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Contributing Editor

Managing next generation crops by genome editing using the precise snipping tool- CRISPR/Cas9

CRISPR-Cas9 systems

A widely used gene editing tool in Molecular Biology, CRISPR-Cas9 or clustered regularly interspaced short palindromic repeat (CRISPR)- associated protein 9 (Cas9) was first discovered by scientists in the fermentation industry in bacteria. This technique has been used successfully on different organisms, including bacteria, fungi, plants and mammals. Bacteria use this sophisticated defense system against invaders, including phages or even foreign plasmid DNA. Certain fragments of the foreign DNA are stored as memory by inserting into the CRISPR-repeat spacer array within the bacterial chromosome (host DNA) to thwart future attacks. Discovery of this uniquely advanced adaptive immune response led to the exploration of non-homologous repair and homologous repair mechanisms in target cells, which eventually resulted in the advent of the CRISPR-Cas9 gene-editing technique- a highly precise and popular tool in the field of Molecular Biology (1).

The CRISPR-Cas9 system constitutes of the mature CRISPR RNA (crRNA) that contains a spacer sequence (complements the foreign sequence) at its 5' end a repeat sequence at the 3' end. This crRNA forms a stable complex with the Cas9 nucleases, and this complex functions in interrogating and destroying invading DNA targets (1, 2, 3). A short 2-5 bp sequence, located on the invading DNA near the target site (PAM sequence), plays an important role in identification and destruction of this foreign invading target DNA. During the complex formation, an additional trans-activating crRNA (tracrRNA) pairs with the crRNA repeat sequence to generate a dual RNA hybrid, this hybrid RNA structure assists the Cas9 to cleave a DNA molecule that contains the complementary target DNA and the protospacer-adjacent motif (PAM sequence). Cas9 nuclease has two essential functional domains: RuvC-like domain and HNH domain; each domain is responsible for cutting one DNA strand of the double-stranded target DNA. The nicks are then

repaired by either the non-homologous end-joining (NHEJ) methods, often leading to small insertions or deletions, or a highly targeted homologous DNA repair, which offers the opportunity of introducing or removing specific gene sequences in the host genome.

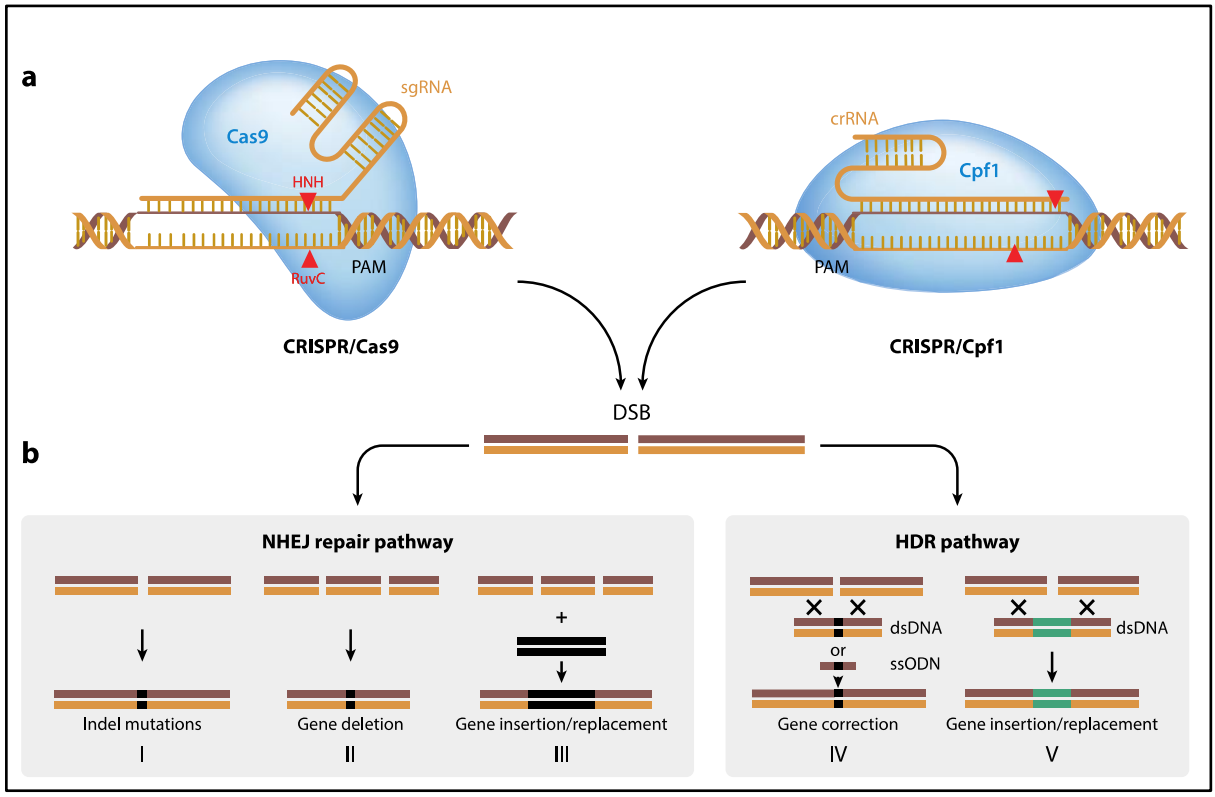

Figure 1. The CRISPR/Cas9 system. a. The Cas9 nuclease binds to the single-guide RNA (sgRNA) and identifying the target DNA sequence, it utilizes its HNH domain and RuvC-like domain to nick either strand of the target DNA. b. The nicks can be repaired either by non-homologous end joining (NHEJ) or homologous DNA repair (HDR). ***[Source:*** *Kunling Chen, et al., Annual Review of Plant Biology, 2019].*

In application, scientists have gravitated towards synthesis of a synthetic guide RNA (sgRNA) molecule of 20 bp, combining the crRNA and the tracrRNA, and the Cas9 protein. There are two major groups of the CRISPR-Cas9 systems: class I (types I, III and IV) form big functional complexes of proteins with the RNA, whereas class II (type II, putative types V and VI) constitutes a single RNA-guided nuclease.

CRISPR-Cas9 genome editing for superior crop management

There are several advantages of the CRISPR-Cas9 gene editing system (second generation gene editing system) over the first-generation gene editing machineries- the TALENs (transcription activator-like effector nucleases) and the ZFNs (zinc-finger nucleases) (4, 5). Both of these technologies are protein-dependent and involve time-consuming and tedious procedures to reach optimum target specificity.

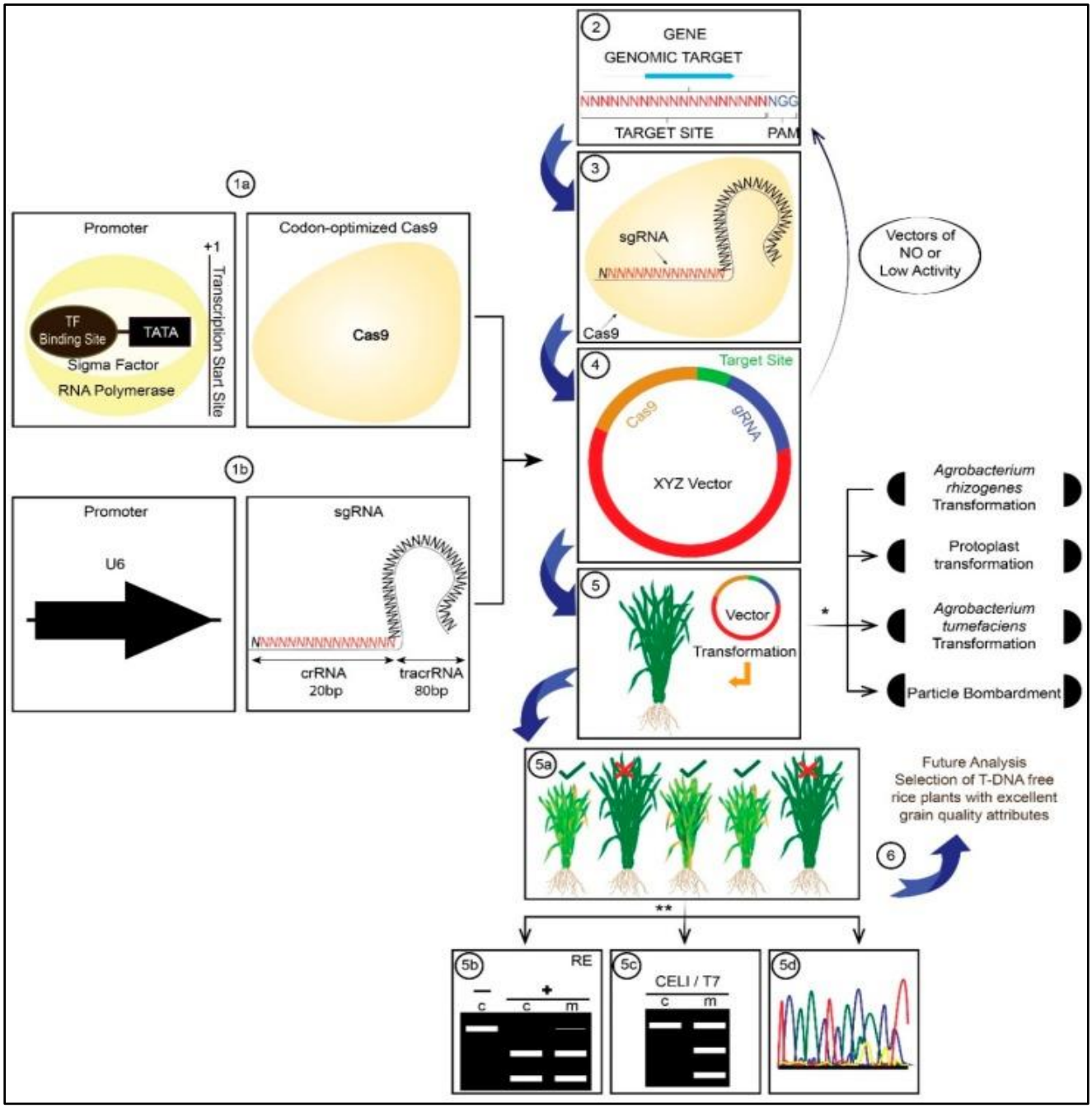

*Figure 2. Flow chart of the steps involved in CRISPR/Cas9-mediated genome editing in plants. The engineered editing complex consists of a single-guide RNA (sgRNA) and the Cas9 endonuclease. The fused crRNA and tracrRNA (trans-activating crRNA) form the stem-loop structure that binds the Cas9 protein. Vector encoding the Cas9 and the sgRNA is transferred to the target plant (rice, maize, wheat, tomato, cotton, etc.) via one of the techniques including protoplast transformation, Agrobacterium-dependent transformation, or particle bombardment. Analyses of the successful gene editing are done on plants selected and screened on the basis of phenotypic changes. [**Source:** Sajid Fiaz, et al., International Journal of Molecular Sciences, 2019].*

CRISPR-Cas9, on the other hand, is a versatile genome editing technique that functions at high efficiency, presents low off-target effects, and offers simplistic synthesis of the sgRNA for the process. Multiplexing by editing multiple loci simultaneously by the Cas9 can be achieved by introducing different targeting sgRNA molecules. Such efforts often lead to major chromosomal alterations in the target genome.

CRISPR-Cas9-dependent genome editing has wide implications in crop management. It presents before us a favorable route for fighting world's hunger. Subtle alterations can result in drastic improvements at several fronts, including resistance to biotic and abiotic stress, stacking favorable mutations, increased yield, high quality (improved nutritional profile), and crops requiring fewer inputs (5).

With a projected population of over 9 billion by 2050, food shortage is inevitable if we fail to increase yield substantially. Genetically modified crops (GM crops) have given the much-needed hope but complex safety concerns (for health and environment) have restricted their application. Instead, subtle alterations produced in the genome by gene editing machinery like CRISPR-Cas9 has revolutionized the crop management extensively. CRISPR-Cas9 is used extensively in both groups of plants- monocots and dicots, including grains, fruits and vegetables.

Monocots

Rice: the major area of focus for this staple crop is stress resistance- abiotic and biotic stress, high yield, and better quality of grains (5, 6, 7). This crop is a favorite among scientists because of the high prevalence of PAM sequence in its genome- 1 in every 10 bp. Scientists have performed knockout experiments to generate successful bacterial blight (OsSWEET13) and blast (OsERF922) disease resistant varieties. Various other findings indicate successful identification and alteration of targets that regulate draught resistance, cold resistance, potassium deficiency tolerance, and glutinous rice grain- to mention a few. Grain quality improvement is key for rice and successful application of CRISPR-Cas9 system has utilized various techniques for transformation, including particle bombardment, protoplast transformation, and Agrobacterium-mediated (A. rhizogenes, A. tumefaciens) gene transfer. Restriction digestion, next-generation sequencing and various confirmation methods are utilized to analyze and proof the successful generation and propagation of the desired traits. In addition to gene knockouts, successful point mutations, deletions, and knockins have played crucial roles in obtaining superior varieties of rice. In addition, CRISPR-Cas9 has been used to modulate transcriptional regulation, thereby imparting favorable characteristics in in rice. Furthermore, forward genetic screens have helped identify important mutations for the purpose of stacking favorable traits. This process also serves well for traits that often require the participation of multiple gene products. Several such quantitative gene loci (or QTLs) are mapped in crop improvement programs and have aided in improving rice grain quality- size and appearance (GS2, GS3,

GS5, Gn1a, GS9, TGW6), nutritional aspects (Wx, SBE1, SBEIIb, ISA1, FLO2, BADH2, FLO5), stress tolerance, and edibility. All these studies have made crucial progress in identifying and modifying contributing characteristics for the survival of the rice crops and improving nutritional output of the grains; however, much needs to be addressed given the wide backgrounds/varieties of rice to provide a common set of applicable gene variations for use.

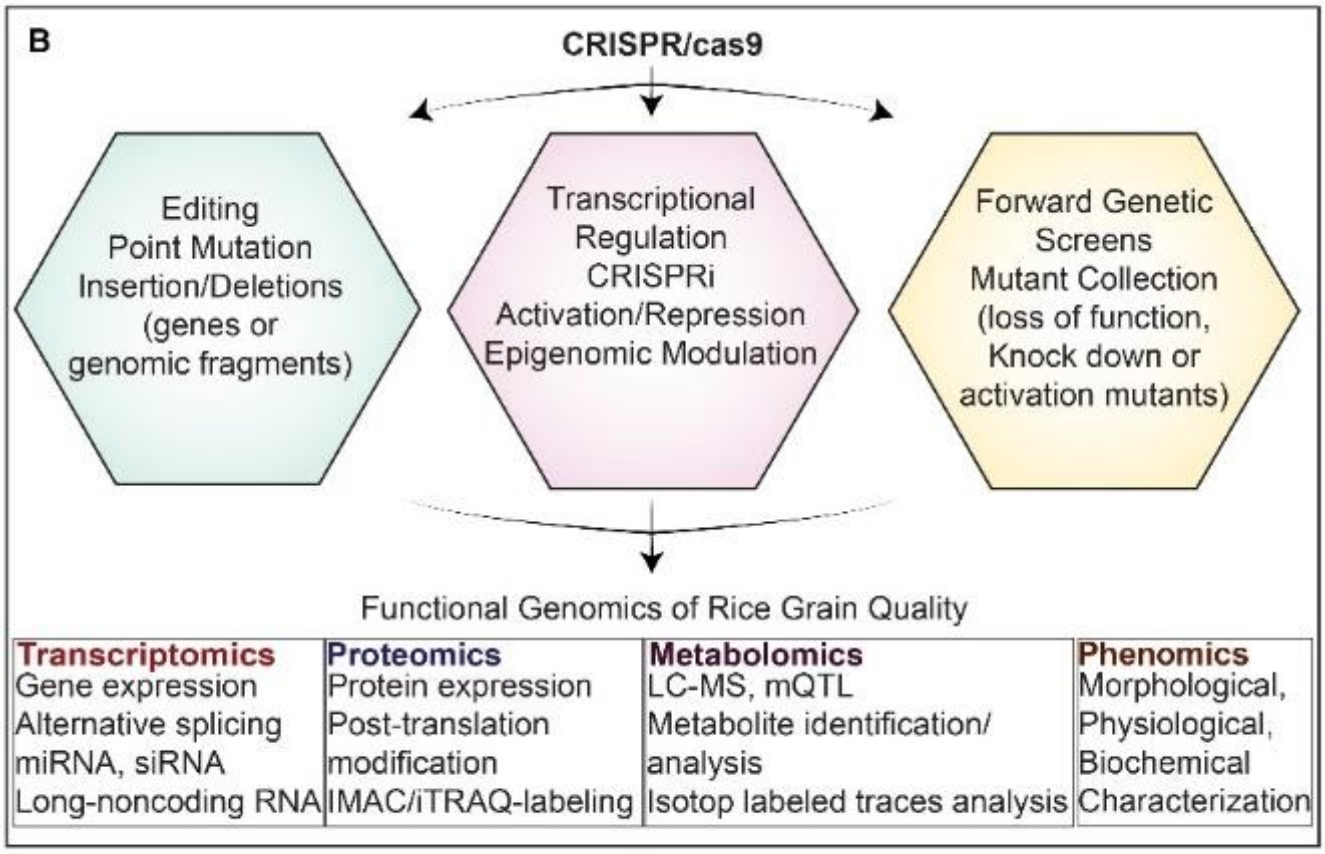

*Figure 3. Application of CRISPR/Cas9 in improving rice grain quality. Introduction of point mutations, deletions, or insertions via CRISPR/Cas9 can cause gene editing via generating point mutations, deletions or insertions, modulate expression by transcriptional or epigenomic regulation (activation/repression), or even create forward genetic screens for studying the molecular basis of rice grain quality. [**Source:** Sajid Fiaz, et al., International Journal of Molecular Sciences, 2019].*

Wheat: the primary focus of utilizing this versatile gene-editing technique in wheat is for developing resistance to powdery mildew disease (6). The TaMLO locus has been targeted for the purpose, the targeted knockout by CRISPR-Cas9 was achieved by particle bombardment in embryo technique. Other abiotic resistance characteristics have been tested in wheat by performing gene editing on TaDREB2 and TaERF3 genes for draught resistance (8). Protoplast transfection was done to achieve the alterations in target genes and about 70% efficiency was observed. Validation is

performed mostly by employing a combination of T7 endonuclease assay, restriction enzyme assay, and Sanger sequencing. Recently, Wang et al. 2018 have attempted to target multiple genes for multiplexing CRISPR-Cas9 gene editing, including TaMLO, TaGW2 (negative regulator in gene quality traits), and TaLpx-1 (disease resistance) (9). Three sgRNAs were used and a polycistronic cassette was generated for the purpose. Agrobacterium-mediated transformation was validated by next generation sequencing. This study provides a promising baseline for multiplexing CRISPR-Cas9 in wheat.

Maize: another staple crop that has gained much attention because of its high content of phytic acid. Phytic acid constitutes about 70% of the grain- considered undigestible by monogastric animals and an environmental pollutant, and scientists have reported that knocking out ZmIPK, ZmIPK1A, and ZmARP4- all involved in phytic acid synthesis- have alleviated the problem substantially (combining TALENs and CRISPR-Cas9 and obtaining comparable results) (5, 6). t-RNA based multiplex gene editing was achieved by Qi et al by generating polycistronic cassette targeting three transfection factors RPL, PPR and lncRNA, whereas simplex editing of MADS, MYBR and AP2 yielded commendable efficiency of about 100%. For stress tolerance, scientists have targeted ARGOS gene- identification of novel allelic variants for increasing draught tolerance have successfully helped generate high-yielding draught-resistant maize. There are plenty of other monocots that have been experimented on for improving yield and quality of grains, and to impart tolerance/resistance to abiotic and biotic stress.

In addition to monocots, a large number of dicots have been tested for genetic trait improvement, using CRISPR-Cas9.

Dicots

Soybean: it is an essential crop, it provides oil and protein for human consumption, and feed for animals. Because of successful application in model dicot Arabidopsis thaliana, several genes have been identified that can be plausible gene editing targets (5, 6, 10). One such gene is the GmFT2a, an integrator in the photoperiod flowering pathway. Though extremely valuable, soybean's sensitivity to seasonal changes to day light limits (photoperiod sensitivity) restricts its geographical expanse of cultivation. CRISPR-Cas9-dependent targeted frameshift mutations (1-bp insertions or short deletions), introduced via Agrobacterium-mediated transformation, in this GmFT2a gene resulted in late flowering, irrespective of long or short day. This widened the prospect of growing soybean at varied climate and geographic regions. Another group targeted the small RNA-directed RNA silencing pathway targets, GmDrb2a and GmDrb2b, both loci were successfully edited by multiple sgRNA constructed in the same vector. Scientists have also generated ALS (required for branched chain amino acid synthesis) gene mutants by site-directed mutagenesis using CRISPR-Cas9 system in soybean to develop herbicide resistant varieties.

Cotton: in cotton, transient and stable transformations (using Agrobacterium) have been successfully tested by scientists, they

have targeted the GhCla1 (chloropastos alterados 1) and GhVP (vacuolar pyrophosphatase) (11). Agrobacterium-mediated transformation of the shoot apex resulted in a successful editing of the two target genes- mostly characterized by deletions with one insertion. Scientists have successfully edited the gene for lateral root formation in cotton (12). Two sgRNAs, specific for the two orthologous cotton arginase gene (GhARG), were generated, stable transformation across generations was achieved via Agrobacterium-dependent transformation. This CRISPR-mediated gene editing greatly improved lateral root formation in cotton under high and low nitric oxide conditions, rendering this essential fiber-yielding crop suitable to grow at variable soil conditions.

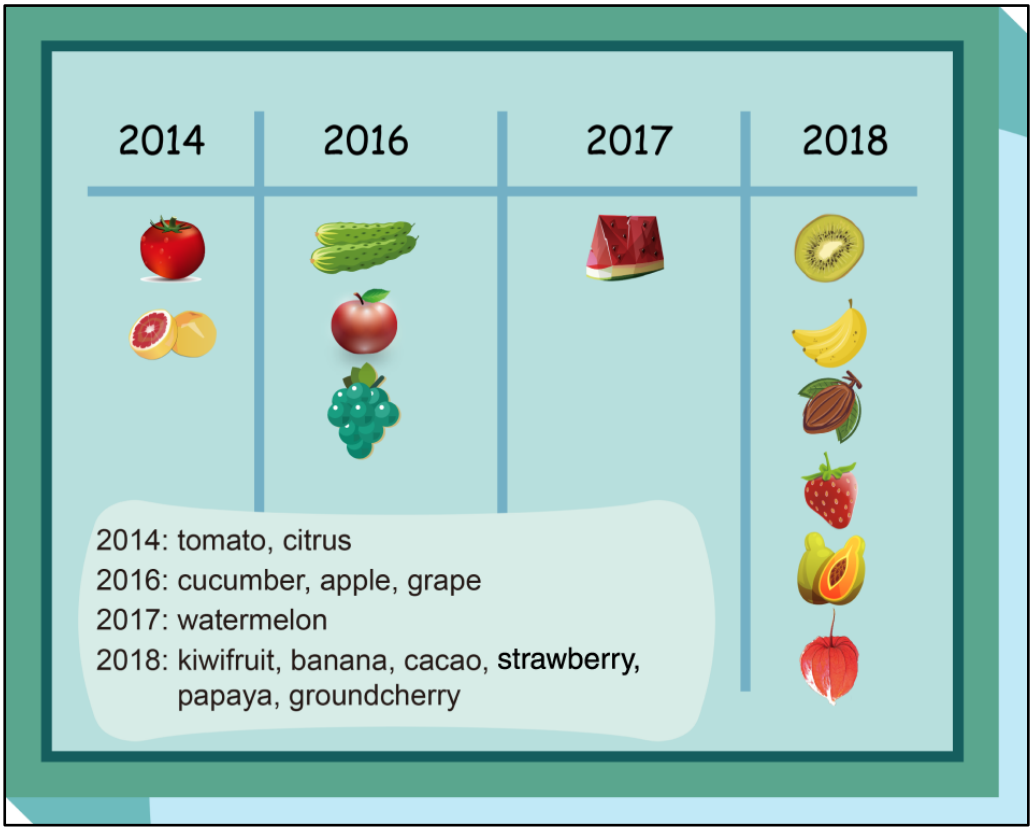

*Figure 4. Timeline for introduction of CRISPR/Cas9 in fruit crops. Initially starting with tomato, this precise genetic editing has a wide application in different fruit crops, as shown above. Most often multiple traits are targeted simultaneously by a single vector constituting of several sgRNAs for various target genes. [**Source:** Tian Wang, et al., Horticulture Research, 2019].*

Tomato: it is a fruit crop that has undergone substantial editing for improving its fruit quality, biotic and abiotic stress resistance, and domestication (13). CRISPR-Cas9 has helped in identifying crucial genes, like DCL2, that render the plant resistant to several different types of viruses, including potato X virus, tomato mosaic virus, and potato mosaic virus. CRISPR-dependent inactivation of DMR6 has resulted in

resistance towards several fungal pathogens too. MLO1 loss-of-function mutation protects tomato plants from mildew disease-causing fungus. Scientists have also addressed the bacterial speck disease of tomato by generating a C-terminal end-lacking mutated JAZ2 repressor. CRISPR-Cas9 has helped identify the gene important for rendering resistance to pre- and post-harvest infection by gray mold disease- knockout of MAPK3 makes tomato plants susceptible to the causal pathogen. In addition to mold disease resistance, MAPK3 also reinforces draught response in tomato crop. Tomato is a cold sensitive plant, and CBF1 was found to protect from cold stress by CRISPR-mediated mutagenesis. The various parameters addressed by scientists to improve tomato fruit quality are- fruit size, color, and texture. Scientists have successfully used CRISPR-Cas9 technology to target CLV3, LC- QTLs (quantitative trait locus) controlling the tomato size to generate large fruits. Several targets including locule number, color and texture have been altered by identification of either individual genes or QTLs. Scientists have been able to use CRISPR-Cas9 to generate variation in fruit colors. Inactivation of RIN or DML by CRISPR also has shown promising results with increasing tomato shelf-life, a major problem with this crop. CRISPR-Cas9 has also aided in producing tomato with high bioactive compounds, including GABA, anthocyanine, malate, and lycopene. Often crops have their wild counterparts that may exhibit favorable characteristics. Abiotic stress may play a significant role in deciding the outcome of the crop. The height of this fruit crop can be controlled by mutating the GAI gene- helps to withstand windy environment. Falling fruits can be a real problem if the crop has to be marketed, and scientists have found a way around- using CRISPR-Cas9, they have identified and tweaked MBP21 gene that regulates the formation of jointed stem/branch. Loss-of-function mutation has enabled the scientists to obtain plants with jointless phenotype. Similarly, scientists are relentlessly characterizing genes in the wild varieties of various fruit crops including tomato- the genes can be useful for increasing their economic value (14). Often, researchers are taking multiple genes to construct the CRISPR-Cas9 sgRNA cassette and using a single cassette to target several loci for enhancing crop productivity.

In addition to tomato, there are several other fruit crops that have been targeted for gene editing, including cucumber, watermelon, banana, grape, apple, strawberry, and kiwifruit. The sole motivation of using CRISPRP-Cas9 is to ensure high yield, less maintenance, and better quality. Given the scope and urgency, CRISPR-Cas9 has a bright future in crop improvement and addressing world hunger. Careful observation of valuable traits in wild relatives of cultivated crops can provide us with the much-needed tools to forward the cause of improved crop development and management. The biggest advantage of this technology is, unlike transgenic plants, it does not pose an ethical issue, and this is key to expedited and rational endeavors towards fulfilling the ultimate goals.

Conclusion

A major aspect of marketing and consuming gene-edited crops is public awareness and

acceptance. Though we have high hopes with this super-efficient editing system, we have to remember that there is still much work to do for fine-tuning the CRISPR-Cas9 technology for widening its application and scope, such as improving its off-target effects. Another point of concern is the chromatin state of the target region; according to certain studies, it may play important role in affecting CRISPR-mediated gene editing. Therefore, in-depth studies in deciphering the detailed mechanism of identification and editing of the target by the CRISPR-Cas9 complex can demystify these problems. Overall, with CRISPR-Cas9 we can hope to obtain answers to several difficult questions plaguing the growing population.

References

1. Jennifer A Doudna and Emmanuelle Charpentier. The new frontier in genome engineering with CRISPR-Cas9, Science, 2014.
2. Haifeng Wang et al. CRISPR/Cas9 in genome editing and beyond. Annual Review of Biochemistry, 2016.
3. Deborah M Thurtle- Schmidt and Te-Wen Lo. Molecular Biology at the cutting edge: a review on CRISPR/Cas9 gene editing for undergraduates. Biochemistry and Molecular Biology Education. 2018.
4. Tariq Shah et al. Genome editing in plants: advancing crop transformation and overview of tools. Plant Physiology and Biochemistry. 2018.
5. Deepa Jaganathan et al. CRISPR for crop improvement: an update review. Frontiers in Plant Science. 2018.
6. Xingliang Ma et al. CRISPR/Cas9 platforms for genome editing in plants: developments and applications. Molecular Plant. 2016.
7. Sajid Faiz et al. Applications of CRISPR/Cas9 system for rice grain quality improvement: perspectives and opportunities. International Journal of Molecular Sciences. 2019.
8. Dongjin Kim et al. CRISPR/Cas9 genome editing in wheat. Functional and Integrative Genomics. 2018.
9. Wei Wang et al. Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. The CRISPR Journal. 2018.
10. Yupeng Cai et al. CRISPR/Cas9-mediated targeted mutagenesis of GmFT2a delays flowering time in soya bean. Plant Biotechnology Journal. 2018.
11. Xiugui Chen et al. Targeted mutagenesis in cotton (Gossypium hirsutum L.) using the CRISPR/Cas9 system. Scientific Reports. 2017.
12. Yanling Wang et al. Increased lateral root formation by CRISPR/Cas9-mediated editing of arginase genes in cotton. Science China. Life Sciences. 2017.
13. Tian Wang et al. CRISPR technology is revolutionizing the improvement of tomato and other fruit crops. Horticulture Research. 2019.
14. Kunling Chen, et al. CRISPR/Cas9 genome editing and precision plant breeding in agriculture. Annual Review of Plant Biology. 2019.