CRISPR/Cas9-Edited Rice: A New Frontier for Sustainable Agriculture

23

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Abstract

With the exponential increase in the world's human population, improving agricultural productivity is among the top of the researchers' agendas till the 2050 deadline. One of the potential solutions to this global issue is genome editing because of the precision, fastness, and probably low cost involved compared to other traditional methods. It is in the spotlight especially from the last decade due to the discovery of sequence-specific-based nuclease technology including CRISPR/Cas9 tool. Initially, this tool was applied only in protoplasts and calli. However, due to the modifications in vectors, Cas9 variants, cassettes, cloning systems, multiplexing, and delivery methods, this platform has revolutionized the plant science field. It has been exploited in such a manner that about 16 crop plants have been already edited in the last few years. Out of all crops, most of the editing has been done in the case of rice (*Oryza sativa* L., Family: Poaceae), a cereal staple food. Therefore, in the current chapter, we have highlighted about

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the CRISPR/Cas9-edited rice for agronomic traits, stress tolerance/resistance, and biofortification. Additionally, we have presented an overview of various tools, databases, and commercial service providers devoted solely to CRISPR/Cas9 genome-editing technology.

Keywords
Plants · Agriculture · Yield · CRISPR/Cas · Online resources · Future crops

23.1 Introduction

In today's world, the human population is increasing exponentially and is expected to cross the whopping mark of 9.7 billion by the year 2050 (Valin et al. 2014; Baltes et al. 2017; Figueroa 2019). Furthermore, the whole scenario is expected to be affected greatly by the need to generate more space, reduce the overexploitation of natural resources, and tackle the uncertainties of climatic conditions and global warming (Cazzolla Gatti 2016; Oldeman et al. 2017; Morton et al. 2017; Subramanian 2018; Philander 2018; Pradinaud et al. 2019). In addition to this challenges, international food security, fighting chronic malnourishment, increasing awareness, and interest for healthier functional foods are at the top of the agendas (Siro et al. 2008; Abuajah et al. 2015; Martirosyan and Singh 2015; Atkins and Bowler 2016; Baltes et al. 2017; Pratim Roy 2019).

As our contemporary agricultural lands are degrading, it necessitates to re-think about the current agricultural practices, generation of elite varieties as well as efficient distribution of food (Wingeyer et al. 2015; Morton et al. 2017; Glenn et al. 2017; Banasik et al. 2017; Zhang et al. 2018; Dillard 2019). Solutions to all these challenges are unlikely to come from cross-breeding and mutation breeding (Kantar et al. 2019; Belkhodja 2018; Chen et al. 2019; Kleter et al. 2019; Mehta et al. 2019a; Singh et al. 2019; Rahman et al. 2019). Cross-breeding takes a large span of years to introduce desirable alleles (Darwin 2010; Scheben et al. 2017). Furthermore, this is limited by greatly reduced genetic variability. On the other hand, mutation breeding usually employs agents like ethyl methanesulfonate (EMS) and gamma rays to expand genetic variation by introducing random mutations (Bado et al. 2015, 2017; Pacher and Puchta 2017; Xuan et al. 2019). However, it is restricted by the large-scale mutant screening, high randomness, low efficiency, and stochastic nature. Furthermore, these approaches cannot keep pace with the whopping demand for increased crop production.

As a result, one of the potent approaches that can withstand the increasing crop productivity is genetic engineering (Marco et al. 2015; Baret and Vanloqueren 2017; Knott and Doudna 2018). It has been the spotlight around the globe to create new crop varieties (Sticklen 2008; Marco et al. 2015; Azadi et al. 2016; Arzani and Ashraf 2016; Kumari et al. 2018; Waltz 2018; Banerjee and Roychoudhury 2019; Zhang et al. 2019). Generally, it is defined as the targeted modification of DNA of any living organism belonging to any kingdom of classification using various tools

(Baltes et al. 2017). In accordance with the current and future scenario challenges, it easily addresses questions like (1) which traits need to be introduced, (2) which crops need to be focused on, (3) which DNA modifications must be done to generate the desired traits in the selected crops, (4) how to introduce these DNA modifications in the crop's genome, (5) how to overcome the bottlenecks of existing tools for crop improvement particularly, and (6) how to shift the agendas in accordance with the changing challenges. Due to the wide-ranging use, the enormous number of application falls under the big umbrella of genome engineering. As a result there is a wide range of potential products that could address food security/quality issues (Hsu et al. 2014; Wu et al. 2016; Nielsen and Keasling 2016; Khalid et al. 2017; Knott and Doudna 2018; Shigaki 2018; Waltz 2018; Pray et al. 2018; Merga et al. 2019; Zhang 2019).

Nonetheless, one of the significant tools that has been used enormously in agriculture is genome editing (Upadhyay et al. 2013; Laible et al. 2015; Alagoz et al. 2016; Ricroch et al. 2017; Gao 2018; Eş et al. 2019; Lassoued et al. 2019; Yin and Qiu 2019). This is truly reflected in numerous improved cultivars which have emerged within the last decade (Laible et al. 2015; Alagoz et al. 2016; Yin et al. 2017; Gao 2018; Yin and Qiu 2019). Here, we have highlighted different types of genome-editing tools for plants. Additionally, we have focused on the CRISPR/Cas9-edited rice for various traits and the current limitations and challenges within this field.

23.2 Genome-Editing Techniques for Plants

Perhaps the availability of numerous tools for DNA/RNA modifications, the sequence-specific nucleases have been the spotlight for the entire last decade (Porteus and Carroll 2005; Wright et al. 2005; Christian et al. 2010; Voytas 2013; Sprink et al. 2015; Zischewski et al. 2017; Waltz 2018; Novak 2019). These nucleases introduce targeted DNA double-strand breaks (DSBs) which are repaired by the cells itself by two evolved pathways, i.e., homologous recombination (HR) and nonhomologous end joining (NHEJ) (Puchta and Fauser 2014). However, in comparison, NHEJ is naturally an error-prone pathway which frequently results in small indels at the repair sites. Therefore, the researchers utilize this for targeted mutagenesis at a locus of interest. This NHEJ pathway exists in somatic, meiotic, and mitotic cells throughout the cell cycle, whereas HR-mediated repair pathway occurs only within the G2 and S phases of cells having mitotic activity (Huang and Puchta 2019; Jun et al. 2019).

In the present scenario, mostly genome editing is done by multiple technologies like meganucleases (Certo et al. 2012; Daboussi et al. 2015; Youssef et al. 2018), zinc-finger nucleases (ZFNs) (Porteus and Carroll 2005; Wright et al. 2005; Bilichak and Eudes 2016; Novak 2019), TALENs (Christian et al. 2010; Bilichak and Eudes 2016; Hensel and Kumlehn 2019), and CRISPR/Cas9 systems in plants (Bilichak and Eudes 2016; Knott and Doudna 2018; Waltz 2018; Huang and Puchta 2019). A detailed comparison of all these editing technologies is tabulated in Table 23.1. For more detailed information, the readers can look for publications by Gaj et al. (2013),

Table 23.1 A tabular comparison of major genome-editing technologies in plants

S. No.	Attributes	ZFNs	TALENs	CRISPR/Cas9
1	Cleavage type	Protein-dependent	Protein-dependent	RNA-dependent
2	Size	Significantly smaller than Cas9 (+)	Comparatively larger than ZFNs (++)	Significantly larger than both ZFNs and TALENs (+++)
3	Components	Zinc-finger domains, nonspecific FokI nuclease domain	TALE DNA-binding domains, nonspecific FokI nuclease domain	Cas9 protein, crRNAs
4	Catalytic domain(s)	FokI endonuclease domain	FokI endonuclease domain	HNH, RUVC
5	Structural components (dimeric/ monomeric)	Dimeric	Dimeric	Monomeric
6	Target sequence length	18–36	24–59	20–22
7	gRNA production required	No	No	Yes
8	Cloning required	Yes	Yes	No
9	Protein engineering steps needed	Yes	Yes	No
10	Mode of action	Induce DSBs in target DNA	Induce DSBs in target DNA	Induce DSBs or single-strand DNA nicks in target DNA
11	Restriction target site	High G	5′T and 3′A	PAM sequence
12	Level of target recognition efficiency	High	High	Very high
13	Targeting	Poor	Good	Very good
14	Mutation rate level	High	Low	Very low
15	Off-target effects	Yes	Yes	Yes, but can be minimized by the selection of unique crRNA sequence
16	Cleavage of methylated DNA possible	No	No	Yes, but it will be explored more
17	Multiplexing enabled	Highly difficult	Highly difficult	Yes
18	Labor intensiveness in experiment setup	Yes	Yes	No

(continued)

Table 23.1 (continued)

S. No.	Attributes	ZFNs	TALENs	CRISPR/Cas9
19	Possible to generate large-scale libraries	No	Yes, but it is highly challenging	Yes
20	Design feasibility	Difficult	Difficult	Easy
21	Technology cost	Very high (£1000–£3000)	High (£40–£350)	Comparatively low (£30–£300)
22	First report in plants	Durai et al. (2005), Lloyd et al. (2005)	Christian et al. (2010)	Feng et al. (2013), Shan et al. (2013), Miao et al. (2013)
23	First report in rice	Kim et al. (2012)	Li et al. (2012)	Jiang et al. (2013), Shan et al. (2013), Miao et al. (2013)

Puchta and Fauser (2014), Sprink et al. (2015), Bilichak and Eudes (2016), Noman et al. (2016), Baltes and Voytas (2015), Baltes et al. (2017), Malzahn et al. (2017), Kamburova et al. (2017), Lino et al. (2018), Shah et al. (2018), and Novak (2019).

23.3 CRISPR/Cas9 System for Fathomless Genetic Engineering

Currently, the most popular genetic cargo technology is CRISPR/Cas9 (Shan et al. 2013; Belhaj et al. 2013; Miao et al. 2013). This system has truly revolutionized the plant science research (Bilichak and Eudes 2016; Knott and Doudna 2018; Waltz 2018; Huang and Puchta 2019). As a result, various articles have been published throughout the last few years (Belhaj et al. 2013; Shan et al. 2014; Gao et al. 2015; Bilichak and Eudes 2016; Liu et al. 2017a, b; Liang et al. 2017; Knott and Doudna 2018; Butt et al. 2018; Abbott and Qi 2018; Huang and Puchta 2019). This is even supported by the fact that the keyword "CRISPR/Cas" in the paper title fetched about 5610 publications in Google Scholar (https://scholar.google.co.in/).

This CRISPR/Cas9 tool is popular due to the advantages such as simplicity, easy design, and easiness in delivery (Upadhyay et al. 2013; Baltes et al. 2017; Langner et al. 2018; Soda et al. 2018; Chen et al. 2019). CRISPR/Cas9 stand for clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated endonuclease 9 (Cas9) (Shan et al. 2013; Baltes et al. 2017). Both are integral components of the adaptive immunity system present within bacteria and archaea for protection against bacteriophages (Horvath and Barrangou 2010; Bondy-Denomy et al. 2013; Sampson et al. 2013; Shan et al. 2014). Based on this immunity mechanism, the CRISPR/Cas9 plant transformation vectors have been designed which carries guide RNA (gRNA) and Cas9 (Cong et al. 2013). In the initial days, it was applied in protoplast, calli, germ cells, and somatic cells (Shan et al. 2013; Feng et al. 2013, 2014a, b; Shen et al. 2014; Xing et al. 2014; Yin et al. 2015; Bhowmik

et al. 2018). Until now, various modifications have been done in CRISPR/Cas9 plant vectors (Shen et al. 2014; Ma et al. 2015; Mikami et al. 2015a, b; Osakabe et al. 2016; Tsutsui and Higashiyama 2017; Wang et al. 2018; Wu et al. 2018; Mahas et al. 2019). This is even supported by the fact that the optimized protocols are available for many plant species (Miao et al. 2013; Xing et al. 2014; Lowder et al. 2015; Char et al. 2017; Bhowmik et al. 2018; Osakabe et al. 2018; Li and Zhang 2019). Additionally, there is a plethora of available tools and databases devoted to the various omics technologies (Anamika et al. 2019) as well as CRISPR/Cas9 (Tables 23.2 and 23.3). Furthermore, there are many commercial service providers in the marketplace which provide many services and products related to the CRISPR/Cas9 technology (Table 23.4).

Furthermore, this CRISPR/Cas9-mediated genome-editing tool has been successfully implied in various plants (Cong et al. 2013; Upadhyay et al. 2013; Feng et al. 2014a, b; Shan et al. 2014; Svitashev et al. 2015; Malnoy et al. 2016; Alagoz et al. 2016; Liu et al. 2017a, b; Soda et al. 2018). For more detailed information, the researchers are advised to look for publication from the Korotkova and group (Korotkova et al. 2017, 2019).

Recently, Korotkova and colleagues published a cataloging article entitled "Current achievements in modifying crop genes using CRISPR/Cas system" (Korotkova et al. 2019). They studied all the published research articles on crop genome modifications from the Scopus database. In their article, they reported CRISPR/Cas-based genome-editing technology has been applied largely to the rice. The probable reason is being an established model plant which simultaneously counted as the highly valued cash crop worldwide (Khush 2005). This is even boosted by the availability of the rice genome sequence, sequence maps and multiple databases (Goff et al. 2002; Yu et al. 2002; Project, I.R.G.S. and Sasaki 2005; Smita et al. 2011; Zhao et al. 2014; Copetti et al. 2015; Zhang et al. 2016; Crossa et al. 2017). This is even supported by the surge in the number of publications related to the CRISPR/Cas9 (Fig. 23.1). Figure 23.2 highlights the key developments in the field of CRISPR/Cas9 technology for rice.

Typically, the CRISPR/Cas9 system success in rice relies mostly on two factors: (1) type of plant transformation vector and (2) the used delivery system. In general, the vectors carry essentially Cas9 (a endonuclease/nickase), T-DNA border region, selectable marker genes (plant and bacterial), ori site, and gRNA(s) (Alok et al. 2018) depending on the type of strategy-employed binary system, co-transformation, and/or multiplexing (Fig. 23.3). For more detailed information about the CRISPR/Cas9 vector components, the readers can look for publication by Alok et al. (2018).

Similarly, the CRISPR/Cas9-editing reagents (DNA/RNa,RNPs) are delivered into plant cells by particle bombardment (Shan et al. 2014; Sun et al. 2016; Li et al. 2016a, b, c, 2019), *Agrobacterium*-mediated transformation (Shan et al. 2013, 2014; Xu et al. 2014; Hu et al. 2016; Lu and Zhu 2017; Wang et al. 2019), or protoplast transfection (Xie and Yang 2013; Tang et al. 2019; Lin et al. 2018). The overall workflow for rice genome editing using CRISPR/Cas9 is depicted in Fig. 23.3.

Table 23.2 Tabular account of available CRISPR/Cas9 tools in plants

S. No.	Tool	Specification	Website URL address	Provider	References
1	CRISPR.mit	Tool to facilitate the design of gRNAs	http://crispr.mit.edu/	Zhang Lab	Hsu et al. (2013)
2	sgRNA designer	Online tool for effective sgRNA designing	http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design	Broad Institute	Doench et al. (2014)
3	E-CRISP	Web application to design gRNA sequences	http://www.ecrisp.org/ECRISP/	German Cancer Research Center	Heigwer et al. (2014)
4	CRISPRseek	Part of R programming package for designing gRNAs	http://www.bioconductor.org/packages/release/bioc/html/CRISP Rseek.html	Bioconductor	Zhu et al. (2014)
5	Cas-OFFinder	Algorithm for identifying potential off-target sites in a genome	http://www.rgenome.net/cas-offinder/	Seoul National University	Bae et al. (2014)
6	CHOPCHOP	Online tool for predicting off-target binding of sgRNAs	http://chopchop.rc.fas.harvard.edu/	Harvard University	Montague et al. (2014)
7	CRISPRscan	sgRNA-scoring algorithm that effectively captures the activity of CRISPR/Cas9 in vivo	http://www.crisprscan.org/	Giraldez Lab (Yale University)	Moreno-Mateos et al. (2015)
8	CRISPRdirect	Web server for selecting rational CRISPR/Cas targets based on input sequence	http://crispr.dbcls.jp/	Database Center for Life Science	Naito et al. (2014)
9	PROTOSPACER	Web interface for finding, evaluating and sharing Cas9 guide-RNA designs	http://www.protospacer.com/	BIHP-Institute Pasteur (France)	MacPherson and Scherf (2015)
10	sgRNA Scorer 1.0	In vivo library methodology to assess sgRNA activity	http://crispr.med.harvard.edu/sgRNAScorerV1/	Wyss Institute for Biologically Inspired Engineering at Harvard	Chari et al. (2015)
11	CRISPR Multi-Targeter	Online tool to find sgRNA targets	http://www.multicrispr.net/	IWK Health Centre and Dalhousie University	Prykhozhij et al. (2015)

(continued)

Table 23.2 (continued)

S. No.	Tool	Specification	Website URL address	Provider	References
12	Off-Spotter	An algorithm to assist in designing optimal gRNAs	http://cm.jefferson.edu/Off-Spotter/	Thomas Jefferson University	Pliatsika and Rigoutsos (2015)
13	WU-CRISPR	Web tool for the genome-wide design of sgRNAs	http://crispr.wustl.edu	Xiaowei Wang Lab	Wong et al. (2015)
14	Breaking-Cas	Web tool to facilitate the design of guide RNA for CRISPR/Cas technique	http://bioinfogp.cnb.csic.es/tools/breakingcas	Spanish National Center for Biotechnology	Oliveros et al. (2016)
15	CHOPCHOP v2	An updated version of CHOPCHOP which improves the targeting power, usability, and efficiency of CHOPCHOP by offering new options for sgRNA design	http://chopchop.cbu.uib.no/	University of Bergen	Labun et al. (2016)
16	CRISPOR	Web tool to find guide RNAs from an input sequence	http://crispor.tefor.net/	University of California (Santa Cruz)	Haeussler et al. (2016)
17	CCTop	Online, intuitive user interface for designing of guide RNAs	http://crispr.cos.uni-heidelberg.de/index.html	University of Heidelberg	Stemmer et al. (2015)
18	sgRNA Scorer 2.0	Tool to identify sgRNA PAM sites for gene sequence	http://crispr.med.harvard.edu/sgRNA ScorerV2/	Wyss Institute for Biologically Inspired Engineering at Harvard	Char et al. (2017)
19	CRISPR-P 2.0	Web-services for computer-aided sgRNA designing with minimal off-target activity	http://crispr.hzau.edu.cn/CRISPR2/	National Key Laboratory of Crop Genetic Improvement and Center for Bioinformatics, Huazhong Agricultural University	Liu et al. (2017a, b)
20	GuideScan	Software for designing gRNA libraries for various genomic regions	http://www.guidescan.com/	Leslie Lab and Ventura Lab	Perez et al. (2017)
21	CRISPR-GE	Convenient, integrated toolkit to expedite all experimental designs and analyses of mutation for CRISPR/Cas/Cpf1-based genome editing in plants and other organisms	http://skl.scau.edu.cn	Liu YG Lab, The Genetic Engineering Laboratory of South China Agricultural University	Xie et al. (2017)

22	CRISPR-Local	High-throughput tool for designing single-guide RNAs in plants and other organisms	http://crispr.hzau.edu.cn/CRISPR-Local/	National Key Laboratory of Crop Genetic Improvement and Center for Bioinformatics, Huazhong Agricultural University	Sun et al. (2019)
23	CRISPR-PLANT v2	Tool to predict off-target sites found in unbiased genome-wide studies	http://www.genome.arizona.edu/crispr2/	Arizona Genomics Institute	Minkenberg et al. (2019)

Table 23.3 List of available CRISPR/Cas9 databases for plant systems

S. No.	Database	Purpose	URL address	Institution name	References
1.	CrisprGE	A central repository for CRISPR/Cas-based editing	http://crdd.osdd.net/servers/crisprge/	CSIR-IMTECH, India	Kaur et al. (2015)
2.	Cas-Database	Genome-wide gRNA library design tool for Cas9 nucleases from *Streptococcus pyogenes*	http://www.rgenome.net/cas-database/	Center for Genome Engineering, Institute for Basic Science, Korea	Park et al. (2016)
3.	Cpf1-Database	Genome-wide gRNA library design tool for Cpf1	http://www.rgenome.net/cpf1-database/	Center for Genome Engineering, Institute for Basic Science, Korea	Park and Bae (2017)
4.	CRISPRlnc	A manually curated database of validated sgRNAs for lncRNAs	https://www.crisprlnc.org/	Bioinformatics Group of XTBG, Chinese Academy of Sciences	Chen et al. (2019)
5.	PGED (Plant Genome Editing Database)	Database for storing information about CRISPR-mediated mutants in any plant species	http://plantcrispr.org/cgi-bin/crispr/index.cgi	Boyce Thompson Institute	Zheng et al. (2019)

23.4 CRISPR/Cas9 in Rice for Increasing Food Production

One way to address the global food demand is to increase the crop yield. However, it affects various factors including selection of high-yielding/stress-tolerant cultivars, modification of existing cultivars, nutrient supply, water supply, and weed–pest management. In the past 5 years, the use of genome editing was in its infancy; however, there are numerous successful reports currently in the literature.

23.4.1 Agronomic Traits Improvement

The most common way to improve the overall yield is to increase the grain number, weight, and size (Sakamoto and Matsuoka 2008; Xing and Zhang 2010; Baltes et al. 2017). Genetically the underlying grain number, weight, and size are directly linked with hundreds of genes and quantitative trait loci. Various major genes/QTLs have been molecularly characterized and edited using the CRISPR/Cas9 system in rice. Gene editing through CRISPR/Cas9 in rice cultivar Zhonghua for loss of function mutation in genes for grain number (Gn1a), grain size (GS3), panicle architecture (DEP1), and plant architecture (IPA1). Mutated rice plants exhibit higher grain

Table 23.4 List of commercial companies available for the implementation of CRISPR/Cas9 technology

S. No.	Commercial companies	Website link	Headquarters
1	System Biosciences	https://www.systembio.com/	California, United States
2	Sigma–Aldrich	https://www.sigmaaldrich.com/	Darmstadt, Germany
3	Integrated DNA Technologies (IDT)	https://eu.idtdna.com/pages/	Iowa, United States
4	New England Bio Labs	https://www.neb.uk.com/	Hertfordshire, England
5	GeneCopoeia	https://www.genecopoeia.com/	Maryland, United States
6	DNA 2.0	https://www.atum.bio	California, United States
7	ORiGene	https://www.origene.com/	Maryland, United States
8	Eurofins Genomics	https://www.eurofinsgenomics.co.in/	Karnataka, India
9	Genscript	https://www.genscript.com/	New Jersey, United States
10	Oxford Genetics	https://www.scienceexchange.com/	California, United States
11	Cellectis	https://www.cellectis.com/en/	New York, United States
12	Pacific Biosciences	https://www.pacb.com/	California, United States
13	Addgene	https://www.addgene.org/	Maryland, United States
14	Macrogen	http://www.macrogen.com/en/main/index.php	Seoul, North Korea
15	ThermoFisherScientific	https://www.thermofisher.com/in/en/home.html	Massachusetts, United States

number, larger grain size, and dense panicle, and ipa1 mutant shows lesser as well as higher panicle number depending on mutation in the target site of miR156 (Li et al. 2016a, b, c). Knockout mutation in Japonica rice, Kitaake cultivar for *LAZY1* gene, exhibits higher tiller number (Miao et al. 2013).*OsCAld5H1* gene knockout by CRISPR/Cas9 in rice leads to enrichment of G units in lignins and reveals its role in the synthesis of non-c-p-coumaroylated S lignin units (Takeda et al. 2019). Mutation in abscisic acid receptor family of genes, PYLs through CRISPR/Cas9 in rice, leads to improved growth and enhanced productivity (Miao et al. 2018). Grain weight in rice is regulated by GW2, GW5, and TGW6. The multiple gene editing of all three genes by CRISPR/Cas9 in rice shows larger grain size as compared to non-edited rice (Xu et al. 2016). Multiplex editing of genes *Hd2*, *Hd4*, and *Hd5* mediated by CRISPR/Cas9 leads to early maturity in rice (Li et al. 2017a, b). All these results together provide information regarding already edited genes in various rice cultivars for enhancing agronomic traits (Table 23.5).

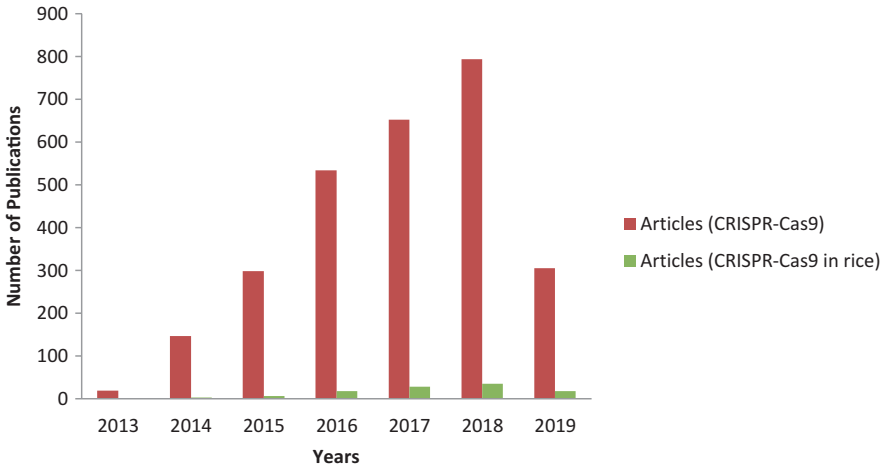

Fig. 23.1 The graph representing the number of publications per year related to CRISPR/Cas9 and CRISPR/Cas9 in rice by years 2013–2019. Keywords used in PubMed search included CRISPR/Cas9 and rice. (Accessed on April 1, 2019)

23.4.2 Enhanced Stress Tolerance/Resistance

A major bottleneck to the current rice productivity is due to the losses incurred by pests, pathogens, and weeds. These biotic stresses are estimated to decline global agricultural productivity by 40% (Mew et al. 1993; Oerke 2006; Savary et al. 2012). In a favorable environment, blast disease causes 60–100% yield loss in rice-growing area (Kihoro et al. 2013). Blast is one of the most devastating diseases in rice caused by *Magnaporthe oryzae* (Zhang et al. 2014). Great efforts were made in the last few decades for developing blast-resistant rice cultivar through the application of genomics tools. Through conventional breeding approaches, blast-resistant rice has been developed (Fukuoka et al. 2014; Ashkani et al. 2015). Conventional breeding approaches are tedious in nature and need a longer duration. Other limitations like the existence of pathogen variability and the emergence of new pathotype cause breakdown of resistance barrier leading to severe disease infestation. Recent advanced technologies like CRISPR/Cas9, TALEN, and ZFNs could be alternative approaches for engineering rice genome for acquiring disease-resistant phenotype. Blast disease-resistant phenotype is reported in rice by the disruption of ethylene-responsive factor 922 (*OsERF922*) gene-mediated through CRISPR/Cas9 (Wang et al. 2016). Targeted mutation through CRISPR/Cas9 in the ethylene responsive factor 922 provides blast disease resistance in rice (Liu et al. 2012). Expression of *OsSWEET13* gene in rice responsible for bacterial blight disease and indica rice, IR24, with improved resistance for bacterial blight disease has been developed through CRISPR/Cas9 knockout targeting promoter of *OsSWEET13* (Zhou et al. 2015). By disrupting, promoter of *OsSWEET14* gene by TALEN technology results in resistance towards bacterial blight in rice (Li et al. 2012). TALEN technology is

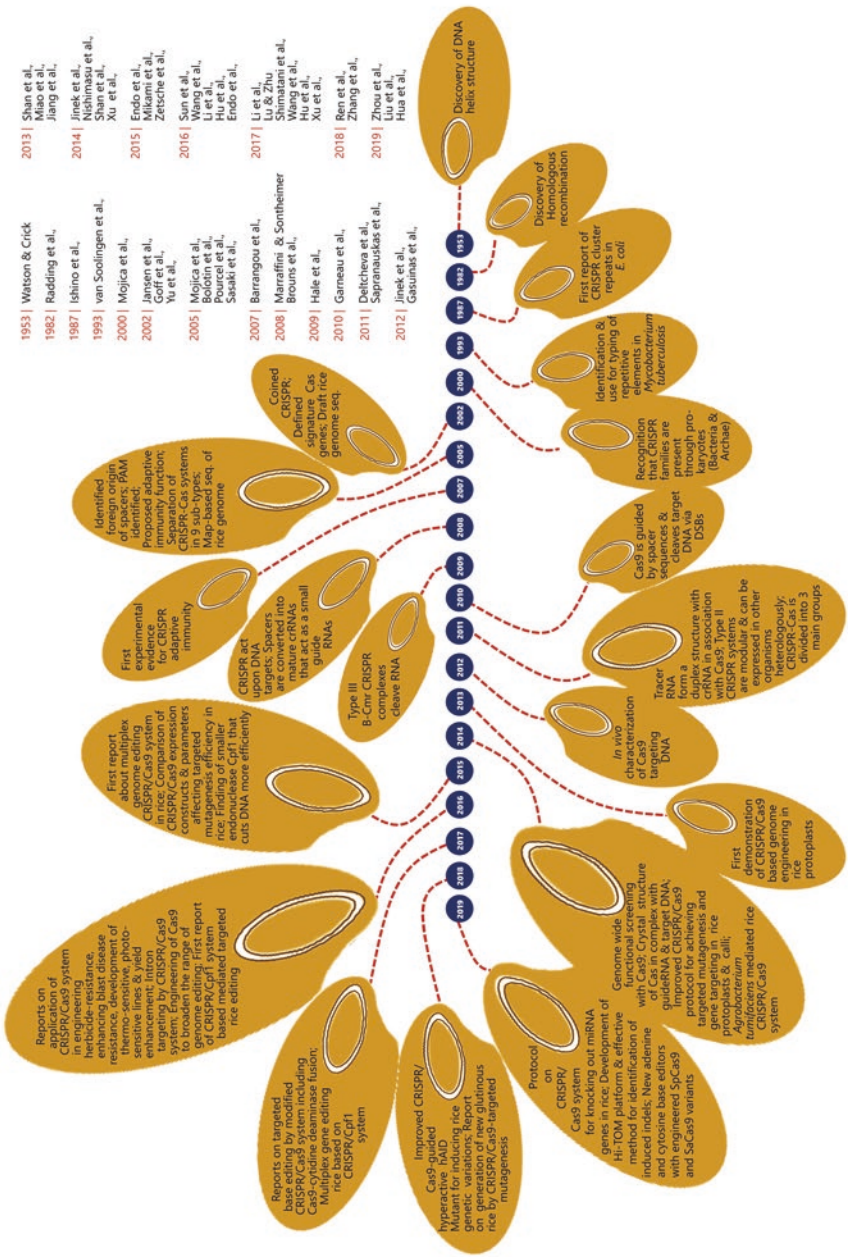

Fig. 23.2 A timeline of key developments of CRISPR/Cas9 technology in rice

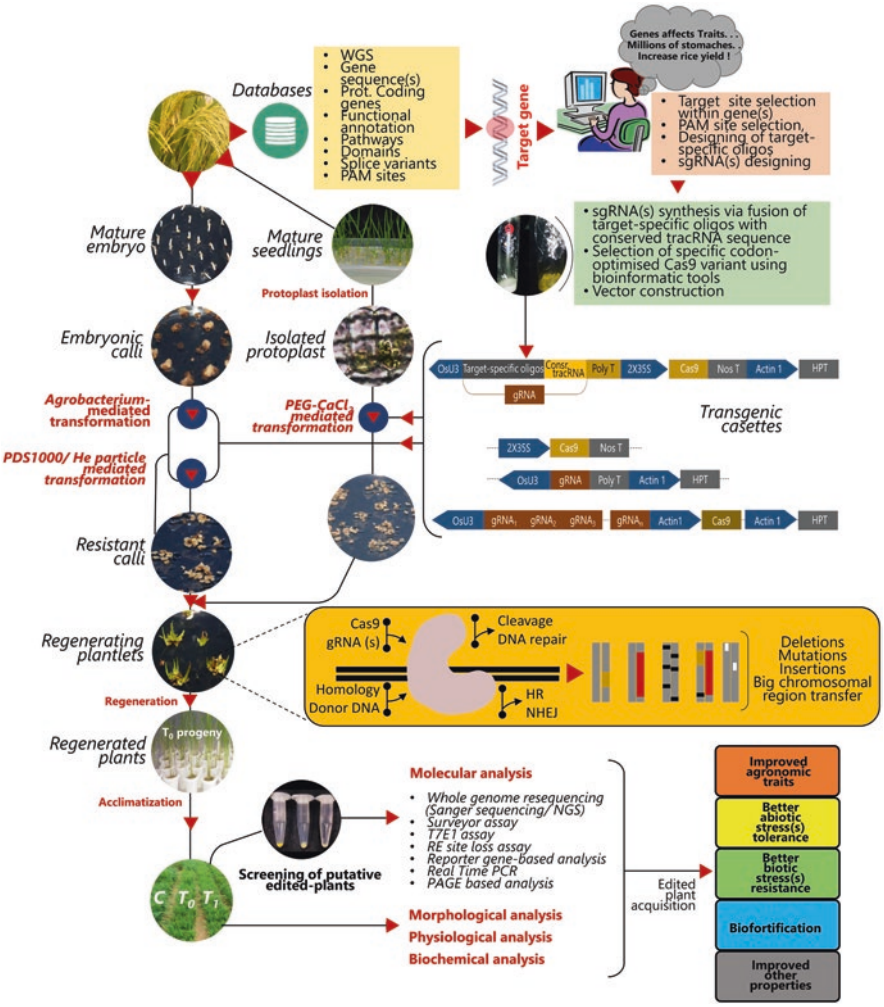

Fig. 23.3 Workflow illustrating the successive steps for rice genome editing using CRISPR/Cas9 technology in rice

employed for modifying the promoter of *Os09g29100* gene to nullifying EBEtal7 interaction, which could provide tolerance to BLB disease in rice (Cai et al. 2017). TALENs targeting effector binding elements (EBEs) of AvrXa7 and Tal5 disrupt their interaction with the susceptible gene *Sweet14*. The edited rice plants were resistant to *Xanthomonas* infection. These technologies are quite helpful in developing rice cultivar with tolerant phenotype for Xoo (Li et al. 2012).

Table 23.5 Summary of CRISPR/Cas9-mediated genome editing in rice for agronomic traits

S. No.	Gene	Gene function	Delivery method	Cultivars	% mutations/ HR	Mutant plant	References
1	*LAZY1*	Tiller number	*Agrobacterium*-mediated	Kitaake (Japonica)	–	More tiller number	Miao et al. (2013)
2	*Gn1a*	Grain number	*Agrobacterium*-mediated	Zhonghua (Japonica)	42.5	Higher grain number	Li et al. (2016a, b, c)
3	*GS3*	Grain size	*Agrobacterium*-mediated	Zhonghua (Japonica)	57.5	Larger grain size	Li et al. (2016a, b, c)
4	*DEP1*	Panicle architecture	*Agrobacterium*-mediated	Zhonghua (Japonica)	67.5	Dense panicle	Li et al. (2016a, b, c)
5	*IPA1*	Plant architecture	*Agrobacterium*-mediated	Zhonghua (Japonica)	27.5	More panicle number	Li et al. (2016a, b, c)
6	*OsCAld5H1*	Lignin synthesis	*Agrobacterium*-mediated	–	94	Enriched G-units in lignin	Takeda et al. (2019)
7	*PYLs*	Abscisic acid receptor	*Agrobacterium*-mediated	Nipponbare (Japonica)	–	Improved growth and productivity	Miao et al. (2018)
8	*GW2*, *GW5*, *TGW6*	Grain width and grain size	*Agrobacterium*-mediated	–	–	Larger grain size	Xu et al. (2016)
9	*Hd2*, *Hd4*, *Hd5*	Suppressor of flowering	*Agrobacterium*-mediated	–	–	Early maturity	Li et al. (2017a, b)

Rice production constrained by viral disease including rice tungro disease (RTD) plays a major role in reducing rice production in rice-growing areas (Azzam and Chancellor 2002; Muralidharan et al. 2003; Chancellor et al. 2006). Through the development of near-isogenic lines (NILs), it is confirmed by the researchers that resistance to RTSV and RTBV depend on the translation and in-frame mutation of initiation factor 4 gamma (*eIF4G*) gene respectively (Lee et al. 2010; Macovei et al. 2018). In-frame mutation in *eIF4G* gene in rice confers resistant phenotype for RTSV (Macovei et al. 2018).

In addition to pathogen resistance, weed management is also considered as a critical factor in optimizing the crop yield. One of the effective ways is the application of herbicides on the field. Herbicide-resistant gene, bentazon-sensitive lethal (BEL) knockout by CRISPR/Cas9, and biallelic mutated rice confer sensitivity to bentazon. This trait could be successfully utilized for hybrid seed production (Xu et al. 2014). CRISPR/Cas9-mediated gene replacement of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) having the desired substitution gives glyphosate-resistant phenotype in rice (Li et al. 2016a, b, c). Herbicide-tolerant rice cultivar is generated by mutation in the *ALS* gene by genome editing (Li et al. 2016a, b, c; Sun et al. 2016). TALEN technology was used for creating double-point mutation mediated through homology-directed repair (HR) in *OsALS* rice gene (Li et al. 2016a, b, c). Rice *ALS* gene also mutated at multiple points using CRISPR/Cas9 HR and edited rice plant shows tolerance to bispyribac sodium (BS) spraying, and wild-type rice died after 36 days of herbicide spray (Sun et al. 2016). A point mutation generated in acetolactate synthase (*ALS*) gene through CRISPR/Cas9 coupled with cytidine deaminase confers tolerance to imazamox herbicide (Shimatani et al. 2017a, b) (Table 23.6).

Next to biotic stress, abiotic stresses are considered a factor that controls the rice productivity (Mehta et al. 2019a, b). It includes flooding, drought, heavy metal stress, metalloid stress, and heat stress (Dhakate et al. 2019). Rice plant is extremely sensitive under low temperature especially during the early stage of development. Therefore, the improvement of rice varieties for cold tolerance could significantly enhance productivity in rice. For enhancing cold tolerance in rice, *TIFY1b* and its homology gene *TIFY1a* were edited through CRISPR/Cas9 (Huang et al. 2017). Osmotic stress/ABA-activated protein kinase 2 (*OsSAPK2*) knockout mutant-mediated by CRISPR/Cas9 exhibits higher sensitivity for drought and reactive oxygen species than control rice plant (Lou et al. 2017).

23.4.3 Biofortification

Next to increasing food production, improving food nutritional value is the biggest hurdle to the researchers. This demand has increased globally with the hike in household incomes and food-related awareness in developing countries. As a result, nowadays consumers require food with properties such as reduced cholesterol,

Table 23.6 Summary of CRISPR/Cas9-mediated genome editing related to various stresses

S. No.	Gene	Gene function	Delivery method	Cultivars	% mutations/ HR	Mutant plant	References
1	*BEL*	Herbicide resistance	*Agrobacterium*-mediated	Rice cultivar Nipponbare	2–16	Sensitive to bentazon	Xu et al. (2014)
2	*SWEET13*	Negative regulator of blast resistance	*Agrobacterium*-mediated	Indica rice IR24	–	Resistance to bacterial blight	Zhou et al. (2015)
3	*ERF922*	Negative regulator of blast resistance	*Agrobacterium*-mediated	Japonica rice variety Kuiku131	42	Enhance blast resistance	Wang et al. (2016)
4	*EPSP*	Tolerance to glyphosate	Biolistic transformation	Rice variety Nipponbare	2	Resistance to glyphosate	Li et al. (2016a, b, c)
5	*ALS*	Tolerance to bispyribac sodium (BS)	*Agrobacterium*-mediated	–	–	Tolerance to bispyribac sodium (BS)	Sun et al. (2016)
6	*TIFY1a/TIFY1b*	Cold tolerance	*Agrobacterium*-mediated	Rice cultivar Nipponbare	35–87.5	Tolerance to cold	Huang et al. (2017)
7	*ALS*	Resistance to herbicide imazamox (IMZ)	*Agrobacterium*-mediated	Rice cultivar Nipponbare	3.41	Resistance to imazamox (IMZ)	Shimatani et al. (2017a, b)
8	*SAPK2*	Tolerance to drought	*Agrobacterium*-mediated	Rice cultivar Nipponbare	–	Sensitive to drought	Lou et al. (2017)
9	*eIF4G*	Susceptibility to rice tungro virus	*Agrobacterium*-mediated	Indica rice IR64	36.0–86.6	Resistance to rice tungro spherical virus (RTSV)	Macovei et al. (2018)

Table 23.7 Successful reports of CRISPR/Cas9-mediated genome editing in rice biofortification

S. No.	Gene	Gene function	Delivery method	Genotype name	% of mutation/HR	Mutant plant	Reference
1.	*SBEI*, *SBEIIb*	Starch debranching enzyme	*Agrobacterium*-mediated	Kitaake (Japonica)	26.7–40	Enhanced amylose content	Sun et al. (2017)
2.	*Nramp5*	Cadmium transporter	*Agrobacterium*-mediated	Indica	70–82.4	Low-grain cadmium content	Tang et al. (2017a, b)
3.	*OsPDS*, *OsSBEIIb*	Phytoene desaturase, starch debranching enzyme	*Agrobacterium*-mediated	–	20	Targeted mutations were generated	Li et al. (2017a, b)
4.	*BADH2*	Betaine aldehyde dehydrogenase	*Agrobacterium*-mediated	–	–	Enhanced fragrance	Shao et al. (2017)
5.	Waxy gene	Starch synthesis	*Agrobacterium*-mediated	Japonica	82.76	Reduced amylose content	Zhang et al. (2018)
6.	*ISA1*	Amylose synthesis	*Agrobacterium*-mediated	Zhonghua11	–	Reduced amylose and amylopectin content	Chao et al. (2019)

biofortified whole grains, and low wax. As a result, various researchers have successfully used CRISPR/Cas9 technology for biofortification especially in rice.

Loss of function mutation through CRISPR/Cas9 of waxy gene in rice has reduced amylose content (Zhang et al. 2018). CRISPR/Cas9-mediated loss-of-function mutation of the starch debranching enzymes SBEI and SBEIIb has higher amylose content and resistant starch (Sun et al. 2017). Knockout of *ISA1* gene through CRISPR/Cas9 in rice exhibit reduced amylose and amylopectin contents. The mutant seeds were altered with shrunken endosperm and lesser grain weight (Chao et al. 2019). Loss-of-function mutation of Nramp5 through CRISPR/Cas9 in rice have low cadmium content when grown in cadmium-contaminated field (Tang et al. 2017a, b). Targeted mutations through modified CRISPR/Cas9 (nCas9 containing cytidine deaminase) for *OsPDS* and *OsSBEIIb* in rice were generated (Li et al. 2017a, b). Knockout of *Badh2* gene mediated by CRISPR/Cas9 in rice exhibits enhanced aroma (Shao et al. 2017). Table 23.7 summarizes the successful reports of rice biofortification.

23.5 Insights into the CRISPR/Cpf1: An Alternative to CRISPR/Cas9

In addition to Cas9, scientists have reported other Cas family members for genome editing in the last 5 years. One of the promising members is Cpf1 (CRISPR from *Prevotella* and *Francisella*1) (Zetsche et al. 2015). The mechanisms of CRISPR/Cpf1 and CRISPR/Cas9 are compared in Table 23.8. In order to draw out more information, the researchers are suggested to refer to the publications by Endo et al. (2016), Wang et al. (2017), Xu et al. (2017), and Jun et al. (2019).

All the successful reports regarding the application of CRISPR/Cpf1 in rice are enlisted in Table 23.9.

Table 23.8 Comparison of Cas9- and Cpf1-mediated editing

Attributes	Cas9	Cpf1 (Cas12a)
gRNA components	tracrRNA and crRNA	crRNA
gRNA length (bp)	≤100	≤43
Type of ends produced	Blunt ends	Sticky ends
Type of overhang generated	No	5′ overhang
Target PAM site	G-rich	T-rich
PAM sequence	5′-NGG-3′	5′-TTTN-3′
Cutting site	3–4 bp upstream to the PAM site	18–24 bp downstream to the PAM site
RNase III required	Yes	No
Off-target effects	Yes, comparatively higher	Yes, comparatively low
Nickase generation	Possible, already done	Impossible

Table 23.9 Summary of CRISPR/Cpf1-employed editing in rice

S. No.	Tool	Gene	Gene function	Cultivars	Delivery method	%mutations/ HR (%)	Mutant plant	Reference
1	CRISPR/ FnCpf1	*DL* (drooping leaf)	Midrib formation	Japonica rice cultivar Nipponbare	*Agrobacterium*-mediated	8.3–60	All mutants show a loss of midrib leading to drooping leaf phenotype	Endo et al. (2016)
2	CRISPR/ FnCpf1	*ALS* (Acetolactate synthase)	Involved in the synthesis of branched chain amino acids	Japonica rice cultivar Nipponbare	*Agrobacterium*-mediated	15–60	Loss of *ALS* activity leading to lethality	Endo et al. (2016)
3	CRISPR/ FnCpf1	*RLK-798, RLK-799, RLK-802, RLK-803*	Receptor like kinases	Japonica rice	–	43.8–75	–	Wang et al. (2017)
4	CRISPR/ FnCpf1	*NAL1*	Phosphate (Pi) accumulation	Japonica Rice	*Agrobacterium*-mediated	–	Enhanced phosphate accumulation	Hu et al. (2017)
5	CRISPR/ FnCpf1	*LG1*	Legule formation	Japonica Rice	*Agrobacterium*-mediated	–	No ligule formation	Hu et al. (2017)
6	CRISPR/ LbCpf1	*BEL-230, BEL-240, BEL-250, BEL-260*	Bentazon-sensitive lethal	Japonica rice	–	40–60	–	Wang et al. (2017)
7	CRISPR/ FnCpf1 and CRISPR/ LbCpf1	*MPK2, MPK5*	Mitogen-activated protein kinase	Japonica rice Zhonghua 11	*Agrobacterium*-mediated	9–32	–	Ding et al. (2018)

8	CRISPR/FnCpf1 and CRISPR/LbCpf1	*PDS*	Phytoene desaturase	Japonica rice Zhonghua 11	*Agrobacterium*-mediated	–	Albino	Ding et al. (2018)
9	CRISPR/FnCpf1 and CRISPR/LbCpf1	*DEP1*	Dense and erect panicle	Rice protoplast	PEG-$CaCl_2$-mediated	90	Scattered panicle	Zhong et al. (2018)
10	CRISPR/FnCpf1 and CRISPR/LbCpf1	*ROC5*	Leaf rolling	Rice protoplast	PEG-mediated	–	Outcurve rolled leaves	Zhong et al. (2018)
11	CRISPR/LbCpf1	*EPFL9*	Regulation of stomatal density and patterning	Indica rice cultivar IR64	*Agrobacterium*-mediated	–	Altered stomatal pattern and density	Yin et al. (2019)

23.6 Conclusion

In the past five decades, crop improvement via traditional breeding has significantly contributed to acquiring food security for the every second whopping human population. However, various developments require more manpower, time duration, efforts along with high chance of failures in getting the "desirable traits". Additionally, other conventional technologies like chemical mutagenesis, somaclonal variation, in vitro tissue culture and physical irradiation have also multiple loopholes. For increasing crop production under the changing climate as well as fulfilling the calorific and nutritional demands of mankind, the most recent, advanced nuclease-based technologies have emerged as the most suitable candidate in many crops including rice. Among all these technologies, the CRISPR/Cas9 tool is more precise, easy to handle, and also employed for avoiding backcrossing of a huge number of inbred lines. The varietal development using CRISPR/Cas9 technology consumes less time and is easy to introduce/restore desired changes in the existing elite rice germ plasm. Recently, multiple genes have been stacked together to get the desired phenotype in rice. Additionally, due to the advances like base editing, gene targeting, and DNA-free genome editing, the rice researchers have affirmatively taken a big leap towards the biggest milestone, i.e., super rice generation. Furthermore, due to the technical advances in the post-genomic era, the researchers have characterized a plethora of negative regulatory genes, SNPs, and QTLs for various traits. Taking these points, we hope that our children will be eating the socially accepted, highly nutritious super rice in the long run in the future.

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