPR? A user's guide to genetic screens. Nat Rev Genet. 2017; 19:67–80.

[9] Hanna RE, Doench JG Design and analysis of CRISPR–Cas experiments. Nat Biotechnol. 2020; 38:813–823.

[10] Radzisheuskaya A, Shlyueva D, Müller I, Helin K. Optimizing sgRNA position markedly improves the efficiency of CRISPR/dCas9-mediated transcriptional repression. Nucleic Acids Res. 2016; 44:e141–e141.

[11] Genome Editing: The Next Big Revolution in Indian Agriculture - TCI (cornell.edu)

[12] How to Design CRISPR Guide RNAs For Highest Editing Efficiencies (synthego.com).

[13] CRISPR Design Tools - Synthego.

[14] Shravanti S. Beginner's guide to CRISPR-Cas9-based gene editing. PhD candidate in the Department of Biochemistry, Biophysics and Molecular Biology at Iowa State University. Biochem (Lond). 2021; 43 (4): 36–40.

[15] Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime editors. Nat. Biotechnol. 2020;**38**: 824–844.

[16] CRISPR/Cas9 guide RNA design checker | IDT (idtdna.com).

[17] Mohr SE, Hu Y, Ewen-Campen B, Housden BE, Viswanatha R, Perrimon N. CRISPR guide RNA design for research applications. FEBS J. 2016;283(17):3232-8.

[18] Abdallah NA, Prakash CS, McHughen AG. Genome editing for crop improvement: challenges and opportunities. GM Cr Food. 2015; 6: 183–205.

[19] Acharya S, Mishra A, Paul D, Hussain A, Azhar M. Kumar M, Rauthan R, Sharma N, Aich M, Sinha D, Sharma S, Jain S, Ray A, Jain S, Ramalingam S, Maiti S, Chakraborty D. Francisella novicida Cas9 interrogates genomic DNA with very high specificity and can be used for mammalian genome editing. ProcNatl Acad Sci. 2019; 116: 20959–20968

[20] Barman A, Deb B, Chakraborty. A glance at genome editing with CRISPR/Cas9 technology. Curr Genet. 2020; 66:447–462

[21] Barrangou R, Marraffini LA. CRISPR-Cas systems: prokaryotes upgrade to adaptive immunity. Mol Cell. 2014; 54:234–244

[22] Bharat SS, Li S, Li J, Yan L, Xia L. Base editing in plants: current status and challenges. Crop J. 2019; 8:384–39.

[23] Bhattacharya A, Parkhi V, Char B (eds). CRISPR/Cas genome editing: strategies and potential for crop improvement. Springer. 2020; https://doi.org/10.1007/978-3-030-42022-2

[24] Bisht DS, Bhatia V, Bhattacharya R. Improving plant-resistance to insect-pests and pathogens: The new opportunities through targeted genome editing. Semin Cell Dev Biol. 2019; 96:65–76.

[25] Chib S, Thangaraj A, Kaul S, Dhar MK, Kaul T. Development of a system for efficient callus production, somatic embryogenesis and gene editing using CRISPR/Cas9 in Saffron (*Crocus sativus* L.). Pl Meth. 2020;16:47

[26] Das A, Sharma N, Prasad M. CRISPR/Cas9: a novel weapon in the arsenal to combat plant diseases. Front Pl Sci. 2019; 9:2008

[27] Debbarma J, Sarki YN, Saikia B, Boruah HPD, Singha DL, Chikkaputtaiah C. Ethylene response factor (ERF) family proteins in abiotic stresses and CRISPR/Cas9 genome editing of ERFs for multiple abiotic stress tolerance in crop plants. Mol Biotechnol. 2019; 61:153–172

[28] Ding Y, Li H, Chen L-L, Xie K. Recent advances in genome editing using CRISPR/Cas9. Front Pl Sci. 2016; 7:703

[29] Farhat S, Jain N, Singh N, Sreevathsa R, Dash PK, Raj R, Yadav S, Ananda PK, Sarkar AK, Jain A, Singh NK, Rai V. CRISPR-Cas9 directed genome engineering for enhancing salt stress tolerance in rice. Semin Cell Dev Biol. 2019; 96:91–99.

[30] Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/ Cas-based methods for genome engineering. Trends Biotechnol. 2013; 31: 397–405.

[31] Gaj T, Sirk SJ, Shui S-I, Liu J. Genome-editing technologies: principles and applications. Cold Spring Harb Perspect Biol. 2016; 8: a023754.

[32] Gao. The future of CRISPR technologies in agriculture. Nat Rev Mol Cell Biol. 2018;19:275–276.

[33] Gatica-Arias A, Valdez-Melara M, Arrieta-Espinoza G, Albertazzi- Castro FJ, Madrigal-Pana J. Consumer attitudes towards food crops developed by CRISPR/Cas9 in Costa Rica. Plant Cell Tissue Organ Cult. 2019; 139:417–427.

[34] Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern- Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. CRISPR-mediated modular RNA- guided regulation of transcription in eukaryotes. Cell. 2013; 154:442–451.

[35] Jyoti A, Kaushik S, Srivastava VK, Datta M, Kumar S, Yugandhar P, Kothari SL, Rai V, Jain A. The potential application of genome editing by using CRISPR/Cas9, and its engineered and ortholog variants for studying the transcription factors involved in the maintenance of phosphate homeostasis in model plants. Sem Cell Develop Biol. 2019; 96:77–90.

[36] Kato-Nitta N, Maeda T, Inagaki Y, Tachikawa M (2019) Expert and public perceptions of gene-edited crops: attitude changes in relation to scientific knowledge. Palgrave Comm. 2019; 5:137.

[37] Kaur N, Shivani AA, Kumar P, Kaur N, Awasthi P, Chaturvedi S, Pandey P, Pandey A, Pandey AK, Tiwari S. CRISPR/Cas9 directed editing of lycopene epsilon-cyclase modulates metabolic flux for β-carotene biosynthesis in banana fruit. Metabolic Eng. 2020; 59:76–86

[38] Kelliher T, Starr D, Su X, Tang G, Chen Z, Carter J, Wittich PE, Dong S, Green J, Burch E, McCuiston J, Gu W, Sun Y, Strebe T, Roberts J, Bate NJ, Que Q. One-step genome editing of elite crop germplasm during haploid induction. Nat Biotechnol. 2019; 37:287–292.

[39] Kumar R, Kaur A, Pandey A, Mamrutha HM, Singh GP. CRISPR- based genome editing in wheat: a comprehensive review and future prospects. Mol Biol Rep. 2019; 46:3557–3569.

[40] Kumar VVS, Verma RK, Yadav SK, Yadav P, Watts A, Rao MV, Chinnusamy V. CRISPR-Cas9 mediated genome editing of drought and salt tolerance (OsDST) gene in indica mega rice cultivar MTU1010. Physiol Mol Biol Plants. 2020; 26:1099–1110.

[41] Lander ES. The heroes of CRISPR. 2016; Cell 164:18–28.

[42] Lassoued R, Macall DM, Hesseln H, Phillips PWB, Smyth SJ. Benefits of genome-edited crops: expert opinion. Transgenic Res. 2019; 28:247–256.

[43] Li C, Zong Y, Wang Y, Jin S, Zhang D, Song Q, Zhang R, Gao C. Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. Genome Biol. 2018; 19:59.

[44] Limera C, Sabbadini S, Sweet JB, Mezzetti B. New biotechnological tools for the genetic improvement of major woody fruit species. Front Pl Sci. 2017; 8:1418

[45] Liu Y, Zou RS, He S, Nihongaki Y, Li X, Razari S, Wu B, Ha T. Very fast CRISPR on demand. Science. 2020; 368:1265–1269.

[46] Lowe K, Wu E, Wang N, Hoerster et al., Morphogenic regulators Baby Boom and Wuschel improve monocot transformation. Plant Cell. 2016; 28:1998–2015.

[47] Maher MF, Nasti RA, Vollbrecht M, Starker CG, Clark MD, Voytas DF. Plant gene editing through de novo induction of meristems. Nat Biotechnol. 2019; 38:84–89.

[48] Mishra R, Joshi RK, Zhao K. Base editing in crops: current advances, limitations and future implications. Plant Biotechnol J. 2019; 18:20–31.

[49] Monsur MB, Shao M, Ahmad S, Wei X, Hu P, Tang S. Base editing: the ever expanding clustered regularly interspaced short palindromic repeats (CRISPR) tool kit for precise genome editing in plants. Genes. 2020; 11:466.

[50] Moon SB, Kim DY, Ko J-H, Kim Y-S. Recent advances in the CRISPR genome editing tool set. Exp Mol Med. 2019; 51:130.

[51] Mout R, Ray M, Lee Y-W, Scaletti F, Rotello VM. In vivo delivery of CRISPR/Cas9 for therapeutic gene editing: progress and chal lenges. Bioconjug Chem. 2017; 28:880–884.

[52] Nagaraj H, Kumar KK, Sudhakar D, Amudha K, Meenakshi SP, Kokiladevi E. Genome engineering for thermo-sensitive genic male sterilty (TGMS) in rice using CRISPR/Cas9 editing system. Madras Agril J Special issue. 2019; 106:150–154.

[53] Wei T, Cheng Q, Min YL, Oslon EN, Siegwart DJ. Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. Nat Commun. 2020; 11:3232.

[54] Whelan AI, Gutti P, Lema M. Gene editing regulation and innovation economics. Front Bioengg Biotech. 2020; 8 https://doi.org/10. 3389/fbioe.2020.00303.

[55] Wu X, Kriz A, Shart PA. Target specificity of the CRISPR-Cas9 system. Quant Biol. 2014; 2:59–70.

[56] Xie K, Minkenberg B, Yang Y. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci U S A. 2015; 112:3570–3575.

[57] Xu J, Hua K, Lang Z. Genome editing for horticultural crop improvement. Hort Res. 2019; 6:113.

[58] Rajyaguru RH, Tomar RS. Use of CRISPR-CAS9 system in groundnut (Arachis hypogaea) transformation targeting ahFAD2 gene. J Oilseed Res. 2020; 37:45.

[59] Parisi C, Tillie P, Rodriguez-Cerezo E. The global pipeline of GM crops out to 2020. Nat Biotechnol. 2016; 34:31–36.

[60] Parkhi V, Bhattacharya A, Choudhary S, Pathak R, Gawade V, Palan B, Alamalakala L, Mikkilineni V, Char B. Demonstration of CRISPR-cas9-mediated pds gene editing in a tomato hybrid parental line. Ind J Genet Plant Breed. 2018; 78:132–13

[61] Rees HA, Liu DR. Base editing: precision chemistry on the genome and transcriptome of living cells. Nat Rev Genet. 2018; 19:770–788.

[62] Saikia B, Singh S, Debbarma J, Velmurugan N, Dekaboruah H, Arunkumar KP, Chikkaputtaia C.Multigene CRISPR/Cas9 genome editing of hybrid proline rich proteins (HyPRPs) for sustainable multi-stress tolerance in crops: The review of a promising approach. Phys Mol Biology. 2020; 26:857–869.

[63] Schmidt SM, Belisle M, Frommer WF. The evolving landscape around genome editing in agriculture. EMBO Rep. 2020; 21:e50680.

[64] Shrestha A, Khan A, Dey N. CRISPR-Cas9-mediated editing of the CYP82E4-nicotine N-demethylase (nnd) gene in tobacco protoplasts. J Pl Biochem Physio. 2019; 7:230.

[65] Standage-Beier K, Tekel SJ, Brookhouser N, Schwarz NT, Wang X, Brafman DA. A transient reporter for editing enrichment (TREE) in human cells. Nucleic Acids Res. 2019; 47:e120.

[66] Thakare SS, Bansal N, Vanchinathan S, Prashat GR, Krishnan V, Sachdev A, Praveen S, Vinutha T. GFP tagging based method to analyze the genome editing efficiency of CRISPR/Cas9-gRNAs through transient expression in N. Benthamiana. J Plant Biochem Biotechnol. 2020; 29:183–192.

[67] Voss-Fels KP, Stahl A, Hickey LT. Q&A: modern crop breeding for future food security. BMC Biol. 2019; 17:18

[68] Wada N, Ueta R, Osakabe Y, Osakabe K. Precision genome editing in plants: state-of-the-art in CRISPR/Cas9-based genome engineering. BMC Plant Biol. 2020; 20:234.

[69] Zimny T, Sowa S, Tyczewska A, Twardowski T. Certain new plant breeding techniques and their marketability in the context of EU GMO legislation – recent developments. New Biotechnol. 2019; 51: 49–56.

[70] Haider S, Singh AP, Panthi B, Sindhu SR, Safa NT, Malik S, Rahimi M. Advances in CRISPR/Cas9 genome editing for crop improvement and global food security. Current Plant Biology. 2026; 46: 100593.

[71] Deres D, Terefe M. Beyond CRISPR/Cas9: Emerging genome editing technologies for next-generation crop improvement. Mol Biol Rep. 2026; 29;53(1):695. doi: 10.1007/s11033-026-11879-8.

[72] Mansi M, Danai P. The emerging impact of CRISPR and gene editing on global crop improvement. Transgenic Res. 2026; 14;35(1):8. doi: 10.1007/s11248-026-00484-x.

[73] **Yıldırım K, Kavas M**. The role of CRISPR in modern plant breeding: overcoming breeding barriers and legislative challenges through transgene-free genome editing. Genome. **2026;** 69: 1–17. dx.doi.org/10.1139/gen-2025-0063.

[74] Rasheed A, Gill RA, Hassan MU, et al. A critical review: recent advancements in the use of CRISPR/Cas9 technology to enhance crops and alleviate global food crises. Curr Issues Mol Biol. 2021;43(3):1950– 1976.

[75] Bekalu ZE, Panting M, Holme IB, et al. Opportunities and challenges of in vitro tissue culture systems in the era of crop genome editing. Int J Mol Sci. 2023;24(15):11920.

[76] Koti P, Bill T. Plant tissue culture and genetic transformation in crop improvement. J. Bacteriol Mycol Open Access. 2025;13(1):61–69.

[77] Tang X, Lowder LG. et al., A CRISPR–Cpf1 system for efficient genome editing and transcriptional repression in plants. Nat. Plants. 2017; 3: 17018.

[78] Tariq A. et al., 2023. Putting CRISPR-Cas system in action: a golden window for efficient and precise genome editing for crop improvement. GM Crops & Food. 2023; 14(1): 1–27.

[79] Tran M. et al., CRISPR/Cas9-based precise excision of SlHyPRP1 domain(s) to obtain salt stress-tolerant tomato. Plant Cell Rep. 2021; 40(6): 999–1011.

[80] Svitashev S. et al., 2016. Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat. Commun. 2016; 7: 13274.

[81] Zhong Y, Liu C, Ma L, Zhang L, Xu C, Li H, Chen Z. CRISPR/dCas-mediated gene activation toolkit development and its application for parthenogenesis induction in maize. Plant Biotechnol. J. 2024; 22(1): 162–174.

[82] Kumar N, Singh A, Priyansha Sarkar PR, Sharma B, Kumar S, Aggarwal N, Arya A. India biotech revolution: the approval of genome-edited rice. The Bioscan. 2025; 20(3): 893–897.

[83] Kumar R, Das SP. et al., Advances in genomic tools for plant breeding: harness- ing DNA molecular markers, genomic selection, and genome editing. Biol. Res. 2024;57(1): 80. doi:10.1186/s40659-024-00562-6.

[84] Karlsson M. et al., CRISPR/Cas9 genome editing of potato StDMR6-1 results in plants less affected by different stress conditions. Hortic. Res. 2024; 11(7): uhae130. doi:10.1093/hr/uhae130.

[85] Kaul T, Thangaraj A. et al., CRISPR/Cas9-mediated homology donor repair base editing system to confer herbicide resistance in maize (Zea mays L.). Plant Physiol. Biochem. 2024; 207: 108374. doi:10.1016/j.plaphy.2024.108374.

[86] Kravchik M, Shnaider Y. et al., Knockout of SlTOM1 and SlTOM3 results in differential resistance to tobamovirus in tomato. Mol. Plant Pathol. 2022; 23(9): 1278– 1289.

[87] Liang Z. et al., Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat. Commun. 2017; 8: 14261. doi:10.1038/ ncomms14261.

[88] Gil-Humanes J. et al.,High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR /Cas9. Plant J. 2017; 89: 1251–1262.

[89] Miladinovic D, Antunes D, Yildirim K, Bakhsh A, Jeromela AM, Hilioti Z. et al., Targeted plant improvement through genome edit- ing: from laboratory to field. Plant Cell Rep. 2021; 40: 935–951.

[90] Okada A, Arndell T. et al., CRISPR/Cas9-mediated knockout of Ms1 enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. Plant Biotechnol. J. 2019; 17: 1905–1913.

[91] Ray DK, Mueller ND, West PC, Foley JA. Yield trends are insufficient to double global crop production by 2050. PLoS One. 2013; 8(6): e66428. doi:10.1371/journal.pone.0066428.

[92] Yıldırım K. et al., Genome editing for healthy crops: traits, tools and impacts. Front. Plant Sci. 2023; 14: 1231013.

[93] Yu D, Zhou T, Xu N, Sun X, Song S, Liu H. et al., Novel CRISPR /Cas9 system assisted by fluorescence marker and pollen killer for high-efficiency isolation of transgene-free edited plants in rice. Plant Biotechnol. J. 2024; 22: 1649–1651.

[94] Yu Q. et al., CRISPR/Cas9-induced targeted mutagenesis and gene replacement to generate long-shelf life tomato lines. Sci. Rep. 2017; 7.doi:10.1038/ s41598-017-12262-1

[95] Ishii T. Consumer choices regarding genome-edited food crops: lessons from Japan. Front Genome Ed. 2025; 7: 1672358. doi:10.3389/fgeed. 2025.1672358.

[96] Jinek M, et al., Structures of Cas9 endonucleases reveal RNA-mediated con- formational activation. Science. 2014; 343(6176): 1247997. doi:10.1126/ science.1247997

[97] Maher MF. et al., Plant gene editing through de novo in- duction of meristems. Nat. Biotechnol. 2020; 38: 84–89.

[98] Malnoy M. et al., DNA-free genetically edited grapevine and apple protoplast us- ing CRISPR/Cas9 ribonucleoproteins. Front. Plant Sci. 2016; 7: 1904. doi:10. 3389/fpls.2016.01904.

[99] Aksoy E, Yıldırım K, Kavas M. General guidelines for CRISPR /Cas–based genome editing in plants. Mol. Biol. Rep. 2022; 49(12): 12151– 12164.

[100] Alam M. et al., CRISPR/Cas9 mediated knockout of the OsbHLH024 transcrip- tion factor improves salt stress resistance in rice (Oryza sativa L.). Plants (Basel). 2022; 11(9): 1184.

[101] Andersson M. et al., Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol. Plant,. 2018;164: 378–384.

[102] Ali Z, Eid A, Ali S, Mahfouz MM. Pea early-browning virus- mediated genome editing via the CRISPR/Cas9 system in *Nicotiana benthamiana* and Arabidopsis.VirusRes. 2018;244: 333–337.

[103] Enciso-Rodriguez F. et al., Overcoming self- incompatibility in diploid potato using CRISPR-Cas9. Front. Plant Sci. 2019; 10.doi:10.3389/fpls.2019.00376.

[104] Cody WB, Scholthof HB, Mirkov TE. Multiplexed gene edit- ing and protein over expression using a tobacco mosaic virus viral vector. Plant Physiol. 2017; 175: 23–35.

[105] Singh M, Kumar M, Albertsen MC, Young JK, Cigan AM. Concurrent modifications in the three homeologs of Ms45 gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (*Triticum aestivum* L.). Plant Mol. Biol. 2018;97: 371–383.

[106] Yıldırım K. et al., Development of highly efficient resistance to beet curly top Iran virus (Becurtovirus) in sugar beet (*B. vulgaris*) via CRISPR/Cas9 system. Int. J. Mol. Sci. 2023; 24: 6515

[107] Hamdan MF. et al., Green revolution to gene revolution: technological advances in agriculture to feed the world. Plants (Basel). 2022; 12(10): 1297.

[108] Liu H. et al., Efficient induction of haploid plants in wheat by editing of TaMTL using an optimized agrobacterium-mediated CRISPR system. J. Exp. Bot. 2020; 71: 1337–1349.

[109] Bhoomika S, Salunkhe SR, Sakthi AR, Saraswathi T, Manonmani S, Raveendran M. et al., CRISPR-Cas9: Unraveling genetic secrets to enhance floral and fruit traits in tomato. Mol. Biotechnol. 2024;67(10): 3786–3799.

[110] Santosh Kumar VV. et al., CRISPR-Cas9 mediated genome editing of drought and salt tolerance (OsDST) gene in indica mega rice cultivar MTU1010. Physiol. Mol. Biol. Plants. 2020; 26(6): 1099–1110.

[111] Yerlikaya BA. et al., Innovations in plant genome editing and biotechnology: ad- dressing global agricultural challenges. Turk. J. Bot. 2024; 48(SI-1): 351–375.

[112] Zhu M. et al., Transgene–Killer– RISPR version 2 (TKC2) eliminates occasional transgene escape by coupling with a RUBY reporter. Plant Biotechnol. J. 2025; Adv. Online Publ. doi:10.1111/pbi.70257

[113] Xie E, Li Y, Tang D, Lv Y, Shen Y, Cheng Z.A strategy for generating rice apomixis by gene editing. J. Integr. Plant Biol. 2019; 61: 911. doi:10.1111/jipb.12785.

[114] [114] Liu F, Jiang T, Tanwir SE. *et al,.* CRISPR/Cas9-mediated *DFR* disruption suggests coordinated changes in flavonoid flux and development in *Petunia* × *hybrida*. Plant Cell Rep*. 2026;* **45**: 216. https://doi.org/10.1007/s00299-026-03903-9.

[115] Abdulla MF, Mostafa K, Kavas M. CRISPR/Cas9-mediated mutagenesis of FT/TFL1 in petunia improves plant architecture and early flowering. Plant Mol Biol 2024; 114:69. https://doi.org/10.1007/s11103-024-01454-9.

[116] Szabados L, Kant K, Rabilu S. *et al.,* Crops exposed to extreme conditions: perspectives of gene editing to improve stress tolerance. Plant Cell Rep. 2026*;* **45**: 207 https://doi.org/10.1007/s00299-026-03891.

[117] Mostafa K, Scarano A, Abdulla MF. *et al.,* A rapid *Agrobacterium rhizogenes*-mediated transient expression for assessing sgRNA efficiency in CRISPR-Act3.0 in tomato. Plant Cell Rep. 2026*;* **45**: 118. https://doi.org/10.1007/s00299-026-03792-y.

[118] Zhao R, Chen J, Li Y. *et al.,* CRISPR/Cas9-mediated α-prolamin gene (*Seita.8G190200*) mutagenesis increases the content of functional amino acids in foxtail millet (*Setaria italica*). Plant Cell Rep. 2026; **45**: 65. https://doi.org/10.1007/s00299-026-03751-7.

[119] Rocha DC, Omoregbee MO, Luo W. *et al.,* Transgene-free genome editing in citrus and poplar meristem tissues via biolistic ribonucleoprotein delivery of CRISPR-Cas9. Plant Cell Rep. 2026; **45**:58. https://doi.org/10.1007/s00299-026-03741-9.

[120] Wang Z, Zhang Y, Xu N. *et al.,* Improving soybean fatty acid profiles by CRISPR/Cas12a-mediated gene editing of *GmFAD2* and *GmFAD3*. Plant Cell Rep. 2025; **44**: 282. https://doi.org/10.1007/s00299-025-03672-x.

[121] Botelho FBS, Nandy S, Srivastava V. CRISPR/Cas9-based modulation of *V-PPase* expression in rice improves grain quality and yield under high nighttime temperature. Plant Cell Rep. 2025*;* **44**: 119. https://doi.org/10.1007/s00299-025-03504-y.

[122] Kaniganti S, Palakolanu SR, Thiombiano B. *et al.,* Developing *Striga* resistance in sorghum by modulating host cues through CRISPR/Cas9 gene editing. Plant Cell Rep. 2025*;* **44**: 90. https://doi.org/10.1007/s00299-025-03474-1.

[123] Kang B, Venkatesh J, Lee JH. *et al.,* CRISPR/Cas9-mediated editing of eukaryotic elongation factor 1B gamma (eEF1Bγ) reduces Tobacco etch virus accumulation in Nicotiana benthamiana. Plant Cell Rep. 2025*;* **44**: 62. https://doi.org/10.1007/s00299-025-03440-x.

[124] Asa H, Kuwabara C, Matsumoto K. *et al.,* Simultaneous site-directed mutagenesis for soybean ß-amyrin synthase genes via DNA-free CRISPR/Cas9 system using a single gRNA. Plant Cell Rep. 2025*;* **44**: 40. https://doi.org/10.1007/s00299-025-03433-w.

[125] Hao Y, Feng J, Li Y. *et al.,* Construction of a Cas9-targeted mutagenesis mini-library in the upland cotton genetic standard line (TM-1). Plant Cell Rep. 2025*;* **44**: 28. https://doi.org/10.1007/s00299-024-03416-3.

[126] Kang GH, Ko Y, Lee JM. Enhancing virus-mediated genome editing for cultivated tomato through low temperature. Plant Cell Rep. 2025*;* **44**: 22. https://doi.org/10.1007/s00299-024-03392-8.

[127] Nareshkumar V, Harish Chandar S, D'Orso F. *et al.,* Genome editing for trait optimization: The role of CRISPR, base editing and prime editing in modern era of plant science. J. Plant Biochem. Biotechnol. 2026;**35**;119–144.

[128] Sreekanth D, Pawar DV, Sahadeo IK. *et al.,* Advancements in CRISPR/Cas technology for generating herbicide tolerant crops: a comprehensive review. J. Plant Biochem. Biotechnol. 2025;**34**, 835–850. https://doi.org/10.1007/s13562-025-00984-7.

[129] Brock N, Kaur N, Halford NG. Advances in genome editing in plants within an evolving regulatory landscape, with a focus on its application in wheat breeding. J. Plant Biochem. Biotechnol. 2025*;* **34**: 599–614. https://doi.org/10.1007/s13562-025-00981-w.

[130] Kumara N, Singhb A, Priyansha, Sarkarc PR, Sharmad B, Kumare S, Aggarwal NK, Aryab A. India Biotech Revolution: The Approval of Genome-Edited Rice. The Bioscan. 2025; 20(3): 893-897.

[131] Kaur N, Qadir M, Francis DV, Alok A, Tiwari S, Ahmed ZFR. CRISPR/Cas9: a sustainable technology to enhance climate resilience in major Staple Crops. Front Genome Ed. 2025 18;7:1533197. doi: 10.3389/fgeed.2025.1533197.

[132] Kaur N, Alok A, Kumar P, Kaur N, Awasthi P, Chaturvedi S. et al., CRISPR/Cas9 directed editing of lycopene epsilon-cyclase modulates metabolic flux for β-carotene biosynthesis in banana fruit. Metab. Eng. 2020; 59: 76–86.

[133] Van VT, Das S, Hensel G, Kim JY. Genome editing and beyond: what does it mean for the future of plant breeding? Planta. 2022; 255: 130. doi:10.1007/s00425- 022-03906-2

[134] Sharma S, Kumar A, Kumari N, Walia A. RNAi as a tool to enhance crop yield and biotic stress management in the plants. PCTOC. 2023; 152: 437–454.

[135] Zheng S, Ye C, Lu J, Liufu J, Lin L, Dong Z. et al., Improving the rice photosynthetic efficiency and yield by editing OsHXK1 via CRISPR/Cas9 system. Int. J. Mol. Sci. 2021; 22: 9554. doi:10.3390/ijms22179554

[136] Zhou J, Zhang R, Jia X, Tang X, Guo Y, Yang H. et al., CRISPR-Cas9 mediated OsMIR168a knockout reveals its pleiotropy in rice. Plant Biotechnol. J. 2024;20: 310–322.

[137] Zhou Y, Xu S, Jiang N, Zhao X, Bai Z, Liu J. et al., Engineering of rice varieties with enhanced resistances to both blast and bacterial blight diseases via CRISPR/Cas9. Plant Biotechnol. J. 2022; 20: 876–885.

[138] Usman B, Nawaz G, Zhao N, Liao S, Qin B, Liu F. et al., Programmed editing of rice (*Oryza sativa* L.) OsSPL16 gene using CRISPR/Cas9 improves grain yield by modulating the expression of pyruvate enzymes and cell cycle proteins. Int. J. Mol. Sci. 2021; 22: 249. doi:10.3390/ijms22010249.

[139] Usman B, Nawaz G, Zhao N, Liu Y, Li R. Generation of high yielding and fragrant rice (*Oryza sativa* L.) lines by CRISPR/Cas9 targeted mutagenesis of three homoeologs of cytochrome P450 gene family and OsBADH2 and transcriptome and proteome profiling of revealed changes triggered by mutations. Plants. 2020; 9: 788. doi:10. 3390/plants9060788

[140] Sony SK, Kaul T, Motelb KFA, Thangaraj A, Bharti J, Kaul R. et al., CRISPR/Cas9-mediated homology donor repair base editing confers glyphosate resistance to rice (*Oryza sativa* L.). Front. Plant Sci. 2023; 14: 1122926. doi:10.3389/fpls. 2023.1122926.

[141] Shah PR, Varanavasiappan S, Kokiladevi E, Ramanathan A, Kumar KK. Genome editing of rice PFT1 gene to study its role in rice sheath blight disease resistance. Int. J. Curr. Microbiol. Appl. Sci. 2019; 8: 2356–2364.

[142] Alam MS, Kong J, Tao R, Ahmed T, Alamin M, Alotaibi SS. et al., CRISPR/Cas9 mediated knockout of the OsbHLH024 transcription factor improves salt stress resistance in rice (*Oryza sativa* L.). Plants. 2022; 11: 1184. doi:10.3390/plants11091184.

[143] Anjala K, Augustine R. Designing of guide RNA constructs for CRISPR/ Cas9-mediated editing of rice transcription factor osmads26 for enhancing drought tolerance. J. Appl. Biol. Biotechnol. 2022; 11: 176. doi:10.7324/jabb.2023.110124

[144] Biswas S, Ibarra O, Shaphek M, Molina-Risco M, Faion-Molina M, Bellinatti Della Gracia M. et al., Increasing the level of resistant starch in 'Presidio' rice through multiplex CRISPR–Cas9 gene editing of starch branching enzyme genes. TPG. 2023; 16, e20225. doi:10.1002/tpg2.20225.

[145] Huang J, Gao L, Luo S, Liu K, Qing D, Pan Y. et al., The genetic editing of GS3 via CRISPR/Cas9 accelerates the breeding of three-line hybrid rice with superior yield and grain quality. Mol. Breed. 2022; 42: 22. doi:10.1007/s11032-022-01290-z

[146] Huang Q. et al., CRISPR/ Cas9-mediated mutagenesis of the susceptibility gene OsHPP04 in rice confers enhanced resistance to rice root-knot nematode. Front. Plant Sci. 2023;14: 1134653. doi:10.3389/fpls.2023.1134653.

[147] Jameel MR. et al., CRISPR/Cas9-Mediated genome editing of soluble starch synthesis enzyme in rice for low glycemic index. Agronomy. 2022; 2: 2206. doi:10.3390/agronomy12092206

[148] Biswal AK, Hernandez LRB, Castillo AIR, Debernardi JM, Dhugga KS. An efficient transformation method for genome editing of elite bread wheat cultivars. Front. Plant Sci. 2023;14, 1135047.

[149] Hyde L, Osman K, Winfield M, Sanchez-Moran E, Higgins JD, Henderson IR. et al., Identification, characterization, and rescue of CRISPR/Cas9 generated wheat SPO11-1 mutants. Plant Biotechnol. J. 2023; 21:405–418.

[150] Ibrahim S, Saleem B, Rehman N, Zafar SA, Naeem MK, Khan MR. CRISPR/Cas9 mediated disruption of Inositol Pentakisphosphate 2-Kinase 1 (TaIPK1) reduces phytic acid and improves iron and zinc accumulation in wheat grains. J. Adv. Res. 2022; 37: 33–41.

[151] Gupta A, Hua L, Zhang Z, Yang B, Li W. CRISPR-induced miRNA156-recognition element mutations in TaSPL13 improve multiple agronomic traits in wheat. Plant Biotechnol. J. 2023; 21: 536–548.

[152] Zhang Z, Hua L, Gupta A, Tricoli D, Edwards KJ, Yang B. et al., Development of an *Agrobacterium*-delivered CRISPR/Cas9 system for wheat genome editing. Plant Biotechnol. J. 2019; 17:1623–1635

[153] Thakur N, Lakhani H, Tiwari S."Detailed insight into various classes of the CRISPR/Cas system to develop future crops," in Gene editing in plants. Editors A. Kumar, S. Arora, S. Ogita, Y. Y. Yau, and K. Mukherjee (Singapore: Springer). 2024;doi:10. 1007/978-981-99-8529-6_9

[154] Singh S, Chaudhary R, Chaturvedi S, Tiwari, S. "Deciphering the role of CRISPR/Cas9 in the amelioration of abiotic and biotic stress conditions," in Gene editing in plants. Editors A. Kumar, S. Arora, S. Ogita, Y. Y. Yau, and K. Mukherjee (Singapore: Springer). 2024; doi:10.1007/978-981-99-8529-6_8

[155] Singh S, Chaudhary R, Lokya V, Tiwari S. Genome editing based trait improvement in crops: Current perspective, challenges and opportunities. Nucleus. 2024; 67: 97–126.

[156] Ahmar S, Gruszka D. CRISPR/Cas9 boosts wheat yield by reducing brassinosteroid signaling. Trends. biochem. Sci. 2023; 48: 917–919.

[157] Alfatih A. et al., Loss of rice PARAQUAT TOLERANCE 3 confers enhanced resistance to abiotic stresses and increases grain yield in field. Plant Cell Environ. 2020; 43: 2743–2754.

[158] An X, Zhang S, Jiang Y, Liu X, Fang C, Wang J. et al., CRISPR/Cas9- based genome editing of 14 lipid metabolic genes reveals a sporopollenin metabolon ZmPKSB-ZmTKPR1-1/-2 required for pollen exine formation in maize. Plant Biotechnol. J. 2024; 22: 216–232.

[159] Doll NM, Gilles LM, Gérentes MF, Richard C, Just J, Fierlej Y. et al., Single and multiple gene knockouts by CRISPR–Cas9 in maize. Plant Cell. Rep. 2019; 38: 487–501.

[160] Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. Nat. Biotechnol. 2020; 38: 824–844.

[161] Chennakesavulu K, Singh H, Trivedi PK, Jain M, Yadav SR. State-of-the-art in CRISPR technology and engineering drought, salinity, and thermo-tolerant crop plants. Plant Cell Rep. 2021; 41, 815–831.

[162] Bhattacharjee P, Warang O, Das S, Das S. Impact of climate change on fruit crops- A review. CWE. 2022; 17: 319–330.

[163] Cai Y, Chen L. et al., CRISPR/Cas9- mediated targeted mutagenesis of GmFT2a delays flowering time in Soya Bean. Plant Biotechnol. J. 2018; 16: 176–185.

[164] Chandra AK, Joshi A, Tripathi A, Kumar A, Pandey S, Singh A. et al., Climate-resilience maize: heat stress, signaling, and molecular interventions. J. Plant Growth Regul. 2023;42; 6349–6366.

[165] Kumara N, Singhb A, Priyansha, Sarkarc PR, Sharmad B, Kumare S, Aggarwal NK, Aryab A. India Biotech Revolution: The Approval of Genome-Edited Rice. The Bioscan. 2025; 20(3): 893-897.

[166] CRISPR Gene Editing Workflow: A Step-by-Step Guide | CD Genomics Blog (cd-genomics.com).

[167] Shiels D, Prestwich BD, Koo O, Kanchiswamy CN, O'Halloran R and Badmi R Hemp Genome Editing—Challenges and Opportunities. Front. Genome Ed. 2022; 4:823486. doi: 10.3389/fgeed.2022.823486.

[168] Hesami M, Yoosefzadeh Najafabadi M, Adamek K, Torkamaneh D, Jones AMP. Synergizing Off-Target Predictions for In Silico Insights of CENH3 Knockout in Cannabis through CRISPR/CAS. Molecules. 2021; 26 (7): 2053. 10.3390/molecules26072053.

[169] Hesami M, Baiton A, Alizadeh M, Pepe M, Torkamaneh D, Jones AMP. Advances and Perspectives in Tissue Culture and Genetic Engineering of Cannabis. Ijms. 2021; 22 (11), 5671. 10.3390/ijms22115671.

[170] Zhu H, Li C, Gao C. Applications of CRISPR–Cas in agriculture and plant biotechnology. Nat. Rev. Mol. Cell Biol. 2020; 21: 661–677.

[171] Zhang X , Xu G , Cheng C , Lei L, Sun J, Xu Y , Deng C, Dai Z, Yang Z , Chen X, Liu C, Tang Q, Su J. Establishment of an *Agrobacterium*-mediated genetic transformation and CRISPR/Cas9-mediated targeted mutagenesis in Hemp (*Cannabis Sativa* L.). Plant Biotechnol J, 2021;10.1111/pbi.13611.