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CRISPR/Cas9-Mediated Gene Editing in Grain Crops

Adil Hussain, Qari Muhammad Imran and Byung-Wook Yun

Abstract

The development of reliable and efficient techniques for making precise targeted changes in the genome of living organisms has been a long-standing objective of researchers throughout the world. In plants, different methods, each with several different variations, have been developed for this purpose, though many of them are hampered either by providing only temporary modification of gene function or unpredictable off-target results. The recent discovery of clustered regularly interspaced short palindromic repeats (CRISPRs) and the CRISPR-associated 9 (Cas9) nucleases started a new era in genome editing. Basically, the CRISPR/Cas system is a natural immune response of prokaryotes to resist foreign genetic elements entering via plasmids and phages. Through this naturally occurring gene editing system, bacteria create DNA segments known as CRISPR arrays that allow them to “remember” foreign genetic material for protection against it and other similar sequences in the future. This system has now been adopted by researchers in laboratory to create a short guide RNA that binds to specific target sequences of DNA in eukaryotic genome, and the Cas9 enzyme cuts the DNA at the targeted location. Once cut, the cell’s endogenous DNA repair machinery is used to add, delete, or replace pieces of genetic material. Though CRISPR/Cas9 technology has been recently developed, it has started to be regularly used for gene editing in plants as well as animals to good success. It has been proved as an efficient transgene-free technique. A simple search on PubMed (NCBI) shows that among all plants, 80 different studies published since 2013 involved CRISPR/Cas9-mediated genome editing in rice. Of these, 20, 13, and 24 papers have been published in 2019, 2018, and 2017, respectively. Furthermore, 20 different studies published since 2014 utilized CRISPR/Cas9 system for gene editing in wheat, where five of these studies were published in 2019 and seven were published in 2018. Genomes of other grain crops edited through this technique include maize, sorghum, barley, etc. This indicates the high utility of this technique for gene editing in grain crops. Here we emphasize on CRISPR/Cas9-mediated gene editing in rice, wheat, and maize.

Keywords: gene editing, cereal crops, CRISPR/Cas9, rice, wheat, maize

1. Introduction

Perhaps one of the most important differences that can define humans apart from others in the animal kingdom is the ability to make choices among good, better, and best. The ability to reason, argue, research, and adopt what is (or at least what *he* thinks is) best for him. Plants, being the primary producers on this planet, have always been the most important resource of life for the human omnivores. We

have always chosen, selected, and adopted plant species that produced either more or good; and hence, driving *plant domestication* to this day. Initially, this domestication was only based upon observations, personal, physical likes and dislikes. Later, it was based on more complex characteristics that tempted the crossing of different plants with desirable characters to make a new variety or cultivar carrying multiple desirable characters. Such classical or conventional breeding methods included selection and hybridization. Afterward, using DNA-level information, scientists used these methods to tag a large number of quantitative trait loci (QTLs) via marker-assisted breeding or selection for combining different traits in a single variety.

With further developments in genetic engineering, genome modification studies were performed in plants [1]. This was specifically aided by the discovery of nuclease enzyme, the utilization of different bacterial plasmids for cloning of target genes in bacteria as well as target plants and by the development of plasmid vectors carrying antibiotic resistance genes, a variety of constitutive, inducible as well as tissue-specific promoters, and different reporter genes that made the cloning and transformation easier. The discovery of the Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and more recently that of the clustered regularly interspaced short palindromic repeats (CRISPRs) in genomes has further aided the genome editing techniques in different plant species.

1.1 Genome editing in nature

Genome editing involves molecular tools that lead to targeted modification of specific DNA sequences inside the genome. These are usually based on the production of double strand breaks (DSBs) at the specific DNA sites that trigger DNA repair of the cell. In plant cells, this generally happens through the process of non-homologous end-joining commonly abbreviated as NHEJ. However, this process is error-prone, thus exploited by scientists to target genes for possible modification. Sometimes, plants repair the DSBs through homologous recombination which is error-free. Hence, it could be used by scientists to precisely edit DNA or insert DNA sequences in a given genome.

Genetic modification or genome editing is already in practice in cells of living organisms and is one of the most magnificent specters of nature. Natural DNA repair mechanisms, losing and acquiring of genetic material in bacteria, transmission of bacteriophages through bacterial transduction, and genetic modification of plant genome by Ti plasmid during infection by *Agrobacterium tumefaciens* are some of the examples of genetic modifications from nature. DSBs have also been shown to be induced naturally by different factors such as various reactive oxygen species (ROS), radiation, and others. Intricate and complex interconnected cellular networks drive post-transcriptional RNA modifications and post-translational protein modifications to accommodate a literally unlimited number of functions from a limited set of genes.

In the last few decades by unraveling the details of mechanisms underlying natural genome editing, scientists have shown that such editing can also be induced by synthetic nucleases and more recently by the CRIPR system adopted from bacterial defense system which is one of the most promising gene editing technologies introduced in 2012.

1.2 The CRISPR/Cas system

Among the different types of defense systems found in prokaryotes and eukaryotes, a unique molecular system known as the CRISPR/Cas not only provides

protection against genetic attack, but also keeps a record or genetic memory of such attacks for future safety. This technique has been employed in several different studies by scientists from around the world for targeted genetic modifications in a plethora of living organisms, but perhaps most ambitiously by the Chinese scientists who not only used the technique for gene editing an armada of plant and animal species but also humans (<https://www.nature.com/articles/d41586-019-00673-1>), which raises certain ethical concerns about the practical application and subsequent implications of such studies.

A key factor for sustainable agriculture is improvement of crops via genetic engineering. It is true that DNA-free editing techniques are now desirable for molecular breeding in crops for which CRISPR/Cas may offer a better option for more complex and controlled genetic rearrangements. CRISPR arrays are characterized by a series of 20- to 50-bp genomic loci, which are unique spacers separated by direct repeats that usually have similar length to preceding AT-rich fragments [2]. CRISPR loci were discovered about two decades ago in *Escherichia coli* following viral infection as part of the immune system through which the bacteria keep a genetic memory of the viral genome fragments in order to defend against future infections if any other foreign DNA sequences match the unique spacer sequences in the memory (also known as protospacers) [2, 3]. Briefly speaking about the chief attributes of the mechanism of action, CRISPR-based defense system can be divided into three distinct events: adaptation, biogenesis of CRISPR-RNAs, and interference. Upon the introduction of a foreign DNA, the machinery selects protospacers that are inserted into the CRISPR locus following which CRISPR-RNAs (CrRNAs) are synthesized through a series of events, each with a corresponding spacer. Then, the Cas proteins interact with CrRNAs to initiate interference and consequent degradation of the foreign DNA by endonucleases [4]. It is now clear that CRISPR/Cas9 action is based on the participation of Cas9 protein and single guide RNA (sgRNA) [5].

Though CRISPR/Cas9 technology is relatively recent, it has started to be regularly used for gene editing in plants as well as animals to good success. It has been proved to be an efficient transgene-free technique. A simple search on PubMed (NCBI) shows that among all plants, 80 different studies published since 2013 involved CRISPR/Cas9-mediated genome editing in rice. Of these, 20, 13, and 24 papers have been published in 2019, 2018, and 2017, respectively. Furthermore, 20 different studies published since 2014 utilized CRISPR/Cas9 system for gene editing in wheat, where five of these studies were published in 2019 and seven were published in 2018. Genomes of other grain crops edited through this technique include maize, sorghum, barley etc. This indicates the high utility of this technique for gene editing in grain crops. Evidence that CRISPR/Cas9 system can be used for gene editing came around 2013 [6, 7] and the next paper showing the use of this technique for gene editing in rice and wheat was published in 2014 [8]. Furthermore, other studies published from 2013 to 2015 showed the use of this technique for gene editing in an array of organisms such as yeast, zebrafish, fruit flies, mosquitoes, nematodes, mice, monkeys, and human embryos, and several plant species, indicating sufficiently fast and easy application of this technique in this crop.

1.3 CRISPR/Cas9 for crop improvement in rice, wheat, and maize

The importance of agriculture for human survival cannot be questioned. As mentioned earlier, plants are the only primary producers on planet earth providing food, fiber, and other raw material generating the bulk of energy required for the growth of human population. However, plants/crops are facing several challenges and now their own survival may well be at stake. In this context, classical breeding

methods may not be suitable for increasing per unit area production relatively quickly. Hence, the rational use of biotechnological tools is of paramount importance. Editing crop genomes is a promising technique to cope with agricultural challenges. Development of these methodologies is useful for genetic improvement of crops [9]. In 2013, the pioneering works published by Lit et al. using *Arabidopsis* [10], Nekrasov et al. using tobacco [11], and Shan et al. using rice and wheat [12] demonstrated the suitability of the CRISPR/Cas9 system for crop genetic improvement. Since then, a plethora of research on crop genome editing via CRISPR/Cas9 has been published. Crops targeted for genetic editing via this system include soybean [13–15], tomato [16–19], potato [20], cucumber [21], maize [22, 23], the oil-seed plant camellia sativa [24], grapes and apple [25], *Brassica oleracea* and barley [26], watermelon [27], sweet orange [28], *Populus tomentosa* [29], and others to various ends.

The maize ARGOS8 variants generated via CRISPR/Cas9 showed significantly improved yield under drought stress conditions [30].

Rice, given its worldwide relevance for food, has been one of the most studied crops in terms of CRISPR/Cas9 application [31–36]. These include genetic modification for increased disease resistance [37], and herbicide resistance [38]. At present, more than 80 different research papers have been published using CRISPR-based genetic editing in rice and more than 15 papers involving wheat. These studies involve applicative use of CRISPR in rice. Li et al. [39] developed photo- and thermo-sensitive male sterile rice lines using this technique to exploit heterosis and speed up breeding [40]. Rice cultivars carrying high genetic resistance to the rice blast disease have been recently developed by Wang et al. [37]. Li et al. [41] at the South China Normal University, China, targeted four yield-related genes *Gn1a*, *DEP1*, *GS3*, and *IPA1* in the rice genome for modification via CRISPR/Cas9 for the improvement of different agronomic traits such as grain number and size, panicle structure indicating the application of this technique for improvement of agronomic traits in rice. Furthermore, the much required herbicide resistance in rice has also been genetically enhanced through CRISPR/Cas9-mediated gene editing [38]. More interestingly, the use of Cpf1 which is an endonuclease (alternative to Cas9) has been successfully used for targeted gene editing in rice and as a transcriptional regulator [42, 43]. More recently, a research group from University of Arkansas, USA, developed a soybean heat shock-inducible promoter CRISPR/Cas9 system for heritable mutations in rice. They transformed the HS-CRISPR/Cas9 vectors in rice and found only a low rate of target mutations before induction by heat shock compared to an increased rate of target mutations after heat shock treatment indicating that CRISPR/Cas9 is a controlled and efficient platform for gene editing in rice. This work is available online as *Pre-print* at BioRxiv [44]. Xu et al. in 2016 [45] mutated three rice genes *GW2*, *GW5*, and *TGW6* to increase seed size by up to 30% in the triple mutant lines.

Both durum and bread wheat have also been the subject of successful CRISPR/Cas9-mediated genetic modification for powdery mildew resistance and other objectives [42, 46–48]. However, in wheat, regeneration of plants from CRISPR-edited protoplasts has been difficult [12]. In addition, the complexity of wheat genome together with time-consuming tissue culture techniques has made it difficult for scientists to undergo ambitious genome editing projects via CRISPR/Cas9 in this important cereal. Researchers at the Chinese Academy of Science obtained the first CRISPR/Cas9-edited wheat plants [48] by editing three homoeoalleles of a hexaploid bread wheat cultivar to confer heritable resistance against powdery mildew of wheat. Later, the same research group obtained transgene-free genome-edited wheat plants using transient expression of CRISPR/Cas9 DNA or RNA [46]. In 2018, Wang et al. [49] knocked out all the wheat homologs of the rice *TaGW2*

(a gene that negatively regulates seed size) in order to significantly increase wheat kernel size. Transgene-free low-gluten wheat has been developed by Sánchez-León et al. via CRISPR/Cas9-mediated genetic engineering [50]. This indicates that CRISPR/Cas9 can be successfully applied to engineer cereal crops for higher yields.

In their book chapter, Chilcoat et al. [51] discuss the use of CRISPR/Cas9 for crop improvement in maize and soybean and have discussed the molecular details of gene editing projects via CRISPR/Cas9 such as those involving *WX1*, *ALS*, and *ARGOS8* genes in maize. Different studies report successful modification of maize genome using CRISPR/Cas technology [22, 23, 30, 52] for editing traits such as

S. no.	Plant/fungus	Gene/locus	Citation
1	Rice blast fungus (<i>Pyricularia oryzae</i>)	RNAP II	[57]
2		<i>TMS5</i>	[58]
3		MPK1 and MPK6	[59]
4		<i>HAK1</i>	[60]
5		<i>CDKA1</i> , <i>CDKA2</i> and <i>CDKB1</i>	[32]
6		<i>IAA23</i>	[61]
7		<i>EPSPS</i>	[62]
8		<i>GN1A</i> , <i>DEP1</i> , <i>GS3</i> and <i>IPA1</i>	[41]
9		<i>GBSSI</i>	[63]
10		<i>OsAPL2</i> and <i>OsAPS2b</i>	[64]
11		<i>TOS17</i>	[65]
12		<i>SBEI</i> and <i>SBEIIb</i>	[66]
13		<i>CALD5H1</i>	[67]
14		<i>NRAMP5</i>	[68]
15		<i>ERF922</i>	[37]
16		<i>EPFL9</i>	[69]
17	Wheat (<i>Triticum aestivum</i>)	<i>DREB2</i> and <i>ERF3</i>	[70]
18		<i>LOX2</i> and <i>UBIL1</i>	[71]
19		<i>MS1</i>	[72]
20		α - <i>GLIADINS</i>	[50]
21		<i>MS45</i>	[73]
22	Maize (<i>Zea mays</i>)	<i>AGO18A</i> and <i>AGO18B</i>	[22]
23		<i>MS8</i>	[49]
24		<i>DMC1</i>	[74]
25		<i>ZB7</i>	[75]
26		<i>ARGOS8</i>	[76]
27	Barley (<i>Hordeum vulgare</i>)	<i>CKX1</i> , <i>CKX3</i>	[77]
28		<i>PAPHY</i>	[78]
29		<i>ENGASE</i>	[79]
30	Sorghum (<i>Sorghum bicolor</i>)	<i>CAD</i> and <i>PDS</i>	[80]

Table 1.
List of studies involving CRISPR/Cas9-mediated gene editing in grain crops.

lignin biosynthesis, sterility, and herbicide resistance, secondary metabolism, RNA metabolism, drought tolerance, and agronomic traits [51]. Male sterility in maize provides a useful tool to harness hybrid vigor. Therefore, generation of male sterile mutant lines is considered to be of paramount importance in the hybrid seed production industry. Chen et al. in 2018 [49] used CRISPR/Cas9 to target the *MS8* gene of maize and obtained transgene-free *ms8* male sterile plants in the F2 generation that could be used for crossing with other elite lines for hybrid seed production. Zhu et al. in 1999 [53] manipulated maize genes *AHAs108* and *AHA109* encoding acetohydroxyacid synthase enzyme using chimeric RNA/DNA oligonucleotides. Similar studies were performed for targeting various genes in cereal crops such as maize, rice, wheat, and barley [54–56].

Table 1 summarizes the list of important studies involving CRISPR/Cas9-mediated gene editing in grain crops.

Conflict of interest

The authors declare no conflict of interest.

Author details

Adil Hussain^{1*}, Qari Muhammad Imran² and Byung-Wook Yun^{2*}

¹ Department of Agriculture, Abdul Wali Khan University, Mardan, Khyber Pakhtunkhwa, Pakistan

² School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Republic of Korea

*Address all correspondence to: adilhussain@awkum.edu.pk and bwyun@knu.ac.kr

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