CRISPR/Cas for Improved Stress Tolerance in Rice

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Abstract

The discovery of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) system has revolutionized genome editing technology. Though in nature it is found in bacteria and archaea as a defense mechanism against viruses, it has been successfully repurposed as an effective and robust genome editing tool in all forms of life, e.g., bacteria, plants, animals, and humans. The utilization of this ingenious tool in agriculture is increasing day by day as it can be used to introduce the gene of interest in a specific site within the genome and to eliminate the expression of a gene of choice through knockout at DNA or RNA level. To date, this technology has effectively installed resistance against both abiotic and biotic stresses in different crops. In this chapter, we have discussed the basic mechanisms of CRISPR/Cas and its latest classification. Further, we discuss the recent successes of this tool in rice breeding, which is the staple food for billions of people around the world. Finally, we highlight the prospects of CRISPR/Cas technology in providing resistance against stresses in rice.

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Keywords

Abiotic stress · Biotic stress · Climate change · Genome editing · Rice · Resistance breeding

12.1 Introduction

12.1.1 Introduction to CRISPR/Cas

In the late 2019, readers of *The Irish Times* declared gene editing as the innovation of the last decade (O'Connell 2019) because it has opened a new dimension to scientists in the field of biological science by allowing them to alter an organism's (humans, plants, microbes, etc.) DNA and consequently enabling them to develop stress-resilient crop varieties (Zafar et al. 2019), treat inherited diseases (Rajeev Rai and Cavazza 2021), understand a gene's function (Martin et al. 2016), and sometimes even detect unknown species in the environment (Baerwald et al. 2020). Among several types of gene-editing tools such as ZFN (zinc finger nucleases), TALEN (transcription activator-like effector nucleases), CRISPR/Cas, etc., the latter one is most conveniently applied due to its robustness, editing efficiency, simplicity, and most importantly, flexibility (Adli 2018). The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas (CRISPR-associated proteins) is a microbial adaptive immune system that cleaves foreign genetic elements by using RNA-guided nucleases and can be utilized to facilitate efficient genome engineering in eukaryotic cells (Ran et al. 2013). This technology involves the engineering of a single guide RNA (sgRNA) and base-pairing between the sgRNA and the target DNA site that remains adjacent to the protospacer adjacent motif (PAM) followed by a double-stranded breakage (DSB) on the genome by Cas endonucleases (Wang et al. 2017a).

The DSB repair machinery and the outcome of the process play a key role in determining the nature of a genome edit (van Overbeek et al. 2016). There are two common pathways, i.e., non-homologous end joining (NHEJ) and homology-directed repair (HDR), either of which may facilitate the DSB repair (Barman et al. 2020). In higher eukaryotes, NHEJ is the leading pathway for DSB repair that may result in either deletions or insertions or substitutions (commonly termed as indels) at the break site (Shen et al. 2017b). On the other hand, the HDR repair pathway induces specific genetic changes to the DSB by the introduction of a homologous DNA repair template and results in precise point mutations, gene deletions, or insertions of genes of interest (Fig. 12.1) (Salsman and Dellaire 2017).

12.1.2 CRISPR/Cas in Agriculture

The global agricultural production needs to be enhanced drastically as, by 2050, the world's population will be around 9.6 billion, increasing the demand for staple crops

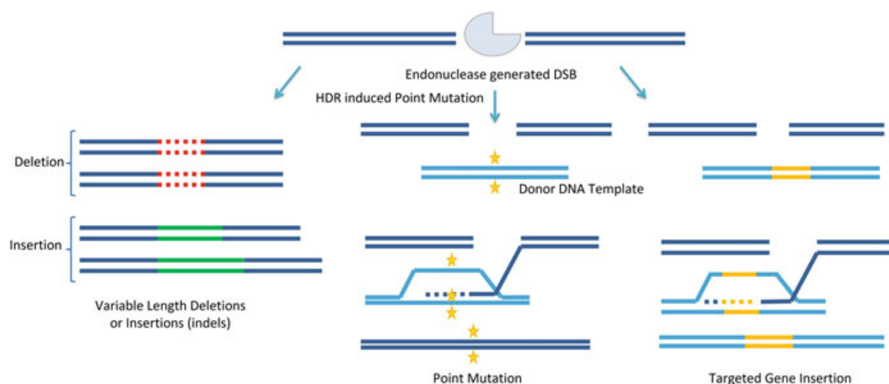

Fig. 12.1 Double-stranded breakage induced by CRISPR/Cas followed by the repair mechanism through non-homologous end joining (NHEJ) or homology-directed repair (HDR). The NHEJ may result in either deletion or insertion (indels) and HDR could be used to introduce a point mutation or insert a gene of interest; a donor template is required to be delivered in cells with CRISPR/Cas machinery for HDR

by 60%. However, the conventional crop breeding techniques alone cannot accomplish this objective as these methods are often time-consuming, laborious, and complicated. Hence, CRISPR/Cas, a rapid and more reliable technology, has been widely used in improving several crop characteristics (yield, quality, disease resistance, herbicide resistance, etc.) in recent years (Zhu et al. 2020). Since its first application in 2012, advancement in CRISPR/Cas technology has revolutionized research in the field of life sciences (Gao 2018) especially in the fields of functional genomics and crop improvement by allowing researchers to develop novel plant varieties with either deletion of harmful traits or addition of desired characteristics (Arora and Narula 2017). CRISPR/Cas has been employed in influencing the genome of different plant species including *Arabidopsis*, *Medicago truncatula*, tomato, potato, wheat, corn, rice, etc. (Afzal et al. 2020).

CRISPR/Cas was first introduced in agriculture in 2013, and since then, it has been successfully implemented in several crop species. One study reported targeted mutagenesis in the tomato *PMR4* gene could generate higher resistance in comparison to RNAi-silenced transgenic plants (Santillán Martínez et al. 2020). In another report, CRISPR/Cas9 gene editing was employed to knock out the *BBL* genes that are responsible for nicotine production in tobacco plants resulting in the development of nicotine-free, non-transgenic plants, thus reducing the risk of death from tobacco use (Schachtsiek and Stehle 2019). CRISPR/Cas9 engineering also demonstrated potential for genetic modification of potato that has high nutritional value and is considered one of the major starch-producing crops. Successful knockout of the *GBSS* gene (responsible for the synthesis of amylose enzyme) of tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts resulted in mutations in all four alleles without stable integration of DNA (Andersson et al. 2017).

The CRISPR/Cas system can induce targeted changes in the genomes of elite crop varieties and is effective in a wide range of major cereal crop species (Scheben et al. 2017), for example, in developing low-gluten, non-transgenic wheat variety (Sánchez-León et al. 2018); generating a novel maize variant that showed improved grain yield under stressful drought condition (Shi et al. 2017); providing resistance in barley against the *wheat dwarf virus* (Kis et al. 2019); contributing to soybean breeding and regional adaptability by the mutagenesis of *GmFT2a* and *GmFT5a* (responsible for flowering activation) but most significantly in rice (Ricroch et al. 2017) to provide either resistance against abiotic stress like salinity tolerance (Zhang et al. 2019a) or biotic stresses, e.g., bacterial blight-resistant variety development (Makarova et al. 2020); conferring resistance to *rice tungro spherical virus* (Macovei et al. 2018); providing resistance to *Xanthomonas oryzae* pv. oryzae (Kim et al. 2019), etc.

12.1.3 Economic Importance of Rice and Production Constraints

Rice is the most common cereal crop and acts as the staple food for approximately half of the world's population. In Asia alone, over 2 billion people obtain 80% of their energy from the consumption of rice. Not only that, reports have been made that rice contains lesser antioxidant molecules along with several other medicinal properties in comparison to other cereal crops, making it an ideal contender for a natural source of antioxidants and exploitation in the pharmaceutical industry (Chaudhari et al. 2018). In addition to providing calories, rice is a potential source of magnesium, phosphorus, manganese, iron, folic acid, selenium, thiamine, and niacin. It also contains low fiber and fat (Fukagawa and Ziska 2019). Moreover, a recent study investigated the antidiabetic activity of purple rice bran and discovered the great potential for its application to improve hepatic insulin signaling and in decreasing hepatic gluconeogenesis (Hlaing et al. 2019).

However, due to various constraints such as biotic factors (insects and pests, weeds, diseases, etc.) and abiotic factors (scarcity of good quality water, salt stress, nutrient imbalance, climatic factors, etc.), overall rice productivity has been harmfully affected (Fahad et al. 2019). A quantification study in Tamil Nadu reported a total loss of 2.73 million tonnes of rice due to various constraints that are about 39.45% of the total production (Shanmugam et al. 2006). Another report suggested that drought alone can be responsible for as much as 40% loss in rice production, reducing income up to 58% in South and Southeast Asia. Furthermore, it has been reported that owing to several rice diseases, more than 40% yield of the total harvest is lost in South Asia where it represents the first source of caloric intake (Savary et al. 2012).

12.1.4 CRISPR/Cas in Rice

It is estimated that by 2030, rice production will need to be increased by one-fourth percent of the current production to meet the demand of the expanding global population (Ansari et al. 2015). Hence, anticipating the immense importance of rice in the present and upcoming future, numerous measures have been undertaken in the previous years to ensure adequate rice production by improving yield (Khan et al. 2015), developing tolerance against biotic (Sreewongchai et al. 2010), and abiotic (Singh et al. 2010) stresses, etc. Since its first application in 2013 (Shan et al. 2013), CRISPR/Cas-mediated genome editing has demonstrated immense potential in rice breeding towards an improved production because of its ease of use, economic nature, and efficiency (Bandyopadhyay et al. 2019).

One of the most prominent applications of CRISPR/Cas technology in rice is the construction of a genome-wide mutant library that can be utilized to find out gene functions, genetic improvement, and functional characterization of unknown genes. Another significant use could be the precise elimination of selective marker genes in transgenic plants to further improve breeding techniques. Besides, CRISPR/Cas can also be applied to rice to provide biotic and abiotic stress tolerance, improving grain yield, replacing alleles efficiently, and thereby, hastening the crop improvement process through the induction of point mutation or base editing or multiplex genome editing or insertion or deletion of a precise region of a gene (Romero and Gatica-Arias 2019). In this chapter, we'll discuss various stresses of rice production and applications of different types of CRISPR/Cas technologies to overcome the hurdles. We'll also discuss different delivery methods of CRISPR/Cas that have previously been employed on rice. Additionally, we'll highlight the success of CRISPR/Cas-mediated gene editing in providing stress tolerance/resistance to date in rice and its future implications for increasing overall rice productivity for a sustainable future.

12.2 Stresses Hampering Rice Production

The global rice production has declined in the past years owing to various constraints that include limited yield potential of high-yielding varieties, pressure from abiotic and biotic stresses, socioeconomic concerns, increasing production costs, etc., and if appropriate measures are not immediately carried out to address and reduce the effects of these factors on the overall rice productivity, then serious food scarcity may occur shortly in different parts of the world (Van Nguyen and Ferrero 2006). Some of the major abiotic and biotic stresses in rice are described below along with stating their potential threats to loss of rice yield and productivity.

12.2.1 Abiotic Stresses in Rice

Abiotic stresses are induced by abiotic factors, i.e., unfavorable environmental conditions (extreme temperature, cold stress, drought stress, salt stress, etc.), causing

significant variation in the ideal production environment of plants and thus resulting in the visible decline of their growth, development, and production (Fahad et al. 2019). Abiotic stresses not only play a crucial role in the yield loss of rice but also are responsible for lower grain quality which in turn causes decreased consumer acceptance (Fahad et al. 2019).

12.2.1.1 High-Temperature Stress

Due to global warming and the greenhouse effect, the global air temperature has significantly increased in the past few decades. This high temperature is responsible for increasing floods, storms, and other adverse calamities worldwide and eventually affecting overall food production. Not only that, but an increase in temperature is also accountable for amplifying the atmospheric CO_2 concentration that possesses a severe threat to crop production (Wheeler et al. 2000). Temperature is a key aspect in controlling several features in rice such as germination, seedling growth, leaf emergence, tillering, heading, plant height, dark respiration, grain filling, grain quality, yield, etc. Therefore, a temperature rise may cause serious alteration in rice structure and a decline in overall productivity. Additionally, high temperature disturbs the water, ion, and organic solute movement across plant membranes and thereby affects photosynthesis and respiration causing a reduction in yield performance (Krishnan et al. 2011).

In many tropical and subtropical countries, high day temperature has caused significant loss of rice production (Fahad et al. 2019). Asia is the biggest global rice contributor accounting for about 87% of the global rice production. Rice exports from Asian regions especially from China and India (about 49% of the world's rice producers) play a key role in maintaining global food security (Bandumula 2018). However, due to climate change and increasing temperature, several reports on yield reduction have been made in major rice-producing regions of Asia such as China (Lv et al. 2018), India (Setiyono et al. 2018), Malaysia (Vaghefi et al. 2011), and Vietnam (Thuy and Saitoh 2017). Similar outcomes of yield reduction with increasing temperature have been reported in Africa, another leading rice-producing region of the world (Adhikari et al. 2015; van Oort and Zwart 2018).

12.2.1.2 Cold Stress

Cold stress concludes both chilling injuries (under 20 °C) and freezing injuries (under 0 °C) and is one of the most significant abiotic stresses reducing production and yield of several major crops such as rice, maize, soybean, and cotton (Thakur et al. 2010). Cold stress possesses an immense threat to rice production affecting both vegetative and reproductive phases of its life cycle with the latter one being more prominent. As a result, abnormal development of anthers, low spikelet fertility, and eventually notable yield losses are observed (Bai et al. 2015).

Recent studies on cold stress on rice production have suggested that low temperatures during long, cold springs in several low-altitude and high-altitude regions of China, Japan, Korea, and other parts of the world can result in inhibition of germination and restrict early seedling growth (Zhang et al. 2014). One study in Southern China recorded the cold stress effect on rice from the year 1981 to 2009

and represented production loss with decreasing temperature (Zhang et al. 2016). It is indicated that if the temperature drops below 15 ° C, the germination and seedling growth of rice will be severely affected (Lv et al. 2019).

12.2.1.3 Drought Stress

Drought is termed as the inadequacy of water for a while due to insignificant rainfall, unavailability of water in the soil, and lack of moisture in the air that in turn causes continuous loss of water from plants through excessive evaporation and transpiration (Singh et al. 2018). Drought is one of the primary stresses in rice, because of which plant growth and development are severely hampered eventually resulting in reduced grain yield of rice (Sharifunnessa and Islam 2017). Severe drought stress in rice causes economic yield loss in both reproductive (48–94%) and grain-filling stage (60%) (Kim et al. 2020).

Drought negatively affects rice production and yield stability and causes rigorous yield loss in many rainfed areas in many Asian countries but most significantly in Eastern India and adjacent parts of Nepal (more than 17 million hectares of rainfed area) which is considered as the largest drought-prone regions of Asia (Palanog et al. 2014). In 1987 and 2002/2003, owing to severe drought, 50% of the total cropped area of India was affected and more than 300 million people had to suffer across the country. Thailand encountered adverse effects of drought in the year 2004 affecting 20% of rice lands and more than eight million people (Wassmann et al. 2009).

12.2.1.4 Salt Stress

Normal salt pH ranges between 4.5 and 7.5 making the soil most favorable for nutrient availability and plant growth. Salt stress is the condition when the soil contains a high concentration of soluble salts such as sodium (Na^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chloride (Cl^-), and sulfate (SO_4^{2-}), etc. creating an environment that negatively impacts plant growth (Hussain et al. 2017). More than 90% of the world's rice is grown in Asia where approximately 60% of the earth's population resides. One of the harshest abiotic stresses that affect rice plants in their early seedling stage and cause serious yield and production loss in Asia is soil salinity (Kumar et al. 2013).

A study on the impact of salt stress on the growth and yield of some native rice cultivars of Kerala in India reported significant height reduction in two cultivars among the seven studied. Further, tiller production was seen to have decreased in three cultivars due to salt stress. Additionally, panicle length, spikelet per panicle, and fertility percentage were found to have reduced due to salinity (Joseph 2013). Rice production and yield in Pakistan also declined due to increased salinity in approximately ten million ha of irrigated land (Zaman et al. 2018).

12.2.2 Biotic Stresses in Rice

Biotic stress refers to the infection of different pathogens as well as herbivore pests in plants under natural conditions that possesses an enormous threat to plant

productivity and yield by causing many diseases (Suzuki et al. 2014). Rice is the second most important cereal crop in the world in terms of productivity and is considered the principal food of most developing countries (Molla et al. 2019). Among various constraints of rice production, diseases are the major factors behind low yields of rice throughout the world. To date, more than 70 diseases caused by bacteria, fungi, viruses, and nematodes have been reported in rice (Singh et al. 2020).

Bacterial leaf blight, primarily reported in Japan during 1884–1885 and then in many other major rice-growing regions of the world (Gnanamanickam 2009), is responsible for serious damage of rice plant and can result in a yield reduction of 20–40% and 50% if infection occurs during tillering stage and early stage, respectively (Chukwu et al. 2019). Bacterial leaf streak can reduce yields of rice up to 8–17% in the wet season and 1–3% in the dry season. However, it can be more severe in certain areas; for instance, studies in India reported yield loss of rice to reach as high as 30% due to the bacterial leaf streak (Kumar et al. 2017). Another major bacterial disease frequently occurring in rice is bacterial sheath rot causing grain sterility and notable yield loss (Rostami et al. 2005). In Indonesia, an extreme yield loss of 72.2% was recorded due to the disease, whereas the highest yield loss in Malaysia was 20%.

Rice blast is a major fungal disease in rice, usually causing 30% yield loss which could be fed to 60 million people, and not only that, the disease has even been regarded as capable of causing 100% yield loss. Some reports on yield loss of rice due to blast disease have been reported in India (5–10%), Korea (8%), China (14%), and the Philippines (50–85%) (Fahad et al. 2019). Another most significant fungal disease of rice is brown spot that can cause serious damage to rice production (up to 90%) and was responsible for the great Bengal famine during 1942. The percentage of yield loss varies depending on the rice cultivar and stage of infection, mostly ranging between 18.75% and 22.50% (Sunder et al. 2014). Rice production has been acutely damaged in tropical regions especially in South Asia due to sheath blight, a fungal disease, whose infection is favored by warm temperature and high humidity. The disease was first reported in Japan in 1910 and can cause a yield loss of up to 45% (Singh et al. 2019).

Among the viral diseases of rice, tungro disease is highly significant as it possesses a great deal of economic and social consequences on the rice production of Asia and Southeast Asia (more than 90% of the world's rice producer and consumer) and is estimated to cause an annual yield loss of 5% to 10% as well as an economic loss of approximately US $1.5 billion (Dai and Beachy 2009). Africa is the second-largest rice importer in the world representing 25% of the world's rice importation (Woin et al. 2010). Rice yellow mottle virus was first reported in Kenya (Bakker 1974), and since then, it has been reported in many countries in East and West Africa. This disease is one of the most damaging diseases of rice in Africa and can cause a yield loss of 10–100% depending on plant age and susceptibility of the rice variety (Kouassi et al. 2005).

Here, Table 12.1 represents the major bacterial, fungal, and viral diseases that are responsible for high quantities of annual yield loss along with their causal organisms, disease symptoms, and commonly occurring regions in the world.

Table 12.1 Different major diseases of rice caused by bacteria, fungi, and bacteria, their causal organisms, typical symptoms, and occurrence

Pathogen	Disease name	Causal organism	Disease symptoms	Occurrence
Bacteria	Bacterial leaf blight	*Xanthomonas oryzae* pv. oryzae	Kresek phase (sudden wilting and death of plant), leaf wilting and upward rolling, leaf color change from grayish-green to yellow, water-soaked lesions, milky bacterial ooze, plant drying	Southeast Asia (Zhou et al. 2013), Africa (Verdier et al. 2012), Australia, Latin America, and the Caribbean (Mew 1989)
	Bacterial leaf streak	*Xanthomonas oryzae* pv. *oryzicola*	Dark-green and water-soaked streaks on interveins, streaks soon turn yellow or orange-brown, infection in the florets and seeds discoloration and death of ovary, stamens, browning of glumes	Southern and Central China, Southeast Asia, and Africa (Wu et al. 2019)
	Brown sheath rot	*Pseudomonas fuscovaginae*	Symptoms on the flag leaf sheath and the panicle, a systemic discoloration spreading to the midrib or veins of the leaves, yellow to brown discoloration of seedlings, necrosis, grain discoloration	Temperate regions of Asia, Africa, South America, and Australia (Kakar et al. 2014)
Fungi	Rice blast	*Magnaporthe oryzae*	White to gray-green lesions or spots, lesions can enlarge and coalesce to kill the entire leaf	China, India, Japan, South Korea, Indonesia (Wang and Valent 2009)
	Brown spot	*Bipolaris oryzae*	Small, circular, yellow-brown or brown lesions, spikelet and floret infection, grain-filling disruption, and grain quality reduction and discoloration	Japan, China, Burma, Sri Lanka, Bangladesh, Iran, Africa, South America, Russia, North America, Philippines, Saudi Arabia, Australia, Malaya, and Thailand (Sunder et al. 2014)

(continued)

Table 12.1 (continued)

Pathogen	Disease name	Causal organism	Disease symptoms	Occurrence
	Sheath blight	*Rhizoctonia solani*	Greenish gray lesions on the leaf sheaths, sclerotia	Africa, Bangladesh, Brazil, Burma, Colombia, China, Cuba, Germany, Fiji, Formosa, India, Indonesia, Iran, Korea, Liberia, Madagascar, Malaya, Malaysia, Netherland, Nigeria, Papua New Guinea, Philippines, Russia, Senegal, Sri Lanka, Surinam, Taiwan, Thailand, Trinidad, Tobago, UK, USA, Venezuela, and Vietnam (Singh et al. 2016)
Virus	Tungro disease	Combine infection of *rice tungro bacilliform virus* (RTBV) and *rice tungro spherical virus* (RTSV)	Presence of leafhoppers, leaf (yellow or orange-yellow) discoloration, leaves show mottled or striped appearance, interveinal necrosis	Philippines, India, Indonesia, Malaysia, Bangladesh, Nepal, Pakistan, Sri Lanka, Vietnam, China, and Japan (Dai and Beachy 2009)
	Rice yellow mottle disease	*Rice yellow mottle virus* (RYMV)	Yellow-green spots on the base of the youngest leaves, mottled and twisted leaves, discoloration and poor panicle exertion, reduced tillers, sterile spikelets, and eventually death	Kenya, Liberia, Nigeria, Sierra Leone, Tanzania, Nigeria, Burkina Faso, Mali, Malawi, Rwanda, Madagascar, Gambia, Guinea Bissau, Senegal, Mauritania, Zanzibar, Cameroon, and Chad (Kouassi et al. 2005)

12.3 Structure of CRISPR/Cas

The CRISPR/Cas system mainly consists of a CRISPR array that includes short direct repeats spaced by short variable DNA sequences termed as a spacer. This CRISPR array is flanked by a variety of *cas* genes. In front of the CRISPR array,

Fig. 12.2 Diagrammatic representation of different components of CRISPR/Cas system. The *cas* genes produce the Cas proteins required for acquiring new spacers from invader DNA, crRNA biogenesis, and interference. The CRISPR array contains palindromic repeats and spacers that transcribe into pre-crRNA. In the upstream of the CRISPR array, there is a leader sequence that contains a promoter for the expression of pre-crRNA

there is a leader sequence that contains the promoters required to transcribe the CRISPR array (Fig. 12.2). There is no open reading frame present in the CRISPR array. The repeats are identical direct repeats in sequence and they can be 21 to 50 nucleotides long. The number of repeats varies from organism to organism ranging from 2 to several hundred (mostly around 50). The spacers, on the other hand, are highly variable in the sequence of similar size ranging from 20 to 84 nucleotides long. These sequences can be identical to sequences from bacteriophages, plasmids, or rarely from chromosomes. The leader sequence is always present upstream of the CRISPR array. It also does not contain any open reading frame and could be several hundred nucleotides long (AT-rich); however, it contains all the necessary promoters and protein binding sites required for the biogenesis of crRNAs (Amitai and Sorek 2016).

12.4 Mechanism of CRISPR/Cas System

The overall mechanism of CRISPR/Cas can be divided into three major steps, e.g., adaptation, crRNA biogenesis, and interference (Fig. 12.3). These major steps are discussed in brief as below.

12.4.1 Adaptation

This is the first step of the CRISPR/Cas mechanism where a complex of Cas proteins functions together to bind to a target DNA, mostly upon recognizing a distinct, short motif called “protospacer-adjacent motif” of simply PAM presented on the upstream or downstream of the target DNA. After binding to the target DNA, the Cas proteins cleave a portion of that target DNA (known as protospacer) and insert it into the spacer of the CRISPR array which is then called the “spacer” (Amitai and Sorek 2016). There are other types of CRISPR/Cas system that acquire such spacer from RNAs through the reverse transcription procedure governed by reverse transcriptase enzymes encoded by the CRISPR/Cas locus (Makarova et al. 2020). Such spacer sequences are stored in the CRISPR array as the immunological memory. The spacer is integrated between the leader sequence and the first repeat of the CRISPR array

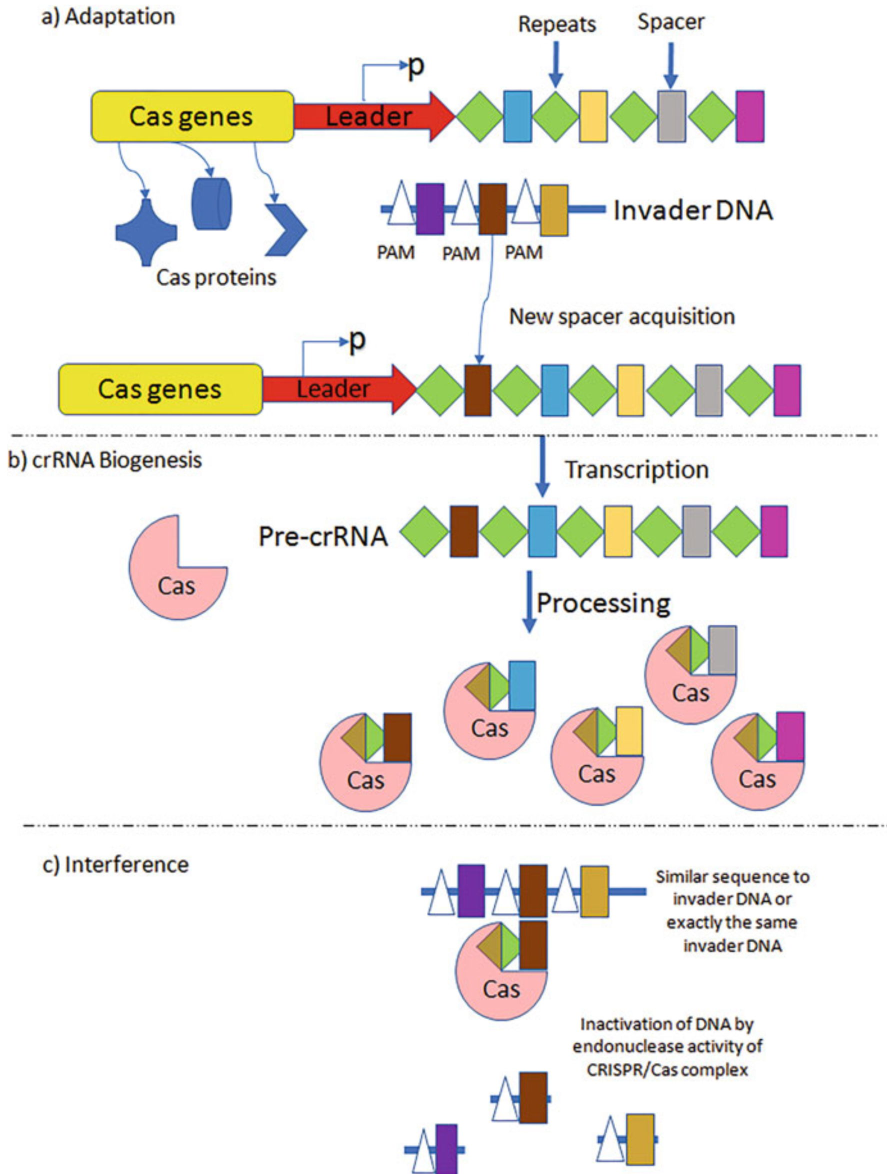

Fig. 12.3 A simplified graphical representation of CRISPR/Cas mechanism. (**a**) The adaptation step: the cas protein complexes recognize an invader DNA acquiring a new "spacer" into the CRISPR array. (**b**) Expression/crRNA biogenesis: the *cas* genes express Cas protein with endonuclease activity and the CRISPR array is expressed into pre-crRNA through transcription using the promoter sequence present in the upstream leader sequence of the CRISPR array. The pre-crRNA is further processed into mature crRNA by Cas protein to form the CRISPR/Cas complex. (**c**) Interference: when an invader DNA is present in the cell having complementary sequence with the CRISPR/Cas complex, the complex binds with the invader DNA at the complementary site and cleaves it into pieces, thus inactivating the invading DNA

and is accompanied by a duplication of the repeat. Several protospacers could be added to the array (each with its repeat) from a single invader to enhance the resistance level.

12.4.2 Expression/crRNA Biogenesis

This step starts with the transcription of the CRISPR array that is driven by a promoter situated in the leader sequence and produces a long precursor CRISPR-RNA (pre-crRNA) that is processed further by the distinct subunit of multiprotein Cas complex or by a single multidomain Cas protein, depending on the variant, into small mature crRNAs. Sometimes, this processing also involves accessory factors, such as non-Cas host RNases (Wimmer and Beisel 2020). Each crRNA contains a part of a repeat on its 5′ side, a part or all spacer, and sometimes also a part of the repeat on the 3′ side.

12.4.3 Interference

Interference takes place when the same foreign nucleic acid tries again to invade the cell itself or the daughter cells. The crRNAs form a complex (Cas-crRNA complex) with the one or multiple Cas protein. These complexes scan the invading nucleic acid and find the protospacer sequence (with the help of the PAM and seed sequences; if this is a system that uses PAM). crRNP inactivates this DNA or RNA by silencing or degradation. Most CRISPR-Cas systems recognize and attack DNA. Some systems attack ssRNA or both DNA and mRNA during transcription.

12.4.4 Distinguishing Between Target and Genomic CRISPR Array

To eliminate the possibility of harming self-DNA, CRISPR/Cas systems need to distinguish between the target DNA and its own CRISPR array. The PAM sequence is found responsible for such safety mechanisms in type I and type II CRISPR/Cas systems. The PAM sequence is situated adjacent to the protospacer sequence that is essential for recognition and cleavage of target DNA. Oppositely, it is absent in the spacer sequence of the CRISPR array. Therefore, the CRISPR/Cas system never cleaves its own CRISPR array. However, in type III system, such self-discrimination does not depend on the PAM. Rather, it utilizes the 5′ handle of the crRNA that interacts with the repeat sequence in the CRISPR locus followed by the inhibition of nuclease recruitment. Thus, the cleavage of self-DNA is prevented (Burmistrz and Pyrć 2015).

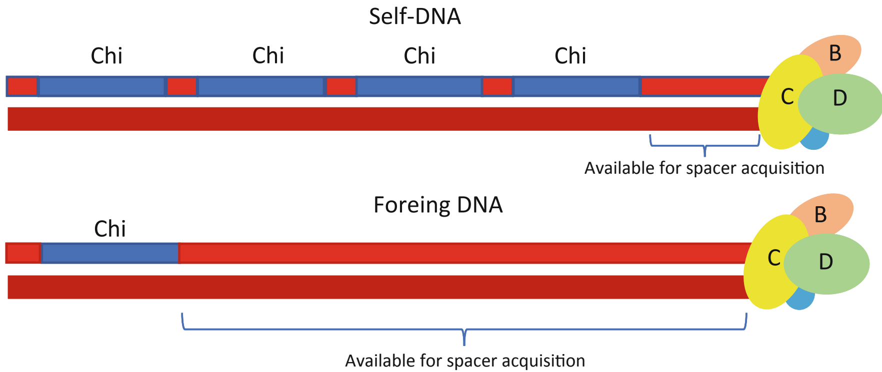

Fig. 12.4 Richness in Chi-site in *E. coli* genome prevents the degradation activity by RecBCD having only a small portion available for spacer acquisition, whereas the foreign DNA has fewer Chi-sites resulting in long-range DNA degradation by RecBCD and having many materials for spacer acquisition

12.4.5 Negligence of Acquiring Spacer from Self-DNA

In nature, the acquisition of spacer from the chromosomal DNA of the organism instead of the invader DNA is detrimental as it leads towards the breakage of self-DNA by the interference mechanism of the CRISPR/Cas system. Such an event leads to CRISPR/Cas autoimmunity. Organisms adopt different changes in their genome to prevent such autoimmunity when accidentally the CRISPR/Cas system obtains a spacer from its own chromosomal DNA. Such changes involve inactivation of the Cas genes through mutation, bringing changes in the sequence of the repeats next to the self-derived spacer or changing the PAM sequence. However, such mutational changes might not always be favorable. Therefore, the CRISPR/Cas system should eliminate the chances of acquiring self-DNA and avoid any unwanted impacts on the organism itself. It has been observed that the CRISPR/Cas system is indeed fond of acquiring spacer from foreign invading DNA rather than from its own chromosomal DNA. Such preference is believed to be based on the RecBCD machinery and Chi-sites. For instance, the *E. coli* genome is high in Chi-sites (once in every 4.6 kb, on average); as a result, even if there is a DSB the RecBCD only degrades a short length of the self-DNA, and the degradation is halted by the adjacent Chi-site. In comparison to the self-DNA in *E. coli*, the Chi-sites in the exogenous DNA are less densely distributed, e.g., around one Chi-site in every 65 kb. As a result, the RecBCD degrades long-length DNA that ultimately generates ample substrates for new spacers (Fig. 12.4). Even if the system acquires spacer from its own genomic DNA, the system may mutate or delete the specific spacer, mutate or delete flanking repeat, mutate or delete the PAM sequence, mutate or delete the *cas* genes, and even sometimes delete the whole system to avoid any kind of detrimental impact (Wimmer and Beisel 2020).

12.5 Classification of CRISPR/Cas and Frequently Used Systems in Rice Genome Editing

To date, there have been six types of CRISPR/Cas system reported which were further classified into two major classes (Makarova et al. 2020). The classification of CRISPR-Cas systems is based primarily on Cas protein composition differences and sequence divergence between the effector modules. The *cas* genes of different types of CRISPR/Cas system can be broadly divided into four categories based on their function; however, some of them might have an overlapping role. The first category, the adaptation module, includes enzymes that are involved in spacer acquisition. Cas1 and Cas2 are common in all the types and Cas4 is seen in Types I, II, and V. In Type III, a reverse transcriptase (RT) enzyme is involved in this function. In all the types of Class 1, the pre-crRNA processing is done by Cas6 enzyme. In Class 2 large effector Cas proteins, Cas9, Cas12, and Cas13 play this role in Types II, V, and VI, respectively. However, in Type II, this function is accompanied by a non-Cas protein, bacterial RNaseIII. In Class 1 systems, the effector module consists of multiple *cas* genes, e.g., Cas3, Cas5–8, Cas10, and Cas11, in different combinations. On the other hand, in Class 2 systems, the effector module is represented by large single Cas proteins – Cas9, Cas12, and Cas13. Besides these Cas proteins, there are several other genes involved in signal transduction or ancillary functions in some of the systems (Table 12.2).

Among all the systems, CRISPR/Cas9 is the most widely used for genome editing in all forms of organisms including rice (Mishra et al. 2018). The CRISPR/Cas12 system has also been used for different purposes in rice (Mishra et al. 2018). The CRISPR/Cas13, however, is being least used in rice. Since this system targets RNA molecule instead of DNA, it has been demonstrated in rice that it can be used to eliminate rice RNA viruses (Yue et al. 2020).

12.5.1 Use of CRISPR/Cas9 System in Rice

This system has two main components, namely, Cas9 and gRNA. The gRNA is around 100-nucleotide (nt) long that contains two parts, e.g., CRISPR RNA (crRNA), a 17–20 nt sequence that is complementary to the target DNA, and tracrRNA that functions as the binding scaffold with the Cas9. Sometimes a single RNA strand contains both the crRNA and tracrRNA and is called single guide RNA (sgRNA). Cas9 is an RNA-dependent DNA endonuclease enzyme that induces double-stranded breakage (DSB). The crRNA is required to recruit Cas9 with the target DNA. This system also requires another sequence in the target DNA called protospacer adjacent motif (PAM). In the Cas9 system, the PAM is situated at the 3′ end of the target DNA. The PAM sequence usually varies in the originating organism, for instance, in *Streptococcus pyogenes*, 5′-NGG-3′ is recognized as the PAM, whereas, in *Staphylococcus aureus* it is 5′-NNGRRT-3′ (Wada et al. 2020).

In rice, the CRISPR/Cas9 has been shown as an effective tool for targeted mutagenesis and functional genomics studies (Char et al. 2019). The orthologue of

Table 12.2 Different types of CRISPR/Cas systems and function of different Cas proteins

		Adaptation	Expression	Interference		Signal transduction/ancillary			
Class	Type	Spacer integration	Pre-crRNA processing	Effector module (crRNA and target binding)	Target cleavage	CoA synthesis	Sensor effector	Ring nuclease	Helper, unknown role
Class 1	Type I	Cas1, Cas2, Cas4	Cas6	Cas7, Cas5, SS, Cas8/LS	Cas3″, Cas3′				
	Type III	Cas1, Cas2, RT	Cas6	Cas7, Cas5, SS, Cas10, or LS	Cas10 or LS	Cas10 or LS	CARF#, HEPN#	CARF#	
	Type IV	Cas1, Cas2	Cas6	Cas7, Cas5, SS, Csf1/LS	–				DinG
Class 2	Type II	Cas1, Cas2, Cas4	RNaseIII, Cas9	Cas9	Cas9			Csn2	Csn2
	Type V	Cas1, Cas2, Cas4	Cas12	Cas12	Cas12				
	Type VI	Cas1, Cas2	Cas13	Cas13	Cas13				

an early developmental gene *EPFL9* (epidermal patterning factor like-9), a positive regulator of stomatal development of *Arabidopsis* in rice, has been knocked out using the CRISPR/Cas9 system to elucidate its function (Yin et al. 2017). Targeted knockout of rice-dominant *Waxy* gene that controls the amylose content showed low amylose content and glutinous characteristics in rice grain (Zhang et al. 2018). Insertion of a 5.2-kb carotenoid biosynthesis cassette in the rice genome is also achieved using the CRISPR/Cas9 system by HDR. This system has been found effective to confer resistance or tolerance against different stresses which is discussed in the later section of this chapter.

12.5.2 Use of CRISPR/Cas12a or Cpf1 in Rice

The CRISPR/Cas12a (previously known as Cpf1) is a comparatively new genome-editing tool than the Cas9 system. There are several benefits of using the Cas12a system over the Cas9. First of all, the PAM sequence in Cas9 is G-rich that sometimes makes it difficult for organisms rich in AT, whereas this system recognized the PAM of AT-rich, e.g., 5′-TTTN-3′ and 5′-TTN-3′ (Mishra et al. 2018). Besides, this system cleaves the DNA in a staggered manner leaving 4–5 nucleotide sticky overhangs; in contrast, the Cas9 creates blunt ends during DSB that makes the Cas12a system more specific and less error-prone. Also, Cas12 cuts DNA as a distal end to the PAM allowing repeated targeting. Unlike Cas9, this system can target and cleave the DNA without the need for tracrRNA. The Cas12a also has RNase activity along with endonuclease activity; thus, it can process its own crRNAs (Zetsche et al. 2015).

CRISPR/Cas12a system was demonstrated as a tool for targeted mutagenesis in rice through mutating *OsPDS* and *OsBEL* genes *(*Xu et al. 2017*).* Multiplex gene editing in rice using this tool was established targeting six sites of three endogenous genes *5-enolpyruvylshikimate 3-phosphate synthase* (*OsEPSPS*, LOC_Os06g04280), *bentazon-sensitive lethal* (*OsBEL*, LOC_Os03g55240), and *phytoene desaturase* (*OsPDS*, LOC_Os03g08570) (Wang et al. 2017c). A separate experiment successfully edited eight genes at a time using this system (Wang et al. 2018). Targeted mutation of *EPFL9* (*epidermal patterning factor*) orthologue in rice through CRISPR/Cas12a was shown to be a heritable change (Yin et al. 2019; Yin et al. 2017). Several other studies are also present where Cpf1 has been reported successful for genome editing (Li et al. 2018). In a comparative study, where the rice *phytoene desaturase* (*PDS*) gene was targeted using both CRISPR/Cas9 and CRISPR/Cas12a, the latter one was found more effective by achieving higher targeted mutagenesis frequency (Banakar et al. 2020).

12.5.3 Use of CRISPR/Cas13 in Rice

The use of CRISPR/Cas was extended from DNA to RNA with the discovery of CRISPR/Cas13 system that belongs to the Type VI of Class 2 (Mahas et al. 2018).

To date, three families of CRISPR/Cas13 have been discovered, namely, Cas13a (earlier termed as C2c2), Cas13b, and Cas13c. The use of this system ranges from RNA knockdown, transcript tracking to editing tools in both animal and plant cells (Abudayyeh et al. 2017; Cox et al. 2017). Recently it is reported that Cas13a could be used as a nucleic acid detection tool (Gootenberg et al. 2017) too. Unlike other Cas proteins, Cas13 contains two higher eukaryotes and prokaryotes nucleotide-binding domains (HEPN) with exclusive RNase activity (Anantharaman et al. 2013). This system has been demonstrated as a successful plant RNA virus controlling tool (Aman et al. 2018) including the *rice stripe mosaic virus* (RSMV) (Zhang et al. 2019b).

12.6 Delivery Methods of CRISPR/Cas System in Rice

Successful delivery of the CRISPR/Cas system into plant cells is the prerequisite of successful genome editing in plants. Scientists have been using mainly two types of genetic transformation methods in plants, namely, (1) direct and (2) indirect methods. The direct method involved the delivery of CRISPR/Cas components directly through physical or chemical means, whereas the indirect method involves stable expression of transgenes using Ti-plasmid-based vectors or modified plant virus-based vectors (Ran et al. 2017).

12.6.1 Indirect Methods

12.6.1.1 *Agrobacterium*-Mediated Transformation

The *Agrobacterium tumefaciens* is a soil-borne plant pathogenic bacterium that causes crown gall disease in dicot plants. This bacterium has a special ability to transfer a portion of its DNA, called T-DNA, of Ti (tumor inducing) plasmid into the plant genome. The Ti plasmid contains virulence (*vir*) genes that are being activated by phenolic compounds, e.g., acetosyringone (Engström et al. 1987). Upon activation, the *vir* genes produce proteins necessary to transfer the T-DNA portion as a single-stranded DNA (ssDNA) to the plant cell and integrate into the plant genome. The T-DNA is flanked by two directly repeated orientations of 25-bp-long highly homologous sequence, termed as the left border (LB) and right border (RB). These sequences are necessary for recognition by the Vir protein and successful transfer and integration of the T-DNA into the plant genome. The T-DNA naturally contains oncogenes that upon integration in the plant genome produce excessive auxin and cytokinin ultimately result in tumor formation (Gelvin 2003). These pathogenic genes of T-DNA can be removed keeping the LB and RB and expression cassette (s) can be integrated to produce heterologous proteins in the plant. Such Ti plasmids without oncogenes are called disarmed Ti plasmids (Tzfira and Citovsky 2006). Another *Agrobacterium* species, *Agrobacterium rhizogenes*, is also used for the same purpose. This bacterium possesses root inducing (Ri) plasmid harboring T-DNA and this species results in hairy root formation (Ream 2009).

Ti-plasmid-based *Agrobacterium*-mediated genetic transformation has been widely used for delivering CRISPR/Cas components in rice for genome editing. In a study, a single binary Ti plasmid harboring single guide RNA (sgRNA) and Cas9 was delivered in rice using *Agrobacterium* where successful silencing of the rice *bentazon-sensitive lethal* (*BEL, LOC_Os03g0760200*) gene was achieved (Xu et al. 2014). Other studies also being successful to enrich amylose content (Sun et al. 2017), understanding the role of *Isoamylase 1* (*ISA1*) in starch synthesis and endosperm development (Shufen et al. 2019), developed low Cd-accumulating *indica* rice by knocking out *OsNramp5* (Tang et al. 2017) where the CRISPR/Cas components were delivered into rice using *Agrobacterium tumefaciens*. Multiplexed targeting of three genes, namely, *GW2*, *GW5*, and *TGW6*, by CRISPR/Cas9 delivered by *Agrobacterium* is also reported successful where grain weight and size of rice have been increased rapidly (Xu et al. 2016). Apart from enhanced nutritional quality and yield increase and understanding the function of genes, *Agrobacterium*-mediated transformation of CRISPR/Cas components is also used to confer resistance/tolerance in rice, e.g., blast resistance (Wang et al. 2016; Zhou et al. 2018), bacterial leaf blight (Zhou et al. 2018), herbicide tolerance (Sun et al. 2016), and cold tolerance (Shen et al. 2017a).

12.6.1.2 Agroinfiltration

One of the major drawbacks of *Agrobacterium*-mediated genetic transformation is that it integrates the gene of interest(s) into the plant genome and plants generated from explants become transgenic, i.e., stable expression of the transgene. Since transgenic plants are always controversial, another way to use *Agrobacterium* to deliver CRISPR/Cas components is agroinfiltration, where *Agrobacterium* harboring T-DNA with CRISPR/Cas components are injected into plant leaves directly that results in only transient expression of sgRNA and Cas protein. However, this method leads to chimeric rather than systematic expression. During writing this book chapter agroinfiltration of CRISPR/Cas components in several citrus plants has been reported but no study in rice was reported yet (Jia et al. 2019; Jia and Wang 2014; Jia et al. 2017).

12.6.1.3 Viral Vector-Based Transformation of CRISPR/Cas Components

The benefit of using genetically modified plant viruses as a vector to transiently express foreign proteins in the plant over agroinfiltration is that it has the ability to systematically infect the whole plant. The plant viruses have a wide range of host specificity and have their own replicating mechanisms. Till date, many viruses have been adopted for genome engineering in a wide range of crops including rice (Zaidi and Mansoor 2017). The use of plant virus as a vector was first demonstrated using *tobacco mosaic virus* (TMV) for virus-induced gene silencing (VIGS) of carotenoid biosynthesis in *Nicotiana benthamiana* (Kumagai et al. 1995). The use of viral vectors for genome engineering was first reported using geminivirus (Baltes et al. 2014). The geminivirus is a single-stranded DNA (ssDNA) virus that can infect a wide range of dicot and monocot plants. Upon infecting plant cells, it requires only one protein, Rep, to initiate DNA replication through rolling circle amplification

(RCA). However, geminiviruses are not a good option to deliver large DNA fragments due to their smaller genome size (2.5–3.0 kb); therefore, it is recommended to use geminiviruses for the production of an increased amount of sgRNA (Yin et al. 2015). A modified genome of geminiviruses expressing guide RNA could be integrated into T-DNA and delivered into transgenic plants expressing the Cas gene through agroinfiltration.

Another class of viral vector that is extensively used to express alien genes is single-stranded RNA viruses of the family Virgaviridae. Viruses of this family can infect more than 400 plant species belonging to 50 families. *Tobacco rattle virus* (TRV) is the most frequently used of this class of viral vector. Though it can carry more foreign DNA than geminiviruses, it is still not suitable to express Cas protein. Therefore, this vector can also be used to guide RNA delivery (Kuluev et al. 2019).

Recently, the geminivirus-based CRISPR/Cas system has been optimized for rice to knock in a gene of interest (Wang et al. 2017b). This study designed expression cassettes based on the *wheat dwarf virus* (WDV) to express gRNA, *ACT1*, and *GST*. Transgenic rice calli expressing Cas9 were used and successful knock-in of *ACT1* and *GST* has been reported. In this study, wild-type rice calli were also used and another expression cassette to express both Cas9 and gRNA along with knocking in cassettes of *ACT1* and *GST*; however, the success rate was lower in the latter approach.

12.6.2 Direct Methods

12.6.2.1 Biolistic or Particle Gun Bombardment

Biolistic or particle gun bombardment method of genetic transformation requires a special machine called "gene gun" or "biolistic gun." In this method particles of gold, silver, or tungsten coated with DNA are used to transfer DNA into plant explants by applying high pressure. After the successful integration of foreign DNA, the explants are regenerated on selective media, i.e., it is a stable expression of a transgene. This method was used to knock out *OsPDS* and *OsBADH2* where rice calli were bombarded with Cas9 plasmid and sgRNA expression plasmid (Shan et al. 2013). CRISPR/Cas9-based targeted insertion of a 52-Kb carotenoid biosynthesis expression cassette in the targeted site in the rice genome has also been achieved through the particle gun bombardment method (Dong et al. 2020). This method has also been adopted to deliver CRISPR reagents as ribonucleoproteins (RNPs). Integration of the CRISPR/Cas system in the plant genome raises ethical concerns and biosafety issues as it causes continuous genome editing and off-target effects in next generations; therefore, DNA-free delivery of CRISPR reagents is the most desirable as RNPs have limited half-life (Liang et al. 2019). However, a recent study showed that biolistic delivery of CRISPR reagents either in the form of DNA or RNPs results in the insertion of random DNA fragments in the targeted site which was not observed in the case of *Agrobacterium*-mediated delivery (Banakar et al. 2019).

12.6.2.2 Protoplast Transfection

Upon enzymatic removal of plant cell walls, DNA, proteins, and other reagents can be directly transformed into naked protoplasts of plants employing electroporation of polyethylene glycol (PEG) treatment. After successful transformation, the protoplast is regenerated into plants on suitable culture media. For dicot plants, mesophyll protoplasts are used where embryonic callus-derived protoplasts are more preferable for monocots (Ran et al. 2017). Transfection of CRISPR RNPs has been demonstrated in protoplasts of rice (Woo et al. 2015) and in vitro derived zygote of rice (Toda et al. 2019). Protoplast transfection is preferred for the delivery of CRISPR/Cas RNPs. The delivery of CRISPR/Cas RNPs has several benefits over the DNA-based delivery as it does not integrate any DNA in the crop genome. The mutated plants derived through transfecting plant protoplasts by CRISPR RNPs are non-transgenic in nature, thus getting rid of the controversies regarding GM crops. Nonetheless, DNA-based delivery of CRISPR/Cas9 reagents in rice has also been achieved to confer blast disease resistance by mutagenesis of the *ERF transcription factor* gene *OsERF922* (Wang et al. 2016). Besides electroporation and PEG, lipoinfection-mediated delivery of Cas9/gRNA RNPs has proven effective as well but not being tried in rice protoplast yet (Liu et al. 2020).

12.7 CRISPR/Cas for Enhancing Resistance Against Biotic Stresses in Rice

12.7.1 CRISPR/Cas in Providing Resistance Against Rice Bacterial Diseases

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. oryzae (Xoo), is one of the most common bacterial diseases of rice frequently observed mostly in Asia and Africa. Many attempts have already been taken to tackle this disease following genome engineering technology including CRISPR/Cas. Immediately after attack by bacteria the plant recognized a pattern in the pathogen, e.g., bacterial flagellin, that triggers immunity in plants, called pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) (Zipfel 2014). However, the pathogen also injects molecules called “effector” that bypasses the PTI system and leads towards effector-triggered susceptibility (ETS). If the plants can recognize the effector molecules, then a second layer of immunity is boosted called effector-triggered immunity (ETI) (Spoel and Dong 2012). The Xoo contains T3SS effectors, transcription activator-like effectors (TALEs), which are inducing the expression of *OsSWEET* family of putative sugar transporter genes that leads towards susceptibility. Disruption of two susceptible genes to TALEs, *OsSWEET11* and *OsSWEET14*, in rice cv. Kitaake showed broad-spectrum resistance against most of the Xoo (Xu et al. 2019). TALEs attacks the host nucleus and binds with the specific promoters and activates their expression, altering the transcriptome of the plant. Rice *OS8N*, also called *OsSWEET11*, was edited in another study that was found to be providing enhanced significant resistance against Xoo in homozygous mutants

(Kim et al. 2019). A similar result was also obtained in another rice cultivar Zhonghua 11 (CR-S14) where a codon region of *OsSWEET14* was edited using CRISPR/Cas9 to disrupt its function without any yield penalty (Zeng et al. 2020a). All other studies being carried out to confer resistance against Xoo strains in different rice cultivars were successful using CRISPR/Cas9 technology when the expression of *OsSWEET11*, *OsSWEET13*, and *OsSWEET14* was disrupted either targeting the promoter or directly the coding region (Blanvillain-Baufumé et al. 2017; Oliva et al. 2019; Varshney et al. 2019; Zafar et al. 2020). Interference or knockdown of another rice gene, *Xa13*, is reported to provide resistance against leaf blight; however, this gene is also involved in anther development. Therefore, disruption of this gene results in a penalty for fertility. Recently, selective deletion of the promoter region of this gene using the CRISPR/Cas9 system has been proven effective in both japonica and indica rice varieties without losing the expression of the gene, i.e., plants remain fertile (Li et al. 2020).

12.7.2 CRISPR/Cas in Providing Resistance Against Rice Fungal Diseases

Among the different fungal pathogens of rice, the blast disease caused by the filamentous Ascomycetes *Magnaporthe oryzae* is the most devastating one. Worldwide this pathogen results in up to 30% yield loss which would be enough to feed 60 million people (Nalley et al. 2016). There are about 100 resistant (R) genes that have been found in rice that confer resistance against this disease; among them 30 are already cloned at the molecular level (Xiao et al. 2019). These major R genes have been deployed to develop resistant rice lines worldwide through resistance breeding and transgenic approaches. Genome editing techniques like CRISPR/Cas9 have also been used for this purpose (Mishra et al. 2021). In the plant cells, the pattern recognition receptors (PRRs) recognize PAMPs and trigger the PTI. Upon triggering of PTI plants produce different hormones like jasmonic acid, salicylic acid, and ethylene that are involved in defense mechanisms. Plant *ethylene-responsive factor*, a subfamily of the *APETELA/ethylene response factor* (*AP2/ERF*) transcription factor superfamily, has been reported to provide resistance in plants. In rice, genes of this family, e.g., *OsBIERF1*, *OsBIERF3*, and *OsBIERF4*, are involved in providing resistance against *M. oryzae* (Cao et al. 2006). Blast resistance was improved in Kuiku131, a japonica type rice widely cultivated in Northern China, following CRISPR/Cas9-mediated knockout of *ERF transcription factor* gene *OsERF922* (Wang et al. 2016). Around 42% mutation was observed in T_0 in the targeted gene site and a stable heredity of this mutation was also observed in T1 and T2. Besides, no significant loss in agronomic performances was observed, revealing that proper editing using CRISPR/Cas technology does not compromise other desired traits. *Pi21* is another broad-spectrum resistance that encodes a proline-rich protein that contains a putative heavy metal binding domain and a putative protein-protein interaction domain. Wild-type *Pi21* shows susceptibility in plants against *M. oryzae*. Using CRISPR/Cas9 this gene was mutated and enhanced

stability and non-race-specific resistance against *M. oryzae* were obtained (Nawaz et al. 2020). The mutation rate was 66%, among which 26% biallelic, 22% homozygous, 12% heterozygous, and 3% chimeric were obtained. They were also able to achieve transgene-free mutants with enhanced resistance. Mutation of the *Pi21* gene did not compromise the agronomic characteristics.

The plant R genes usually encode for proteins with nucleotide-binding site-leucine-rich repeat (NLR) domains. Using CRISPR/Cas9, a study showed that Ptr, an R gene, provides broad-spectrum resistance against blast disease. Using CRISPR/Cas technology this gene can be knocked in in other susceptible rice varieties to develop resistance against blast disease (Zhao et al. 2018). Another study found another potential gene that plays a role in resistance against *M. oryzae*, the *OsSEC3A*, which is an important unit of the exocyst complex of the rice. Through disrupting this gene using CRISPR/Cas9, enhanced resistance against *M. oryzae* was obtained. Such disruption is linked with enhanced salicylic acid synthesis, thus providing more resistance against *M. oryzae*. However, this came with the penalty of dwarf structure and lesion-mimic phenotype (Ma et al. 2018). Further optimization on the mutating of this gene may result in better resistance against blast disease without any unwanted penalty.

12.7.3 CRISPR/Cas in Providing Resistance Against Rice Viral Diseases

The plant viruses cause a severe economic loss around the world and greatly alter the agronomic traits and physiological functions in crops (Nicaise 2014). Controlling plant viruses greatly depends on controlling their vectors through applying synthetic pesticides. Identification of different resistant (R) genes has also made it possible to control plant viruses through molecular breeding though it is a time-consuming technique (Khan et al. 2018). Besides, several transgenic approaches have been proven effective including the RNAi mechanism. However, such promising techniques of controlling plant viruses come with many hurdles, such as, for DNA viruses, RNAi mechanism can suppress the expression of genes at the post-transcription level rather than eliminating the virus itself (Voinnet 2005). It is proven that the viruses also develop a counter-mechanism of plants' RNAi mechanism (Pumplin and Voinnet 2013). CRISPR/Cas technology can be used as an alternative to resistance breeding or RNAi mechanism to control viral diseases of rice.

The major viral disease of rice is rice tungro disease (RTD), a severe production constraint mainly in tropical Asia. Two viruses act jointly to cause this disease, e.g., the single-stranded RNA virus, namely, *rice tungro spherical virus* (RTSV) and the double-stranded DNA virus named *rice tungro bacilliform virus* (RTBV) (Chancellor et al. 2006). In a recent study, a susceptible (S) gene, *eIF4G*, in rice was mutated using CRISPR/Cas9 and achieved resistance against this disease in an RTD susceptible rice variety, IR-64 (Macovei et al. 2018). They obtained a mutation frequency of 36 to 86% and no potential off-target issue was observed. Analyzing the sequences in mutant lines, it was observed that among all the obtained mutations,

the resistance was conferred by the in-frame mutation in the SVLFPNLAGKS residues (mainly NL), nearby the YUV residues. Such technology could be used to develop rice varieties suitable for cultivating in RTD-prone areas to achieve the targeted yield. SNP in the codon for $Val^{1060-1061}$ of the *eIF4G* gene in rice is also reported to be associated with resistance against RTSV (Lee et al. 2010). Recently, the CRISPR/Cas system has also been optimized for base editing to introduce point mutations (Kantor et al. 2020).

Another study obtained RTD resistant lines using RNAi technology targeting the ORF IV of RTBV (Valarmathi et al. 2016). Alternatively, CRISPR/Cas13 could be used to control RNA viruses in rice at the post-transcriptional level by inhibiting translation (Cao et al. 2020). The benefit of using CRISPR/Cas13 over CRISPR/Cas9 or CRISPR/Cas12 is that it targets RNAs rather than DNA that turns this into an effective tool to control plant RNA viruses (Khan et al. 2018). *Southern rice black-streaked dwarf virus* (SRBSDV), a major virus infecting rice plants in several East Asian countries, was successfully controlled using CRISPR/Cas13 (Zhang et al. 2019b). This study designed three crRNAs to target the double-stranded RNA genome. They also targeted a single-stranded RNA virus of rice, named *rice stripe mosaic virus* (RMSV). Therefore, stable expression of CRISPR/Cas13 in rice can be a better option to control RNA viruses, e.g., it can also be implemented to control RTD.

Other approaches to using CRISPR/Cas are base editing and prime editing. For DNA base editing a Cas enzyme for programmable DNA binding is required with a single-stranded DNA-modifying enzyme for single nucleotide base alteration. There are two types of base editor present, e.g., cytosine base editors (C → T, T → C) and adenine base editors (A → G, and G → A) (Kantor et al. 2020). Recently, C → G transversion using the CRISPR/Cas system is also being reported (Kurt et al. 2021). The limitations of these base editors have been omitted very recently with another technique that does not require any DSB or donor DNA. This method directly alters the DNA information in a specific site of a targeted DNA using a catalytically impaired Cas9 (Cas9 nickase-nCas9) protein coupled with an engineered reverse transcriptase, programmed with prime editing guide RNA (pegRNA) that both specifies the target site and encodes the desired edit (Anzalone et al. 2019). This prime genome editing technology has also been adopted for rice too (Lin et al. 2020).

12.7.4 CRISPR/Cas in Providing Tolerance Against Abiotic Stresses of Rice

Several abiotic stresses result in rice yield reduction including salinity, cold stress, drought, and so on. Among them, the development of saline tolerant rice lines is a promising approach to increase the rice yield as it will permit the cultivation of rice in areas that are saline-prone. Usually, rice is a saline-sensitive crop and cannot be grown in saline-prone land; however, many saline-tolerant genes have been cloned, namely, *SKC1*, *DST*, *OsRR22*, *OsHAL3*, *P5CS*, *SNAC2*, and *OsNAP*. Among them, *OsRR22* gene is reported to be linked with enhanced saline tolerance in rice when its

natural function is disrupted. With this aim, CRISPR/Cas9 was used to mutate this gene and the saline tolerance increased significantly from the seedling stage in rice (Zhang et al. 2019a). Sequence analysis of this study reported six mutation types at the target sites that are proven to be linked with saline tolerance.

Another important environmental factor that limits rice production is cold stress and developing cold stress-tolerant lines would allow cultivating rice in areas with low temperature. Several cold stress tolerance genes have been identified and cloned, such as *COLD1*, *OsSRFP1*, *SGD1*, and *OsMYB30*. Several cold stress-mutant rice lines with improved agronomic characteristics have been developed using CRISPR/Cas9 targeting *OsPIN5b* (a panicle length gene), *GS3* (a grain size gene), and *OsMYB30* (cold tolerance gene) genes in Nipponbare, a japonica rice (Zeng et al. 2020b).

CRISPR/Cas9 technology has been employed to develop herbicide resistance in rice (Sun et al. 2016). Targeting the rice ALS gene, this study used two guide RNAs, a Cas9 enzyme and a 476-bp donor template, to bring several point mutations in the targeted gene (W548L and S627I substitutions). The donor DNA also had some other features like several synonymous substitutions that did not change the amino acid sequence but restricted the Cas9 enzyme from further targeting the gene. They used the particle gun bombardment method to deliver the donor DNA and CRISPR reagents. Herbicide tolerance in rice has also been achieved via prime editing (Butt et al. 2020). In this study, three different gene loci were targeted, termed as *ACETOLACTATE SYNTHASE (OsALS)*, *IDEAL PLANT ARCHITECTURE 1 (OsIPA1)*, and *TEOSINTE BRANCHED 1 (OsTB1)*; however, the authors suggested further studies on using this technology.

12.8 Conclusion and Future Implications

The production of rice is estimated to increase by 1% annually to meet the demand of the growing population (Normile 2008) and the total production must be increased by 40% by 2050 (Milovanovic and Smutka 2017). Biotic and abiotic stresses are the major constraints of lower rice yield worldwide and these issues must be addressed to meet the targeted yield (Stallworth et al. 2020). The development of resistant or tolerant rice lines has always been a continuous process worldwide in rice improvement projects. Developing resistance or tolerance in crops has always been carried out through bringing changes in the genetic constitution by natural mutation followed by selection, hybridization, mutation breeding, or genetic engineering. Conventional breeding is always time-consuming and somewhat becomes static due to the unavailability of genetic variation and loss of genetic diversity due to crop domestication. Natural mutation is a slow process and always needs to rely on fate, and artificial mutagenesis by means of physical (e.g., radiation) and chemical (e.g., ethyl methylsulfonate) mutagens is random, time-consuming, and labor intensive. In contrast, the genetic engineering approach is always less labor intensive, time-saving, and precise and allows transferring of genes from distantly related organisms, for instance, Golden Rice, a genetically modified rice that contains

precursor genes to produce β-carotene that is naturally lacking in rice grains (Paine et al. 2005). With the discovery of CRISPR/Cas system and its establishment as a genome editing tool, it has become the most powerful tool for crop genetic modification because of its high efficiency, preciseness, easiness, and cost-effectiveness (Manghwar et al. 2019). However, this system is not free from limitations. The biggest limitation of the CRISPR/Cas system is the continuous expression of Cas proteins and potential off-targets that may be detrimental to changes in organisms. Though new techniques are being implemented to address this issue, in our opinion DNA-free delivery method of CRISPR/Cas ribonucleoprotein complex in rice zygote or protoplast is the most effective option (Banakar et al. 2019; Toda et al. 2019). Such methods avoid the integration of CRISPR machinery into the genome of the organism and eliminate the potential risks of off-target. More recently, another technique has been developed to address the off-target issue using light-induced degradation of sgRNA named as CRISPRoff (Carlson-Stevermer et al. 2020). This CRISPRoff sgRNA was synthesized artificially using solid-phase synthesis, and photocleavable residues containing o-nitrobenzyl groups were incorporated at specific positions that undergo degradation when exposed to UV light. Upon exposure to UV light, these sgRNAs did not form any complex with Cas9 protein. The authors also demonstrated that these sgRNAs were cleaved within cells when exposed to light. In contrast, the cell that was kept in dark had an abundance of sgRNA. They also successfully showed that sgRNAs could be cleaved in specific tissues via selective illumination. However, this study is yet confined within human cells and no study has been published on plant cells. Development and optimization of CRISPRoff for plant cells including rice have great potential in genome editing for crop improvement.

Another major limitation of the CRISPR/Cas system is PAM. Due to the high specificity of the PAM requirement in successful interference, it limits the number of targets. For instance, the CRISPR/Cas9 system only recognized GC-rich PAMs that limit its application in AT-rich genomes. Through the discovery of the CRISPR/Cas12a system, AT-rich genomes are now also available to be edited using CRISPR/Cas. Besides, new variants and modified systems of CRISPR/Cas are being discovered and developed regularly that are increasing the range of the target sequences (Mishra et al. 2018). Another CRISPR/Cas system-based technique that must be mentioned that can address all the limitations and issues regarding genome editing is "prime editing" which is PAM independent and DSB-free. This method is precise enough to introduce point mutations. Recently, another Cas protein has been discovered, namely, Cas14, that can be used for targeted cleavage of single-stranded DNA (ssDNA) and independent of PAM requirement. In addition, this system is also compact (950 to 1400 amino acids; half of the Cas12a protein) (Harrington et al. 2018). This compact-sized Cas protein can also be used in viral-based vectors. As the viral-based vectors cannot carry large size gene fragments, expression of Cas9 or Cas12a protein has remained impossible and is only limited to CRISPR array expression. Therefore, there is the scope of investigating Cas14 as a potential genome editing tool in rice as well as using viral-based vectors for its transient expression.

Most of the resistance or tolerance is generally achieved through gain-of-function mutation; therefore, knock-in of the desired gene to provide resistance or tolerance against biotic and abiotic stresses is most desired. However, due to the low efficiency of HR in plants, CRISPR/Cas-based knock-in of a gene of interest in plants has remained a major challenge. Therefore, a better delivery method of donor DNAs along with CRISPR/Cas systems will always be admired.

Apart from all the technical issues, other major issues that limit the genome-edited crops being cultivated in the farmers' fields are the regulatory issue and biosafety issue. Genetically modified crops are always a topic of controversy and a great political issue. Contradictory opinions regarding GM crops among different nations and different regulation policies have always made it difficult to be cultivated. Therefore, a proper and unified regulation is required for the whole world that how CRISPR/Cas-edited crops will be treated. Also, identification of a method through which a genome-edited crop will be free of all kind of controversies is a must.

In conclusion, with the advancement of sequencing techniques, more and more information on the function of genes is being revealed. By achieving such genomic data and with advancement of CRISPR/Cas-based genome editing techniques, developing stress resistance and tolerance in rice is becoming easier day by day. Through this, food security could be achieved for the ever-growing population of the world. Therefore, we must be prepared and address all the issues regarding genome editing techniques and regulations to successfully achieve the "Zero Hunger" sustainable development goal of the United Nations.

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