GENOME ENGINEERING FOR CROP IMPROVEMENT

EDITED BY

SANTOSH KUMAR UPADHYAY

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Genome Engineering
for Crop Improvement

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Edited by

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This book is dedicated to all the Corona warriors for their endless efforts to save the human life.

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Preface

About half of the world population is suffering from nutritional deficiency, which is due to either improper diet uptake, or non-availability and unaffordability of balanced nutritive food. Therefore, nutritional enrichment of common food items has been of great interest to fulfill the demands of nutritional deficiency. For instance, development of carotenoids-rich banana and tomato, and iron-rich rice and wheat has been proposed to solve major issues of vitamin A deficiency and anemia. Further, the distribution of important mineral elements in grains is also a major challenge for their bioavailability. For example, the majority of elements like iron are enriched in the aleurone layer of wheat grain and, therefore, it is not in flour for consumers. These are the major challenges which can be addressed by utilizing genome-engineering mechanisms.

Genome engineering has been evolved as an efficient method for targeted mutation and modification in the genome of various organisms. During the last decade, genome engineering has been utilized for numerous trait improvement programs in several important crop plants. Genome-engineering tools like ZFNs, TALENs, and CRISPR-Cas system have been used for *in-vivo* gene editing, as well as much biological pathway engineering for the nutritional improvement of various agricultural and horticultural crop plants. They have been of greater interest due to their precise editing mechanisms and very high specificity. Over the past few years, they have been utilized in numerous agricultural and horticultural crop plants including rice, wheat, legumes, tomato, potato, banana, grapes, etc. They are found to be highly specific and precise in their function. They have also been used for the engineering of numerous metabolic pathways to develop nutrient-rich produce. They are used to decrease the anti-nutrients in crop plants to improve bioavailability of minerals and vitamins, development of zero calorie/ sugar-free potato, indigestible starch-rich grains, allergenic gluten-free wheat and various other processes. Further, they can also be used for the mobilization of minerals from unavailable locations to the bioavailable location for grains.

Researchers have been working hard to increase the nutritional value of various important crop species by increasing the nutraceuticals and minerals such as carotenoids and flavonoid-rich cereals, tomato and fruit crops, iron-enriched rice, maize, and wheat, etc. On the other hand, people are also working to decrease the anti-nutrients such as phytic acids and other chelating elements in crops to increase the bioavailability of the available micronutrients. Further, development of sugar-free potato and gluten-free wheat, resistant starch rich rice and wheat is in great demand by people suffering from various diseases or as preventive measures.

The development of new strategies to cope up with the various challenges has always been a top priority over recent years. Agronomical practices, chemical applications, biofortifications and transgenic expression of protein-coding genes have been explored to alleviate these problems to some extent. However, new and specific technology is always in demand. Genome engineering can be the best alternative for nutritional improvement of agricultural and horticultural crops. Further, it will be highly specific to a particular gene and plant, therefore, it has minimal bio-safety risk. Taking this, together with progress made in crop improvement using genome engineering methods, this book has covered all the recent knowledge and development in the area related to the nutritional value, mineral and nutrient localization, and engineering for stress resistance as well as nutritional improvement of various crop plants. None of the currently available books have covered this topic at such a broad scale. Further, this area is of tremendous importance, not only in the present scenario but also for future decades.

Much effort has been made by all those involved to provide a well-informed and knowledgeable book. I hope that this stimulates discussion and inspires future research and discussion on such an important topic.

About the Editor

Dr. Santosh Kumar Upadhyay is currently working as an Assistant Professor at the Department of Botany, Panjab University, Chandigarh, India. Prior to this, Dr. Upadhyay was DST-INSPIRE faculty at the National Agri-Food Biotechnology Institute, Mohali, Punjab, India. He did his doctoral work at the CSIR-National Botanical Research Institute, Lucknow and received his PhD in Biotechnology from UP Technical University, Lucknow, India. He has been working in the field of Plant Biotechnology for more than 14 years. His present research focuses in the area of functional genomics. He is involved in the characterization of various insect toxic proteins from plant biodiversity, and defense and stress-signaling genes in bread wheat. His research group at PU has characterized numerous important gene families and long non-coding RNAs related to the abiotic and biotic stress tolerance and signaling in bread wheat. He has also established the method for genome editing in bread wheat using CRISPR-Cas system and developed a tool, SSinder, for CRISPR target-site prediction. His research contribution led to the publication of more than 55 research papers in leading journals of international repute. Further, there are more than five national and international patents,17 book chapters and four books to his credit.

In recognition of his strong research record he has been awarded NAAS Young scientist award (2017–2018) and NAAS-Associate (2018) from the National Academy of Agricultural Sciences, India, INSA Medal for Young Scientist (2013) from the Indian National Science Academy, India, NASI- Young Scientist Platinum Jubilee Award (2012) from the National Academy of Sciences, India, and Altech Young Scientist Award (2011). He has also been the recipient of the prestigious DST-INSPIRE Faculty Fellowship (2012), and SERB-Early Career Research Award, (2016) from the Ministry of Science and Technology, Government of India. Dr. Upadhyay also serves as a member of the editorial board and reviewer of a number of peer-reviewed international journals.

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1

An Overview of Genome-Engineering Methods

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CHAPTER MENU

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1.1 Introduction

Agricultural practices, combined with advanced plant breeding and modern technologies, provided food security to millions of people. However, increasing global population demands significant increase in world food production (Parry and Hawkesford 2012). Nevertheless, climate change, depletion of natural resources, increased pollution, and political instabilities are a threat to the food and nutritional security for future generations in the twenty-first century. Unfortunately, the amount of remaining arable land is limited, necessitating an increase in food production on currently-used land. Compounding these challenges are the predicted crop losses due to extreme temperatures, pest attacks, and pathogen outbreaks. A powerful approach that may help overcome these challenges is to modify DNA sequences within plant chromosomes for trait improvement (Sedeek et al. 2019). Further, plants can be engineered to have increased tolerance to environmental stresses and pathogens (Han and Kim 2019; Ji et al. 2015; Makarova et al. 2011). In addition to improving the genetic makeup of the crops to meet increasing food demands and control crop loss, genome engineering can also be used to produce valuable plants or products for non-agricultural purposes (Chen et al. 2019). For example, there is great

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potential for plants to be used as bioreactors for pharmaceutical proteins. Genetic engineering for increasing the secondary metabolite production in plants would be another use of this technology which would help the perfumery, cosmetic and medical industries, as the secondary metabolites produced from plants have a number of uses (El-Mounadi et al. 2020). However, to realize the potential benefits of these applications, we must generate effective tools and approaches for editing plant DNA (Miroshnichenko et al. 2019; Tang and Tang 2017).

Introduction of programmed sequence-specific nucleases (SSNs) and their applications in precise genome editing unfurled a new dimension in genome engineering (Kim and Kim 2014; Voytas 2013). Over the last few decades, researchers reported a few important SSNs, which could be easily engineered and reprogrammed to create double-stranded breaks (DSBs) at the desired location inside the chromosome. There are three major genome engineering methods, ZFNs, TALENs, and CRISPR-Cas system (Figure 1.1A) (Jang and Joung 2019; Mahfouz et al. 2014), that have been utilized so far for a variety of purposes, and these have been discussed in detail in the coming sections. Further, we have also described the recently added CRISPR-Cpf1 system of genome engineering.

1.2 ZFNs

Zinc-finger nucleases are chimeric fusion proteins consisting of a DNA-binding domain and a DNA-cleavage domain. The DNA-binding domain is composed of a set of Cys2His2 zinc fingers (usually three to six). Each zinc finger primarily contacts 3 bp of DNA and a set of three to six fingers recognize 9–18 bp, respectively. The DNA-cleavage domain is derived from the cleavage domain of the FokI restriction enzyme. FokI activity requires dimerization; therefore, to site-specifically cleave DNA, two zinc-finger nucleases are designed in a tail-to-tail orientation (Kim et al. 1996).

Zinc-finger nucleases can be remodified to recognize different DNA sequences. However, one limitation with redirecting targeting is that it depends on the context of the host. For example, a zinc finger that recognizes GGG may not recognize this sequence when fused to other zinc fingers. As a result, the modular assembly of zinc fingers has had limited success (Ramirez et al. 2008). One of the more successful methods for redirecting targeting involves generating a library of three zinc-finger variants from a pre-selected pool of zinc-finger monomers (Maeder et al. 2008). The resulting library of zinc-finger arrays can then be interrogated using a bacterial two- hybrid screen, where binding of the zinc-finger array to a pre-determined sequence results in the expression of a selectable marker gene. This method has generated highly-active zinc-finger nuclease (ZFN) pairs for sites within animal and plant genomes. Since the development of ZFN technology, several studies have been done to engineer specific zinc-finger modules for each of the 64 codon triplets (Bae et al. 2003; Dreier et al. 2001; Pabo et al. 2001). Until now, several ZFNs have been designed and used in numerous species. The developments for more specific and efficient technologies also gave rise to fewer off-target effects. There are three most commonly available tools for engineering the ZF domains: context-dependent Assembly (CoDA), Oligomerized Pool Engineering (OPEN), and Modular Assembly (MA). Several softwares are available for designing engineered ZFs (ZiFiT), containing the database of ZFs (ZiFDB) and

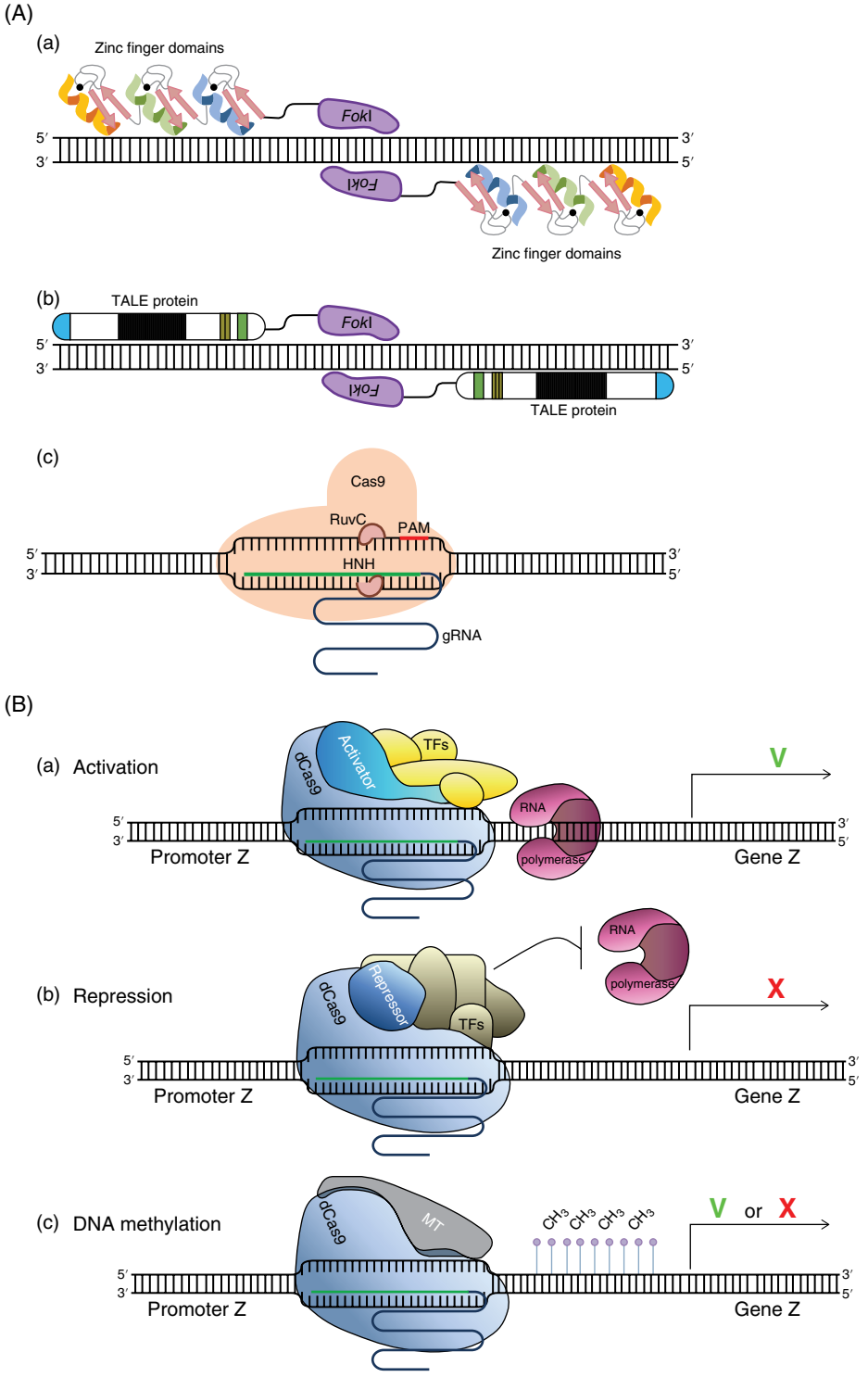

Figure 1.1 (A) Diagrammatic representation of (a) Zinc-finger nucleases (ZFNs), (b) Transcription activator-like effector nucleases (TALENs) and (c) Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 mediates DSBs formation. (B) dCas9-based targeted genome regulation by (a) activation of gene expression, (b) repression of gene expression and (c) DNA methylation. *Source:* Adapted from Mahfouz et al. (2014) © 2014. Reproduced with the permission of John Wiley & Sons.

identification of potential targets for ZFNs in several model organisms (ZFNGenome) (Kim et al. 2009; Mandell and Barbas 2006; Sander et al. 2007).

Zinc-finger nucleases have been widely used for plant genome engineering. Plant species that have been modified using zinc-finger nucleases include, Arabidopsis, maize, soybean, tobacco, etc. (Ainley et al. 2013; Cai et al. 2009; Curtin et al. 2011; Lloyd et al. 2005; Marton et al. 2010; Osakabe et al. 2010; Shukla et al. 2009; Townsend et al. 2009; Wright et al. 2005; Zhang et al. 2010). With their relatively small size (~300 amino acids per zinc-finger nuclease monomer), and the further advancements in methods for redirecting targeting (Sander et al. 2011a), zinc-finger nucleases should continue to be an effective technology for editing plant.

1.3 TALENs

Transcription activator-like effectors nucleases (TALENs) are fusion proteins, consisting of a DNA-binding domain and a DNA-cleavage domain. Whereas the DNA-cleavage domain is the same between zinc-finger nucleases and TALENs (the catalytic portion of FokI), the DNA binding domains are different. The TALEN DNA-binding domain is derived from TALE proteins found in the plant pathogen Xanthomonas. These proteins are composed of direct repeats of 33–35 amino acids, and nearly all arrays found in Xanthomonas contain a final, half repeat, consisting of the first 20 amino acids from the normal repeat. Two amino acids within these repeats (positions 12 and 13) are responsible for recognizing a single nucleotide base (these amino acids are referred to as repeat-variable diresidues; RVDs). When the TALE effector code was broken (i.e. the relationship between the RVD and corresponding target base) (Boch et al. 2009; Moscou and Bogdanove 2009), the ability to redirect targeting, and their use as a genome engineering tool was realized (Christian et al. 2010; Li et al. 2011; Mahfouz et al. 2011). To make TALENs useful in gene targeting, the basic requirement is the modular assembly of repeat sequences containing the appropriate RVD corresponding to the nucleotide target. The most widely used RVDs and their nucleotide targets are HD, cytosine; NG, thymine; NI, adenine; NN, guanine, and adenine; NS, adenine, cytosine, and guanine; N*, all four nucleotides. This one-to-one correspondence of a single RVD to a single DNA base has eliminated construction challenges due to context-dependency seen with zinc-fingers and meganucleases. However, one limitation when using TALENs is that the target sequence must have thymine at the −1 position (Boch et al. 2009). Further, the long and repetitive nature of TALENs puts a strain on delivery methods where cargo capacity or stability is a limitation.

The assembly of engineered TALE repeat arrays can be challenging from nearly similar repeat sequences; therefore, a number of platforms have been designed to facilitate this assembly. These can be classified into three categories: standard restriction enzyme and ligation-based cloning methods (Huang et al. 2011; Sander et al. 2011); Golden Gate assembly methods (Briggs et al. 2012; Cermak et al. 2011; Engler et al. 2008) and solid-phase assembly methods (Heigwer et al. 2013; Wang et al. 2012).

Several online tools are available for designing TALE effectors to target specific gene sequence and off-target analysis. For example- E-TALEN (Lin et al. 2014), Scoring Algorithm for Predicting TALEN Activity (SAPTA) (Neff et al. 2013), Mojo-hand (Coordinators 2013),

TAL Effector-Nucleotide Targeter (TALE-NT), etc. TALE-NT is a collection of versatile web-based tools like-TALEN Targeter, TAL Effector Targeter, Target finder, Paired Target Finder, and TALEN Targeter Off-Target Counter (Christian et al. 2013).

Several studies have demonstrated the usefulness of TALENs in different plant species, including Arabidopsis (Zhang et al. 2013), tobacco (Wang et al. 2012; Wendt et al. 2013), barley (Li et al. 2012), rice (Shan et al. 2013a) and Brachypodium (Reyon et al. 2011). Taken together, the modular nature of TALE repeats, along with efficient methods for assembling repetitive DNA sequences (Garneau et al. 2010; Wang et al. 2012), have enabled TALENs to become one of the premier tools for plant genome engineering.

1.4 CRISPR-Cas System

The most recent addition to the SSN family is the CRISPR/Cas system that is normally present within bacteria and archaea, and provides an adaptive immunity against invading plasmids or viruses. CRISPR/Cas system functions to destroy invading nucleic acids by introducing targeted DNA breaks (Garneau et al. 2010).

There are three major types of CRISPR/Cas system: Types I – III (Makarova et al. 2011). The Type II system was adopted for genome engineering a few years ago (Cong et al. 2013; Zhang et al. 2011). In this system, two components enable targeted DNA cleavage: a Cas9 protein and an RNA complex consisting of a CRISPR RNA (crRNA; contains 20 nucleotides of RNA that are homologous to the target site) and a trans-activating CRISPR RNA (tracrRNA). Cas9 protein causes double-stranded DNA break at the sequences homologous to the crRNA sequence and upstream of a protospacer-adjacent motif (PAM) (PAM; e.g. NGG for *Streptococcus pyogenes* Cas9). For genome engineering purposes, the complexity of the system was reduced by fusing the crRNA and tracrRNA to generate a single-guide RNA (gRNA). Moreover, off-target cleavage is a limitation of the CRISPR/Cas system (Cho et al. 2014; Fu et al. 2013).

The target site recognition in CRISPR-Cas system is facilitated through RNA: DNA interaction (as opposed to a protein: DNA interaction used by meganucleases, zinc-finger nucleases, and TALENs). Redirecting of Cas9 targets involves modification of 20 nucleotides within the crRNA or gRNA. These 20 nucleotides are used to direct Cas9 binding and cleavage, the system has been shown to tolerate mismatches, with a higher tolerance closer to the 5′ end of the target sequence (Fu et al. 2013). Results from recent studies suggest the first 8–12 nucleotides, in addition to the PAM sequence, are most critical for target site recognition (Sternberg et al. 2014; Wu et al. 2014). To reduce off-targeting, several methods have been developed, including dual-nicking of DNA (Mali et al. 2013; Ran et al. 2013), a fusion of catalytically-dead Cas9 to FokI (Guilinger et al. 2014; Tsai et al. 2014) and shortening of gRNA sequence (Fu et al. 2014). Several softwares and programs have been developed in recent years for the identification of target sequences in the genome and the design of specific gRNA, which are listed in Table 1.1.

The Cas9 is an endonuclease consisting of two discrete nuclease domains: the HNH domain which is responsible for the cleavage of the DNA strand complementary to the guide RNA sequence (target strand) and the RuvC-like domain that cleaves the DNA strand opposite the complementary strand (Chen et al. 2014; Gasiunas et al. 2012; Jinek et al. 2012).

Table 1.1 List of available softwares and programs for designing gRNA.

Software	Features	Link	References
Cas-OFFinder	Identifies gRNA target sequence from an input sequence and checks off-target binding site	http://www.rgenome.net/cas-offinder	Bae et al. (2014)
Cas-Designer	Identifies gRNA target sequence from an input with low probability of off-target effect	http://www.rgenome.net/cas-designer/	Park et al. (2015)
Cas9 Design	Designs gRNA	http://cas9.cbi.pku.edu.cn/database.jsp	Ma et al. (2013)
E-CRISP	Designs gRNA	http://www.e-crisp.org/E-CRISP/designcrispr.html	Heigwer et al. (2014)
CRISPR-P	Designs gRNA	http://cbi.hzau.edu.cn/crispr2/	Lei et al. (2014)
CHOP	Identifies target site	https://chopchop.rc.fas.harvard.edu/	Montague et al. (2014)
CRISPR-PLANT	Designs gRNA	http://www.genome.arizona.edu/crispr/	Xie et al. (2014)
CCTop	Identifies candidate gRNA target sites with reduced off-target quality	http://crispr.cos.uni-heidelberg.de/	Stemmer et al. (2015)
CRISPRdirect	Identifies candidate gRNA target sequences	http://crispr.dbcls.jp/	Naito et al. (2015)
COSMID	Identifies target sites	https://crispr.bme.gatech.edu	Cradick et al. (2014)
CRISPR Finder	Identifies CRISPR	http://crispr.u-psud.fr/Server	Grissa et al. (2007)
CrisprGE	Identifies target sites	http://crdd.osdd.net/servers/crisprge	Kaur et al. (2015)
CRISPR Multitargeter	Identifies target sites	http://www.multicrispr.net	Prykhozhij et al. (2015)
CRISPRseek	Identifies target specific guide RNAs	http://www.bioconductor.org/packages/release/bioc/html/CRISPRseek.html	Zhu et al. (2014)
flyCRISPR	Identifies target sites and evaluate its specificity	http://flycrispr.molbio.wisc.edu	Gratz et al. (2014)
GT-SCAN	Identifies target sites and ranking them with their potential off target sites	http://flycrispr.molbio.wisc.edu	O'Brien and Bailey (2014)
sgRNAcas9	Identifies target sites with their potential off target sites	www.biootools.com	Xie et al. (2014)
SSFinder	Identifies target sites	https://code.google.com/p/ssfinder	Upadhyay and Sharma (2014)
ZiFiT	Identifies target sites	http://zifit.partners.org/ZiFiT	Mandell and Barbas (2006)
sgRNA Designer	Guide RNA design based on efficiency score	http://broadinstitute.org/rnai/public/analysis-tools/sgrna-design	Doench et al. (2014)