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CRISPR/Cas9 In Rice: A Preview Of Transformative Genetic Advances

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***Abstract* :** Rice (*Oryza sativa* L.) is an essential global staple grown worldwide owing to its ability to thrive under various environmental conditions. It is among the most economically significant crops and provides sustenance to more than half of the global population. However, increasing population , biotic and abiotic stresses have substantially challenged rice cultivation. Its compact genome size and genetic similarities with other cereal crops make rice an ideal model for functional genomics. Advances in genome editing technologies, such as zinc-finger nucleases (ZFNs) transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)- associated protein 9 (Cas9), have enabled the development of new elite plant varieties to alleviate food insecurity owing to their high efficiency, speed, and accuracy. Among these methods, CRISPR-Cas technology stands out for its precision and user-friendly nature, making it a promising solution for addressing various challenges in increasing rice production. This article explores the potential use of CRISPR/Cas9 role in promoting sustainable rice production amidst evolving challenges.

***keywords* - Genome editing, CRISPR-Cas9, precision, user-friendly nature, sustainable rice cultivation.**

I. Introduction

Rice (*Oryza sativa* L.) is a major cereal crop primarily produced in Asia, with substantial production in America and Africa. It can thrive in diverse environments, but climate change and adverse conditions such as drought and salinity significantly impact its production. The unprecedented population growth experienced in the mid-90s resulted in a severe food crisis, prompting the implementation of a green agricultural revolution. The primary goal of this revolutionary movement was to attain self-sufficiency in food grains by developing crop varieties that are capable of producing high yields. This initiative played a crucial role in addressing food scarcity by introducing advanced farming techniques and innovative crop varieties. To address the pressing challenges of population growth, environmental changes, and food scarcity, it is essential to develop rice (*Oryza sativa*) varieties with increased yields and enhanced tolerance to environmental stress. Traditional hybrid breeders have encountered difficulties in achieving a stable and predictable inheritance of the beneficial characteristics of hybrid crops. Over the past two decades, remarkable advancements have been made in next-generation sequencing (NGS). These innovations have resulted in a substantial reduction in sequencing costs, making them more accessible to researchers to delve into the intricacies of plant genomes. This groundbreaking advancement has unveiled an array of thrilling opportunities for conducting comprehensive exploration and in-depth analysis of plant genetic information. The advent of genome sequencing and editing has revolutionized research in rice breeding. This groundbreaking advancement has unveiled an array of thrilling opportunities for conducting comprehensive exploration and in-depth analysis of plant genetic information. In addition to conventional techniques, the integration of molecular approaches such as marker-assisted selection, QTL mapping, association mapping, and genomic selection has revolutionized the breeding process, significantly boosting its efficiency. The demand for a more precise breeding method has emerged in response to the imprecise nature of traditional mutation breeding. Genome editing is an innovative approach that involves making precise changes to the DNA of an organism.

Targeted mutagenesis allows the expression of desirable traits in specific genotypes. One of the key distinguishing features of genome editing is that it does not involve the introduction of genes from foreign organisms, setting it apart from the traditional transgenic methods. This can be achieved using various techniques, including transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs) (Kim et al., 1996), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), and cutting-edge advancements such as base editing and prime editing. TALENs and ZFNs function by creating double-strand breaks in DNA at specific locations, which can then be repaired to introduce desired changes (Wyman et al., 2006). CRISPR, on the other hand, uses RNA-guided Cas9 nucleases to target specific DNA sequences for modification (Jinek et al., 2012). Base editing enables the direct conversion of one DNA base pair into another (Kurt et al., 2021) whereas prime editing allows for precise insertion, deletion, or replacement of DNA sequences without requiring double-strand breaks (Anzalone et al., 2019). The use of these molecular tools in conjunction with traditional breeding methods has revolutionized the field of research, leading to numerous unexplored opportunities in rice research. These advancements have allowed researchers to gain deeper insights into the genetic makeup of rice varieties, leading to the discovery of novel avenues for improving the quality, yield, and resilience of rice crops. Although these tools have greatly aided in uncovering existing genetic variations, their impact on crop improvement has been static. To overcome this limitation, researchers have turned to mutation breeding as a means of introducing novel genetic variations into the gene pool, thereby establishing it as the ultimate source of variation in crop improvement strategies. Researchers have used CRISPR genome-editing technology to precisely modify the genetic material of plants, allowing them to gain a deeper understanding of plant biology and to address agricultural challenges. This cutting-edge approach enables researchers to investigate how specific genes influence plant traits such as disease resistance, yield, and nutrient content. By creating genome-edited plants, researchers can develop new varieties with improved characteristics, potentially leading to more resilient and productive crops. Innovative technology is regarded as a beacon of hope, offering valuable guidance for steering the future of agriculture toward success.

II. Genome editing and engineering

Genome editing, a revolutionary technology, has been heralded as a promising solution to various challenges in fields, such as agriculture, medicine, and biotechnology. This highly precise innovation in genetic manipulation holds great promise for various applications including agriculture, medicine, and biotechnology. The principle of gene editing relies on genome editing, which relies on the production of site-specific double-strand DNA breaks (DSBs) and the functioning of sequence-specific nucleases, which are enzymes that create specific changes in the DNA sequence. Following a double-strand break, the host repair machinery engages in an intricate process of homology-directed repair (HDR) or non-homologous end joining (NHEJ). NHEJ is a form of repair that operates independently of the donor template, whereas HDR requires the presence of a donor template to mend the DSB. Notably, HDR occurs at a lower frequency than NHEJ, posing significant challenges for the effective use of HDR in plants for generating genomic alterations, gene knockouts, and gene insertions (Puchta et al., 2005). These modifications include the addition, deletion, or substitution of nucleotides, resulting in targeted mutations. These mutations play a pivotal role in conferring favorable traits to organisms, rendering gene editing an indispensable tool for augmenting agricultural productivity and enhancing disease resistance. Genome editing in plants is rapidly transforming the agricultural field. By leveraging advanced techniques to make precise modifications to plant DNA, scientists can enhance crop traits, such as yield, disease resistance, and environmental adaptability with unprecedented accuracy. The development of genome-editing technologies, particularly CRISPR-Cas9, has unlocked new possibilities to improve food security, sustainability, and resilience in the face of climate change. This revolutionary approach allows for targeted alterations in specific genes, leading to the potential for the development of crops with improved nutritional content and enhanced stress tolerance. Furthermore, the precise nature of genome editing minimizes unintended genetic changes and addresses concerns regarding the potential environmental impact of genetically modified organisms.

1) Tools of genome editing

Genome editing tools are constantly evolving, offering various methods to precisely modify DNA, such as engineered endonucleases/mega-nucleases (EMNs), zinc-finger nucleases(ZFNs), TAL effector nucleases (TALENs), prime editing, base editing, and clustered regularly interspaced short palindromic repeats (CRISPR), allowing reengineering of future crops (Ahmad et al., 2021). Meganucleases stand out among endonucleases because of their distinctive feature of a wide recognition site spanning approximately 12–40 base pairs. The specificity and length of their recognition sites make them highly precise restriction enzymes (Mishra et al., 2018).

The challenge arises from the intricate interconnection between the DNA-binding domains and catalytic domain of meganucleases, making it difficult to modify them alongside other genome-targeting techniques. This difficulty stems from the inability to detach DNA-binding domains from the catalytic domain owing to their complex integration (Puchta et al., 2005). The current approach requires further improvement, particularly because manipulation of mega-nucleases is challenging. Consequently, researchers have focused on alternative, more effective, precise, and straightforward gene editing techniques, including Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). Zinc-finger nucleases (ZFNs) are highly efficient tools for editing genomes by targeting double-strand breaks (Durai et al., 2005). Zinc finger nucleases (ZFNs), the first truly targeting protein reagents, have significantly transformed the field of genome manipulation. Zinc finger nucleases (ZFNs) are specialized proteins that act as DNA-binding domains, enabling them to precisely recognize and interact with specific sequences of three base pairs at designated target sites within the DNA molecule (Rai et al., 2005). ZFNs have been used to specifically target IPK1 in rice. This gene plays a crucial role in the synthesis of high-quality phytic acids in rice grains (Cassandri et al., 2017). The TALENs system represents a site-driven mutagenesis genome-editing approach that was initially characterized in plant pathogenic bacteria, specifically Xanthomonas. This innovative system is conceptually similar to ZFNs, and has shown great potential in the field of genome editing. The operation of TALENs depends on their ability to activate or identify distinct transcription factors within the plant promoters. This process is facilitated by a series of tandem repeats that form the fundamental basis of the functionality. TALENs exhibit a higher level of precision than ZFNs because of their ability to target a single nucleotide at the designated site, as opposed to the three nucleotides targeted by ZFNs (Boch et al., 2009). Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALENs) employ a gene-editing strategy involving the cutting and alteration of genes. However, these techniques also result in unintended consequences such as non-specific and off-target gene mutations, which are significant areas of concern. CRISPR-Cas9 represents a major breakthrough in genetic engineering, offering unprecedented precision and efficiency in genome editing. Its versatility and wide range of applications, from basic research to medicine and agriculture, have made it a transformative tool in modern science.

2) Overview of CRISPR-Cas Technology

The scientific community actively sought innovative techniques that would not only be efficient, precise, and reliable but also possess the potential to address the limitations of previous approaches. Consequently, the CRISPR/Cas system has emerged as a promising solution. The term CRISPR was first coined in 2002 (Jansen et al., 2002), where CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats, and refers to tandem repeats flanked by non-repetitive DNA stretches that were first observed downstream of Escherichia coli (alkaline phosphatase isozyme) genes (Ishino et al., 1987). This offers a more cost-effective approach and has reduced the likelihood of off-target mutations (Bortesi et al., 2015; Zhang et al., 2020). The Behold the remarkable natural defense mechanisms found in bacteria and archaea, safeguarding them against invasion by foreign organisms. This incredible protective barrier is a testament to the resilience and ingenuity of the microorganisms. Originally, this methodology was designed for prokaryotic organisms because of the lack of efficient genome-editing techniques for eukaryotic organisms at specific loci. However, with the introduction of eukaryotic genome editing, CRISPR technology has dramatically enhanced our ability to introduce specific modifications to crop genomes (Butt et al., 2020). The first reported use of this technique was in 2012 when it was used as a tool for modifying genes in animals and fungi. The CRISPR/Cas9 system was initially used in rice and wheat species, and marked a significant milestone in genetic editing and crop improvement (Shan et al., 2014). This exciting tool uses guide RNA to bind to the target sequence, allowing the Cas9 enzyme to cut the DNA. Cell repair mechanisms either introduce small changes through non-homologous end joining (NHEJ) or make specific changes using homology-directed repair (HDR) if a template is provided. Cas9 is a CRISPR-associated protein 9 enzyme that is capable of cleaving DNA. Together, they form the CRISPR-Cas9 system, a revolutionary molecular tool that utilizes natural defence mechanisms to enable precise gene modifications in living organisms. The CRISPR-Cas9 system can be compared to a pair of molecular scissors guided by GPS, which is represented by guide RNA. Genome editing with CRISPR-Cas9 is an advanced process in which a carefully designed short synthetic oligonucleotide RNA sequence of 20 nucleotides, known as Guide RNA (gRNA), is used to match the specific DNA sequence requiring editing. This guide RNA directs the Cas9 enzyme to a precise location in the genome, where the editing should be performed, and is complementary to the target DNA sequence. Cas9, a nuclease enzyme, cleaves DNA at a location specified by guide RNA. Once the guide RNA binds to the matching DNA

sequence, Cas9 creates a precise double-stranded DNA (dsDNA) break. Cas9 nuclease is composed of two domains: (a) RuvC-like domains and (b) an HNH domain, with each domain cutting one DNA strand. Based on the number of protein subunits and crRNA in the ribonucleoprotein (RNP) complex, the CRISPR-Cas system is classified into two classes, class I and class II, which are further classified into three types. Types I, III, and IV are in classes I and II, and types V and VI are in class II (Makarova et al., 2015). The class I system contains multiple protein subunits along with crRNA, whereas the class II system contains only one protein and crRNA to target invading viral RNAs (Shmakov et al., 2015). The Cas9 protein in the type II CRISPR-Cas system processes the pre-crRNA with the help of tracer RNA and RNase III, whereas the Cas12 and Cas13 proteins in the type V and VI systems process the pre-crRNA themselves (Deltcheva et al., 2011; Dong et al., 2016; Dong et al., 2017). When foreign organisms invade the host organism, their DNA is fragmented by Cas proteins and the fragments are integrated into the crisper as new spacers. Once the same foreign organism attacks again, it is recognized quickly with the help of crRNA, and pairing occurs between them. This paring will guide the Cas protein to cleave the target sequences of foreign in advance which will result in the early protection of the host (Takahashi et al., 1955) Following DNA cleavage by Cas9, the natural repair mechanisms of cells come into play. Cells can repair this breakthrough non-homologous end-joining (NHEJ), which often results in small insertions or deletions (indels) that can disrupt or "knock out" the gene at the targeted site. Alternatively, cells can use Homology-Directed Repair (HDR) if template DNA is provided, allowing for more precise editing by inserting or replacing genetic material at the site of the break. This groundbreaking technology has emerged as a vital tool poised to revolutionize and drive the progress of agriculture toward unprecedented levels of success.

III. Role of CRISPR-Cas in Crop Improvement

CRISPR-Cas technology has significantly transformed crop improvement, providing a wide array of applications that can improve agricultural productivity and sustainability. Its diverse and influential applications address the numerous challenges encountered in modern agriculture. With the ability to precisely modify plant genomes, CRISPR-Cas technology shows tremendous potential for enhancing food security, nutritional quality, and environmental sustainability in light of our rapidly changing world. CRISPR-Cas technology has various applications in crop improvement, including disease resistance, drought tolerance, nutritional enhancement, yield improvement, quality improvement, herbicide resistance, improved photosynthesis, salt tolerance, enhanced shelf life, and non-GMO traits.

1. CRISPR-Cas as a Catalyst for Grain Yield

By harnessing the power of the CRISPR-Cas9 technology, we have the potential to revolutionize rice production by enhancing its yield and boosting its ability to withstand stress. The rice yield is intricately linked to several key agricultural traits. These include the number of panicles per plant, which influences the potential for grain production, and the number of grains per panicle. Additionally, the weight of a thousand grains is an important factor that contributes to overall rice yield. Grain yield is a complex, yet crucial trait for rice improvement. It is influenced by a multitude of genes known as quantitative trait loci (QTLs). With the advent of CRISPR-Cas9 technology, there have been significant strides in efforts to enhance grain yield in rice. Rice productivity has been enhanced through the inactivation of specific genes, such as GS3, DEP1, GS5, GW2, Gn1a, and TGW6, which are recognized as suppressors of grain size, number, and weight (Zeng et al., 2020). Yield-related genes, such as Gn1a (which is responsible for controlling grain number), DEP1 (DENSE AND ERECT PANICLE1, which regulates panicle architecture), GS3 (involved in regulating grain size), and IPA1 (which influences plant architecture), were specifically targeted for mutations using the CRISPR-CAS technique. The resulting mutants exhibited an increased grain number, denser and more upright panicles, and larger grain size, all of which contributed to a substantial boost in the overall yield. Triple mutants of three yield-related QTL genes (OsGS3, OsGW2, and OsGn1a) showed a 30–68% increase in yield per panicle. Mutant alleles of two yield-regulating genes, Gn1a and DEP1, were engineered to cultivate superior alleles in rice. The modified alleles of Gn1a and DEP1 have been shown to produce higher yields than the natural high-yield alleles (Huang et al., 2018). Mutations in genes encoding the ABA receptors PYRABACTIN RESISTANCE 1-LIKE 1 (PYL1), PYL4, and PYL6 led to the creation of a rice line that exhibited a remarkable increase in grain production. In field tests, this genetically modified rice variety produced up to 31% more grains than the original rice variety did. This groundbreaking work underscores the potential of the genetic modification of ABA receptors to effectively control growth and significantly improve rice yields, offering promising prospects for addressing food security and agricultural sustainability. (Miao et al., 2018). OsMYB30 is a nuclear protein that acts as a negative regulator of cold tolerance in rice. The ospin5b/gs3/osmyb30

triple mutant exhibited both higher yield and better cold tolerance than the wild type. Mutagenesis of the RGG2 gene, which is known to negatively impact rice plant growth and organ size resulted in phenotypic changes including elongated internodes, a significant increase in 1000-grain weight and overall plant biomass, as well as a notable boost in grain yield per plant. Targeted mutagenesis of OsFWL4, an adverse functional rice gene for tiller number and plant yield, enhanced tiller number, and plant yield, offers compelling evidence of the potential for genetic modification to improve rice productivity. Mutagenesis of POLYAMINE OXIDASE 5 (OsPAO5), a negative regulator of mesocotyl elongation, results in direct seeding of rice with markedly increased grain weight, grain number, and yield potential (Lyu et al., 2020). Enhancing rice yield through disruption of miRNA regulators holds great promise. miR396 intricately governs a myriad of essential agronomic traits. These traits include regulation of grain size, enhancement of grain quality, optimization of nitrogen use efficiency, modulation of responses to abiotic stresses, and prevention of seed shattering. MIR396d (Y. Tang et al., 2018), MIR529a (Y. Yan et al., 2021) along with MIR530 (W. Sun et al., 2020), were identified as crucial targets for the enhancement of high-yielding rice through the utilization of advanced genome-editing technologies. When MIR396e and MIR396f were knocked out, there was an exciting increase in both grain size and panicle branching and a significant increase in grain yield. (C.B. Miao et al., 2020), (J. Zhang et al., 2020) . Mutations in OsGRF4 disrupt the regulation of OsGRF4 by miR396, resulting in the development of plants with larger grain sizes and higher grain yields (S. Li et al., 2016).

2. Tailoring Rice for good quality and nutrition

Genome editing has been used to enhance the quality and nutritional conent of rice. This encompasses modifying oil and starch compositions, managing fragrances, decreasing the accumulation of heavy metals, and striving for biofortification. Utilizing the CRISPR/Cas9 model to enhance the nutritional quality of rice grains holds promise for addressing the challenges of malnourishment.

2.1 Micronutrient rich Rice:

During research, it was determined that GW2, which encodes a RING-type E3 ubiquitin ligase, plays a crucial role in controlling grain weight in cereal crops. The endosperm of GW2 knockout mutants created using CRISPR/Cas9 technology in the Indica variety MTU1010 exhibited substantial thickening of the aleurone layer, leading to a notable increase in the grain protein content. Loss of function of OsGW2 leads to the accumulation of essential dietary minerals including iron (Fe), zinc (Zn), potassium (K), phosphorus (P), and calcium (Ca) in the endosperm of rice grains. This provides additional nutritional benefits derived from genetic modification (Achary et al. 2021).

2.2 Creating high oleic/low linoleic rice

Rice bran oil, which is well known for its health benefits, contains a significant amount of oleic acid. This valuable component not only contributes to improving overall health, but also plays a crucial role in preventing various diseases. In plants, oleic acid is enzymatically converted to linoleic acid by fatty acid desaturase 2 (FAD2). Enhancing the oleic acid content of rice bran oil is essential to improve its nutritional quality and overall health benefits. Rice possesses three functional FAD2 genes, among which, OsFAD2-1 has the highest expression in seeds. By manipulating the expression of this gene, rice bran oil can be produced, with elevated levels of oleic acid and reduced levels of linoleic acid. The use of CRISPR/Cas9 technology to introduce targeted mutations in OsFAD2-1 resulted in the development of knockout rice plants. These modified plants displayed a notable 2-fold increase in oleic acid levels and a complete absence of linoleic acid in their composition. As a result, the fatty acid profile of rice bran oil is significantly enhanced (Abe et al. 2018).

2.3 Aromatic Rice:

Rice aroma is an additional superior quality that enhances the value of the product, allowing it to command a higher price. Therefore, it is important to introduce aromatic compounds into rice. The gene known as Betaine aldehyde dehydrogenase 2 (OsBADH2) is a key regulator of the biosynthesis pathway of 2-acetyl-1-pyrroline (2-AP), a compound that is crucial for imparting the alluring aroma in fragrant rice varieties. Using CRISPR/Cas9 gene editing technology, scientists have successfully modified the Badh2 gene to create fragrant rice within the genetic background of Zhonghua 11, an indica rice variety. This involved the addition of an extra base (T) into the first exon of Badh2, leading to a notable increase in 2AP levels, which contributed to the fragrance of rice. When

the activity of Badh2 is suppressed, it triggers a biochemical process that leads to the enhanced biosynthesis of 2-acetyl-1-pyrroline (2AP), infusing rice with an enticing aroma. (Shan et al., 2015; Hui et al., 2022).

2.4 Boosting the β-carotene content in rice

Enhancing the nutritional value of rice by increasing β-carotene levels is crucial for rice enhancement and human health improvement. The Osor gene, which is similar to the Orange (Or) gene found in cauliflower, was specifically modified in rice using CRISPR/Cas9. The Or gene is known to play a role in the accumulation of β-carotene in the cauliflower curd. Using CRISPR/Cas9-based genome editing to target the Osor gene, researchers were able to increase the accumulation of β-carotene in rice Calli (Endo et al., 2019).

2.5 Resistant Starch Rice

Resistant Starch Rice Cereals rich in amylose are an excellent source of resistant starch (Regina et al., 2006). Amylose and amylopectin are essential components of starch. Amylose is a linear glucose polymer, whereas amylopectin is a branched polymer. When starch contains a high proportion of unbranched amylopectin, it leads to the formation of what is known as resistant starch. Resistant starch offers potential health benefits, such as supporting gut health and helping regulate blood sugar levels. Rice plants that underwent editing of the starch branching enzyme IIb (SBEII) gene displayed significant increases in amylose and resistant starch contents, with levels increasing by up to 25% and 9.8%, respectively (Sun et al., 2017). These results demonstrate the effectiveness of the CRISPR/Cas9 system in producing high-amylose rice by modifying SBEIIb. Certain types of rice, particularly indica hybrids, are often deemed of low quality in certain markets because of their elevated amylose levels. Consequently, it is highly desirable to decrease the amylose content to produce glutinous rice. Using the CRISPR/Cas9 gene editing technique, researchers have successfully knocked out the waxy gene in rice. This targeted gene modification has been observed to significantly decrease the amylose content in rice grains. Therefore, modified rice has the potential to exhibit characteristics of glutinous rice, thereby altering its culinary and nutritional properties (Zhang et al., 2019).

2.6 Red rice

The majority of rice varieties commonly cultivated worldwide feature white pericarps, such as the well-known jasmine and basmati rice. However, lesser-known varieties exhibit a spectrum of hues including brown, red, and purple/black pericarps. These colorful rice varieties harbor potent pigments containing elevated levels of proanthocyanidins and anthocyanins, which are known for their health-enhancing properties. Proanthocyanidins, a class of flavonoids, possess antioxidant properties, whereas anthocyanins are responsible for the vibrant red, purple, and blue pigmentation in numerous fruits, vegetables, and grains. Colored rice varieties are cultivated in distinct regions of the world, each with its own culinary and cultural significance. For instance, red rice is prevalent in the Himalayan region and is renowned for its nutty flavor and slightly chewy texture. The nutritional significance of colored rice, combined with its diverse culinary applications, underscores its importance in the global food culture. In the case of Oryza rufipogon, the wild ancestor of cultivated rice, the vibrant red color of the pericarp tissue is a result of the combined action of the Rc and Rd genes. However, throughout evolution, a significant genetic event occurred: a frameshift deletion of 14 base pairs in the 7th exon of the Rc gene. This genetic alteration ultimately led to fascinating transformation of cultivated rice species, resulting in the emergence of white rice grains. CRISPR-Cas9, a cutting-edge gene-editing technology, has been utilized to reverse frame-shift deletions and restore the recessive Rc allele, effectively converting white pericarp rice varieties into vibrant red ones (Zhu et al., 2019). The resulting red grains from genetically modified mutants showed markedly elevated levels of proanthocyanidins and anthocyanidins, which are known for their antioxidant properties and potential health-promoting effects. This groundbreaking advancement in genetic modification not only holds promise for enhancing the nutritional profile of rice, but also introduces visually striking changes that could have far-reaching implications in agriculture and the food industry.

2.7 Rice Bran

Rice bran oil (RBO), a by-product of rice milling, is rich in dietary fiber and contains health-promoting components such as oleic acid. The balance between oleic acid and linoleic acid affects their utility in various applications. FAD2, also known as fatty acid desaturase 2, is pivotal for the conversion of oleic acid to linoleic acid. Within the rice genome, there exist four FAD2 genes, with FAD2-1 exhibiting the highest expression in rice seeds. Therefore, targeting the FAD2-1 gene for knockout using the CRISPR/Cas system could potentially result in the development of a rice variety with high oleic acid content. In homozygous mutants with the FAD2-1 gene knocked out (fad2-1), the oleic acid content surpassed that of the wild type. Linoleic acid was not detected in these mutants. This underscores the substantial impact of FAD2-1 knockout on altering the fatty acid composition of rice seeds, potentially paving the way for the creation of healthier and more nutritionally advantageous rice varieties (Abe et al., 2018).

3. Tolerance to biotic stress:

3.1 Resistance to X. oryzae

Bacterial blight is a prevalent disease caused by the pathogen, Xanthomonas oryzae pv. oryzae, significantly affecting annual rice yields, resulting in a substantial 10-20% reduction in production. This disease poses a significant threat to rice cultivation (Zhang et al. 2013). The SWEET family of sugar transporters is the most extensively studied group of S genes activated by TAL effectors (Chen, 2014). The induction of just three plant SWEET genes, which encode putative sugar transporters, occurs in response to transcription activator-like (TAL) effectors originating from the rice Xoo pathogen (Streubel et al., 2013). The gene OsSWEET13, known as the susceptibility (S) gene, encodes a sucrose transporter that has a significant impact on the interaction between plants and pathogens. When X. oryzae produces the effector protein PthXo2, it triggers the expression of OsSWEET13 in the host plant, leading to increased susceptibility. However, in experiments with rice plants, reducing the activity of the OsSWEET13 promoter resulted in bacterial blight resistance. (Zhou et al., 2015). Specifically, they focused on modifying the promoter fragments of OsSWEET genes using CRISPR-Cas9 (Oliva et al., 2019; Xu et al., 2019). Following the design of a single guide RNA (sgRNA) targeting the first exon of the gene, the researchers utilized Agrobacterium-mediated transformation was used to introduce genetic modifications into rice calli, specifically in the Kitaake variety. This resulted in the identification of two mutant lines, each carrying deletions of the 4th and 11th bases in the coding region of OsSWEETB. By harnessing the power of the NHEJ pathway, scientists have successfully modified the SWEET14 promoter region in Super Basmati rice, resulting in resistance to bacterial blight (Zafar et al., 2020a). These mutant lines showed increased resistance to the harmful bacterium X. oryzae, reducing lesion length by approximately 90% compared with wild-type plants (Zhou et al., 2015). This precise genetic manipulation aimed to bolster natural defenses against bacterial blight in rice.

3.2 Resistance to rice tungro spherical virus (RTSV)

Rice tungro disease (RTD) presents a significant hurdle to rice cultivation in tropical regions. This disease arises from the intricate interplay between the rice tungro spherical virus (RTSV) and rice tungro bacilliform virus. RTSV induces yellowing symptoms in infected rice plants, whereas the bacilliform virus amplifies RTSV's pathogenicity of RTSV. These combined effects severely diminish both the yield and quality of rice crops, underscoring the pressing need for farmers and researchers to devise effective strategies for managing this detrimental disease. In an effort to create new defenses against Rice Tungro Disease (RTD), mutations were deliberately introduced into eIF4G using the CRISPR/Cas9 gene-editing technique (Macovei et al., 2018) in the RTSV-susceptible rice cultivar IR64. The observed mutation rates ranged from 36.0% to 86.6%. These mutations have the potential to impart resistance to RTD, and may play a significant role in the development of RTSV-resistant rice strains (Li et al., 2017; Miao et al., 2013; Xu et al., 2015; Zhang et al., 2014).

3.3 Resistance to Rice blast Rice blast

Resistance to Rice blast Rice blast, caused by the fungus Pyricularia oryzae, is one of the most critical diseases affecting rice (Miah et al. 2013, Srivastava et al. 2017). In 1892, Cavara established the name Pyricularia oryzae and in 1970, Magnaporthe oryzae was renamed. (Couch and Kohn 2002, Zhang et al. 2016). Conventional breeding can be improved using biotechnology to develop resistance to pests and diseases (Miah et al. 2013, Ashkani et al. 2015). CRISPR-CAS was used to install mutations in three broad-spectrum blast-resistant genes, Bsr-d1, Pi21, and ERF922, with single or triple mutants that confer enhanced resistance to blast (Zhou et al., 2022).

CRISPR/Cas9-targeted knockout of the ERF transcription factor gene OsERF922 in Kuiku131, a japonica rice variety widely cultivated in northern China displayed improved rice blast resistance. Mutants with ERF922 displayed the highest level of resistance to blast, comparable to that of the triple mutants. (Wang et al. 2016). The two exons of the OsSEC3A gene, a subunit of exocyst complex 3A, were precisely targeted using CRISPR-Cas technology along with two specific guide RNAs (sgRNAs). The resulting edited rice plants exhibited significantly enhanced immunological responses, particularly against blast diseases (Ma et al. (2018).

4. Tolerance to Abiotic stresses:

4.1 Resistance to pests:

Pests harm crops by feeding on plants, causing damage, and spreading plant diseases. This can lead to reduced crop yield and quality, affecting farmers and food supply. The use of pesticides can harm the environment; therefore, so we need a safe way to control crop pests is required. Increasing salicylic acid levels by disrupting OsCYP71A1 can improve rice resistance to plant hoppers and stem borers (Lu et al., 2018). The gmcdpk38 mutant with Hap3 knockout using CRISPR/Cas9 showed high resistance to common cutworms (Li et al., 2022).

4.2 Enhancing Salinity Tolerance

The CRISPR/Cas9 approach was used to create a gene-edited mutant to study the function of OsbHLH024 in salt-stressed rice. The mutant A91 was found to have a deletion of the A nucleotide base in the osbhlh024 gene. The A91 mutant exhibited strong tolerance to high salinity and showed reduced accumulation of reactive oxygen species (ROS), malondialdehyde (MDA), and sodium (Na+), while displaying increased levels of potassium (K+). Additionally, the mutant maintained a balanced nutritional profile in both the shoots and roots. These findings suggest that OsbHLH024 acts as a negative regulator and its knockout enhances salt stress tolerance in rice. (Alam et al., 2022). Extensive screening of ethyl methanesulfonate (EMS) mutants in japonica rice cv. Zhonghua11 (ZH11), researchers discovered a drought and salt tolerance (dst) mutant of rice. This mutant was found to be associated with OsDST, which encodes a zinc finger transcription factor. Further investigation revealed that the dst mutant carried two substitutions, resulting in changes in the amino acid sequence of the dst protein, specifically N69D and A162T. Subsequent transgenic complementation analysis confirmed that the N69D mutation was the primary cause of the dst-mutant phenotype. (Santosh Kumar et al., 2020). Recent studies have highlighted the significant potential of OsRR22 to expedite the enhancement of salinity tolerance in rice breeding. Research has indicated a substantial increase in salt tolerance following knockout of the OsRR22 gene, which encodes a 696 amino acid B-type response regulator transcription factor. This finding underscores the pivotal role of OsRR22 in modulating salinity tolerance, and presents a promising avenue for further research and application in rice breeding programs. (Takagi et al., 2015). OsmiR535 is a miRNA found in plants, specifically in the miR156/miR529/miR535 superfamily. The suppression or elimination of OsmiR535 in rice has the potential to increase the ability of plants to withstand NaCl, ABA, dehydration, and PEG stresses.CRISPR/Cas9 technology has been used to perform genome editing, resulting in the creation of a homozygous 5 base pair deletion in the coding sequence of OsmiR535. This successful editing demonstrates the potential of OsmiR535 as a valuable target for genetic manipulation aimed at enhancing drought and salinity tolerance in Oryza sativa. Additionally, modified OsmiR535 can serve as a new molecular marker for use in breeding programs for Oryza sativa (Yue et al., 2020).

4.3 Overcoming Drought stress

Drought stress is a significant factor leading to substantial decreases in the yield and productivity of major crops (Joshi et al., 2020). The use of CRISPR/Cas9 technology to induce mutations in OsERA1 has led to the development of drought-tolerant rice varieties (Ogata et al., 2020). CRISPR/Cas9 was used to edit the OsDST gene in the indica mega rice cultivar MTU1010, resulting in a mutant with wider leaves, lower stomatal density, and an improved ability to retain water in the leaves during drought stress (Kumar et al., 2020). A mutant strain of rice known as ospyl9 developed using CRISPR/Cas9 technology conferred ability to withstand drought conditions, while also increasing their overall yield. (Usman et al., 2020). The CRISPR/Cas9 gene editing tool was employed to introduce targeted mutations in the Semi-rolled leaf1 (SRL1) and Semi-rolled leaf2 (SRL2) genes in rice, leading to the development of a distinctive curled leaf phenotype and conferred enhanced drought tolerance. The mutations induced changes in the expression patterns of specific proteins, leading to improved ROS scavenging of reactive oxygen species. This molecular manipulation resulted in desired physiological and

morphological changes, ultimately contributing to the enhanced resilience of rice plants to drought stress. (Liao et al., 2019).

4.4 Heat-Resilient Rice

The combination of prolonged drought and escalating heat stress, attributed to unmitigated climate change, poses a significant threat to global food security, particularly concerning rice production. Elevated temperatures profoundly disrupt the entire growth cycle of crops, exerting the greatest impact on the developmental phases of plants (Jagadish et al., 2021). CRISPR/Cas9 gene editing was employed to create a triple knockout rice variety lacking the PYRABACTIN RESISTANCE-LIKE 1/4/6 (OsPYL1/4/6) genes. This mutant rice strain exhibited improved yield, enhanced tolerance to high temperatures, and reduced pre-harvest germination compared with the wild-type rice variety (Miao et al., 2018). The knockout of the OsNAC006 gene was achieved using the precise genome editing tool CRISPR-Cas9, which led to the manifestation of heat sensitivity in rice plants. This breakthrough provides valuable insights into the significant involvement of OsNAC006 in orchestrating heat stress responses in rice, thereby offering potential avenues for leveraging this knowledge in future endeavors aimed at enhancing crop resilience and productivity. (Wang et al. 2020).

4.5 Engineering Cold Resistance

Cold stress, which includes chilling (<20℃) and freezing (<0℃) temperatures, inhibits the growth and development of plants, and seriously restricts plant spatial distribution and agricultural productivity Rice cultivation in tropical and subtropical zones faces a significant challenge (Van Nguyen, 2006). Low temperatures have a direct impact on the metabolic response of plants, leading to the induction of osmotic stress, oxidative stress, and other forms of stress. Through the use of CRISPR/Cas9, mutants such as PIN-FORMED 5b (OsPIN5b), GRAIN SIZE (GS3), and V-MYB AVIAN MYELOBLASTOSIS VIRAL ONCOGENE HOMOLOG 30 (OsMYB30) have been created. These mutants have shown increased spike length, grain size, and enhanced cold tolerance (Zeng et al., 2019). The CRISPR/Cas9 gene editing technique was employed to specifically target the cDNA encoding the proline-rich protein in a rice gene known as OsPRP1. The resulting knockout mutants exhibited an increased sensitivity to cold conditions. These findings indicate that OsPRP1 plays a crucial role in enhancing cold tolerance by regulating antioxidants and facilitating communication within signaling pathways. Consequently, OsPRP1 holds significant promise for enhancing cold tolerance in rice. The use of CRISPR/Cas9 technology has proved invaluable in elucidating the functional role of this gene by analyzing the phenotypic traits of the generated knockout mutants. This study confirms the pivotal role of OsPRP1 in bolstering cold tolerance, signifying a promising avenue for future research and potential application in breeding programs aimed at enhancing cold tolerance in rice. (Nawaz et al., 2019).

4.6 Combating Heavy metals stress

Heavy metals pose a significant threat to the growth and productivity of plants and present a major obstacle to sustainable agricultural development. These toxic elements exert detrimental effects on plants, leading to reduced crop yields and compromised plant health. This highlights the urgent need for effective strategies to mitigate heavy-metal toxicity in agricultural settings (Hoque et al., 2021). The use of CRISPR/Cas9 technology to knock down OsNramp5 and OsLCT1 has been found to effectively decrease the accumulation of cadmium (Cd) in rice plants (Songmei et al., 2019). scientists have employed the CRISPR/Cas9 gene editing technique to deactivate the NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 1 (OsNRAMP1) gene in mutant rice plants. Because of this genetic modification, the levels of cadmium (Cd) and lead (Pb) in rice grains were notably reduced. This groundbreaking discovery has substantial implications for the advancement of safer and more environmentally sustainable rice varieties (Chang et al., 2020; Chu et al., 2022). The roles of OsNRAMP5 and OsNRAMP1 in reducing Cd accumulation do not overlap (Wang et al. 2017). To develop rice plants with reduced levels of cesium (Cs), scientists utilized CRISPR/Cas technology to disable the K+ transporter known as HIGH-AFFINITY POTASSIUM TRANSPORTER 1 (OSHAK1) (Nieves-Cordones et al., 2017). The research findings indicated that the CRISPR/Cas9-mediated elimination of the R2R3 MYB transcription factor ARSENITE-RESPONSIVE MYB1 (OsARM1) noticeably inhibited the absorption and transport of arsenic in rice. This suggests the potential of targeted genetic modifications to mitigate As accumulation in rice plants (Wang et al., 2017).

4.7 Submergence Tolerance

The majority of coastal rice-growing regions in the tropics and subtropics experience frequent inundation, particularly low-lying deltas along the coasts of Southeast Asia. These regions play a crucial role in rice production, contributing 34–70% of the total rice production in their respective countries. This inundation is a key factor shaping the unique agricultural and ecological characteristics of these areas, making them vital for global rice production. It is crucial to recognize the potential ramifications of the decline in rice production caused by an increase in the frequency of flooding. This situation could lead to a shortage of rice, impacting food security on a global scale, and potentially leading to increased food prices and scarcity in many regions (Wassmann et al., 2009). Through CRISPR/Cas9 gene editing, significant progress has been achieved in the modification of the SUBMERGENCE 1A-1 gene within the Ciherang-Sub1 rice variety, which has resulted in the regeneration of indica rice. These advancements represent a pivotal milestone in the enhancement of indica-resistant rice varieties, providing a swift and precise avenue for genetic enhancement through CRISPR/Cas9 gene editing (Liang et al., 2021).

4.8 Resistance to herbicides :

Severe weed infestation causes delays in growth and loss of grain quality and yield. Herbicides are widely used in agriculture to control unwanted weeds; however, their application can have significant effects on non-target plants and ecosystems. While designed to eliminate specific weed species, herbicides can sometimes drift or leach into adjacent areas, impacting beneficial flora. This unintentional exposure can result in phytotoxicity, causing symptoms, such as leaf discoloration, stunted growth, and even plant death. Additionally, herbicides may disrupt soil microbiomes, thereby affecting nutrient cycling and soil health. Different herbicides are applied in the field to mitigate the negative effects of weeds. Along with weeds, these herbicides also affect cultivated rice. Among the array of herbicides, bispyribac-sodium (BS) and chlorsulfuron are utilized for the management of a diverse spectrum of grasses and weeds. However, it is essential to acknowledge that these herbicides also possess the capacity to target the acetolactate synthase (ALS) gene of rice, which is intricately involved in the biosynthetic pathway responsible for the production of branched-chain amino acids (Chipman et al. 1998; McCourt and Duggleby, 2006). Researchers have successfully performed gene replacement in the rice ALS gene using the homology-directed repair (HDR) pathway with the CRISPR/Cas9 system (Sun et al., 2016). The fragment involving two amino acid residues (W548L/S627I) in the rice ALS gene was deleted using two sgRNAs (Li et al., 2018a). This gene-editing tool was employed to modify the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene, leading to the insertion of two distinct amino acid substitutions (T102I and P106S). This genetic alteration results in the acquisition of resistance to the potent herbicide glyphosate (Li et al., 2016). Manipulation of OsPUT1/2/3 using CRISPR/Cas9 significantly increased the resistance of rice to paraquat without causing any noticeable reduction in yield (Lyu et al., 2022).

5. Rice Early Maturing and Long Day Flowering

The timing of rice (Oryza sativa L.) flowers is a crucial factor in their adaptation to different regions and in determining grain yield. To enhance the rice breeding process, breeders aim to achieve a more precise and controlled flowering period for the elite cultivars. In a recent study, researchers used CRISPR/Cas9 genome-editing to modify four homozygous rice lines. Specifically, they targeted Hd2 uORFs to enhance the expression of flowering repressors to achieve a more efficient and modulated blooming period in rice plants. The CRISPR/Cas9 gene-editing technique was employed to induce a mutation in OsCIPK3, resulting in a delayed heading date under long-day (LD) conditions, while the heading date remained normal under short-day (SD) conditions. These findings suggest that OsCIPK3 plays a role in the phosphorylation of OsFD1, thereby promoting the formation of RFT1-containing florigen activation complexes. This, in turn, leads to the induction of flowering in rice, specifically under long-day conditions (Peng et al., 2021).

6. Hybrid Rice Breeding: Creating Male Sterile Lines

A key component in the successful development of hybrid rice is the creation of a sterile male line, which is essential for effective cross-breeding. This method has proven to be instrumental in boosting rice production and the overall agricultural output. Scientists have employed the cutting-edge gene editing technology CRISPR/Cas9 to induce specific mutations in genes associated with male sterility, including TMS5, Xa13, and Pi21. The resulting transgene-free mutants observed in the T1 generation displayed characteristics consistent with thermosensitive

genic male sterility, a condition where plants fail to produce viable pollen under high-temperature conditions. Moreover, these mutants exhibited increased resistance to two prevalent rice diseases, rice blast and bacterial blight. This groundbreaking approach provides a means to convert breeding materials into thermosensitive genic male-sterile lines through gene editing. Ultimately, this strategy has the potential to accelerate the breeding process by leveraging sterile male lines, resulting in enhanced crop varieties (Li et al., 2019b).

IV. Conclusion

Future studies should focus on the growing population's need for food and the increased challenges of climate change affecting crop growth. Therefore, it is necessary to increase food production to meet the needs of the future population. Rice, one of the primary crops, provides essential nutrition to over half of the world's population. The advent of various genome-editing tools, including Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALENs), and CRISPR/Cas9, has significantly expanded the landscape of research in the field of rice genetics. These tools have unlocked new avenues for developing novel rice varieties with enhanced productivity and improved quality, thereby revolutionizing the potential of rice cultivation. The innovative CRISPR-Cas system has completely transformed the crop breeding landscape. Its remarkable simplicity, exceptional efficiency, high specificity, and adaptability to multiplexing have revolutionized the breeding approach. Naturally occurring components, including genes, promoters, cis-regulatory elements, and epigenetic modifications, can be harnessed as valuable sources for the development of innovative regulatory components. This, in turn, could enable the precise manipulation of metabolic and regulatory pathways, allowing for the enhancement of crop plants' ability to tolerate both biotic and abiotic stressors. This groundbreaking technology has not only changed the methods of crop breeding but has also laid the groundwork for the next generation of breeding practices, promising significant advancements in the field.

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