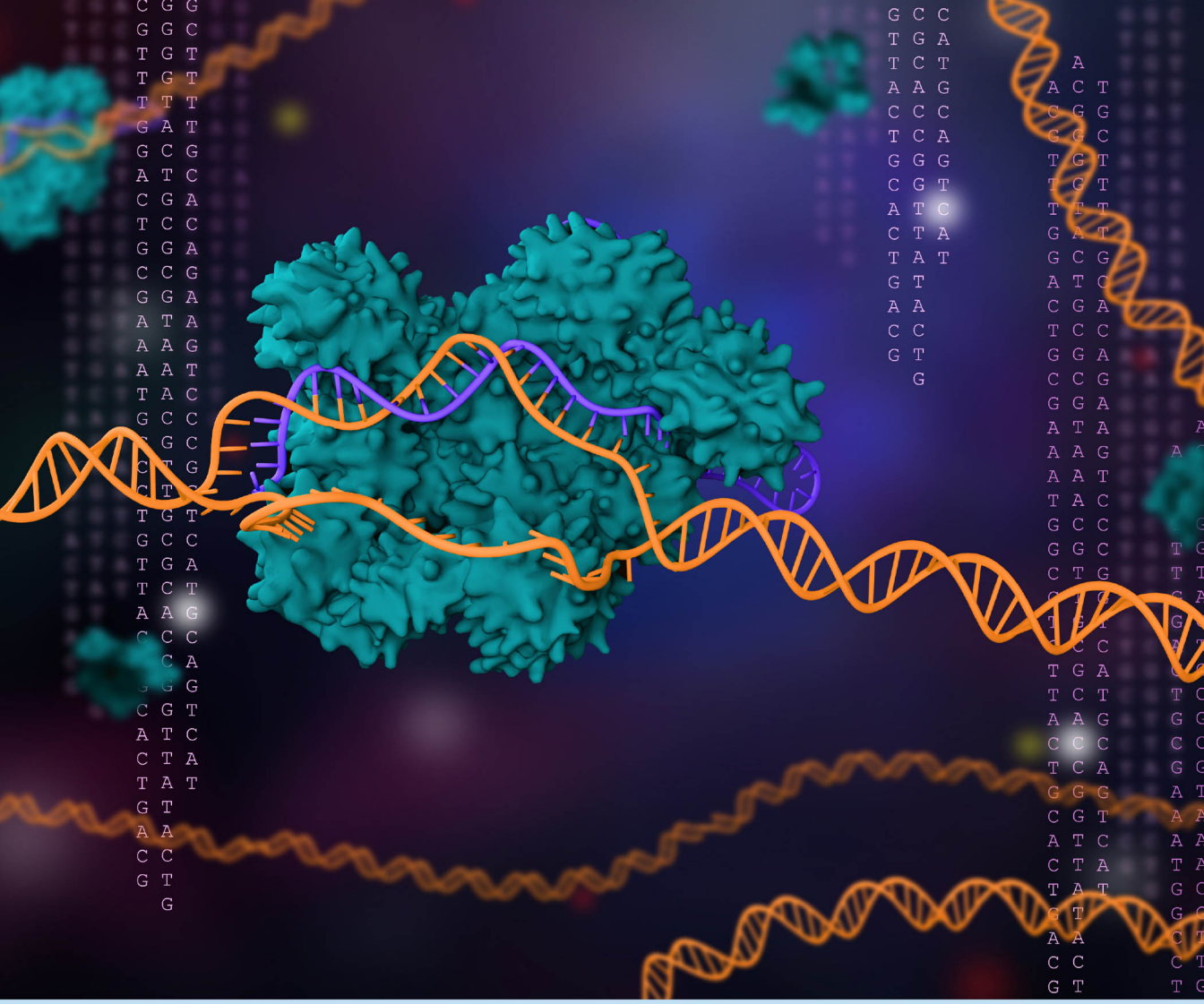

CRISPR AND PLANT FUNCTIONAL GENOMICS

Edited by
Jen-Tsung Chen

CRC Press
Taylor & Francis Group

CRISPR and Plant Functional Genomics

CRISPR is a crucial technology in plant physiology and molecular biology, resulting in more sustainable agricultural practices, including outcomes of better plant stress tolerance and crop improvement. *CRISPR and Plant Functional Genomics* explores ways to release the potential of plant functional genomics, one of the prevailing topics in plant biology and a critical technology for speed and precision crop breeding. This book presents achievements in plant functional genomics and features information on diverse applications using emerging CRISPR-based genome editing technologies producing high-yield, disease-resistant, and climate-smart crops. It also includes theories on organizing strategies for upgrading the CRISPR system to increase efficiency, avoid off-target effects, and produce transgene-free edited crops.

Features:

- Presents CRISPR-based technologies, releasing the potential of plant functional genomics
- Provides methods and applications of CRISPR/Cas-based plant genome editing technologies
- Summarizes achievements of speed and precision crop breeding using CRISPR-based technologies
- Illustrates strategies to upgrade the CRISPR system
- Supports the UN's sustainable development goals to develop future climate-resilient crops

CRISPR and Plant Functional Genomics provides extensive knowledge of CRISPR-based technologies and plant functional genomics and is an ideal reference for researchers, graduate students, and practitioners in the field of plant sciences as well as agronomy and agriculture.

CRISPR and Plant Functional Genomics

Edited by
Jen-Tsung Chen

CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

First edition published 2024
by CRC Press
2385 NW Executive Center Drive, Suite 320, Boca Raton FL 33431

and by CRC Press
4 Park Square, Milton Park, Abingdon, Oxon, OX14 4RN

CRC Press is an imprint of Taylor & Francis Group, LLC

ISBN: 9781032469492 (hbk)
ISBN: 9781032480343 (pbk)
ISBN: 9781003387060 (ebk)

DOI: 10.1201/9781003387060

Typeset in Times
by codeMantra

Contents

Preface....ix
Editor....xi
Contributors....xiii

Chapter 1 Advances in CRISPR/Cas-based Genome Editing: Break New Ground for Plant Functional Genomics....1

Hao Hu and Fengqun Yu

Chapter 2 Functional Genome Analysis and Genome Editing in Plants: An Updated Overview....20

Karma Landup Bhutia, Sarita Kumari, Bharti Lap, Kumari Anjani, Limasunep Longkumer, M. James, Anima Kisku, Bhavna Borah, Prabhakar Nishi, Kanshouwa Modunshim, Vinay Kumar Sharma, Nangsol Dolma Bhutia, Barasha Rani Deka, and Akbar Hossain

Chapter 3 Recent Tools of Genome Editing and Functional Genomics in Plants: Present and Future Applications on Crop Improvement....40

Johni Debbarma, Mamta Bhattacharjee, Trishna Konwar, Channakeshavaiah Chikkaputtaiah, and Dhanawantari L. Singha

Chapter 4 Strategies and Applications of Genomic Editing in Plants with CRISPR/Cas9....59

Ishfaq Majid Hurrah, Tabasum Mohiuddin, Sayanti Mandal, Mimosa Ghorai, Sayan Bhattacharya, Potshangbam Nongdam, Abdel Rahman Al-Tawaha, Ercan Bursal, Mahipal S Shekhawat, Devendra Kumar Pandey, and Abhijit Dey

Chapter 5 Virus-Induced Genome Editing: Methods and Applications in Plant Breeding....81

Mireia Uranga

Chapter 6 CRISPR/Cas9: The Revolutionary Genome Editing Tools for Crop Genetic Tweak to Render It 'Climate Smart'....108

Abira Chaudhuri, Koushik Halder, Malik Z. Abdin, and Asis Datta

Chapter 7 Functional Genomics for Abiotic Stress Tolerance in Crops....124

Muhammad Khuram Razzaq, Guangnan Xing, Ghulam Raza, Muhammad Basit Shehzad, and Reena Rani

Chapter 8 CRISPR/Cas9 for Enhancing Resilience of Crops to Abiotic Stresses....133

Gyanendra Kumar Rai, Danish Mushtaq Khanday, Gayatri Jamwal, and Monika Singh

Chapter 9 CRISPR-Based Genome Editing for Improving Nutrient Use Efficiency and Functional Genomics of Nutrient Stress Adaptation in Plants..... 144

Lekshmy Sathee, Sinto Antoo, Balaji Balamurugan, Asif Ali Vadakkethil, Gadpayale Durgeshwari Prabhakar, Arpitha S R, Sudhir Kumar, Archana Watts, and Viswanathan Chinnusamy

Chapter 10 CRISPR-Cas Genome Modification for Non-Transgenic Disease-Resistant, High Yielding and High-Nutritional Quality Wheat 175

S. M. Hisam Al Rabbi, Israt Nadia, and Tofazzal Islam

Chapter 11 Using CRISPR to Unlock the Full Potential of Plant Immunity..... 189

Mumin Ibrahim Tek, Abdul Razak Ahmed, Ozer Calis, and Hakan Fidan

Chapter 12 Advancement in Gene Editing for Crop Improvement Highlighting the Application of Pangenomes.....206

Biswajit Pramanik, Anamika Das, and Sandip Debnath

Chapter 13 Characterization of CRISPR-Associated Protein for Epigenetic Manipulation in Plants 223

Ayyadurai Pavithra, Chinnasamy Sashtika, Senthil Kalaiselvi, Santhanu Krishnapriya, Natchiappan Senthilkumar, and Arumugam Vijaya Anand

Chapter 14 Advancement in Bioinformatics Tools in the Era of Genome Editing-Based Functional Genomics 239

Karma Landup Bhutia, Bharti Lap, Sarita Kumari, Kumari Anjani, Bhavna Borah, Ponnuchamy Mugudeshwari, Anuradha, Vinay Kumar Sharma, Anima Kisku, Nangsol Dolma Bhutia, Rajalingam Amutha Sudhan, Mahtab Ahmad, and Akbar Hossain

Chapter 15 Gene Editing Using CRISPR/Cas9 System: Methods and Applications 258

Gopika, Boro Arthi, Natchiappan Senthilkumar, Senthil Kalaiselvi, Santhanu Krishnapriya, and Arumugam Vijaya Anand

Chapter 16 CRISPR/Cas13 for the Control of Plant Viruses..... 271

Joana A. Ribeiro, Carla M. R. Varanda, Patrick Materatski, Maria Doroteia Campos, Mariana Patanita, André Albuquerque, Nicolás Garrido, Tomás Monteiro, Filipa Santos, and Maria do Rosário Félix

Chapter 17 Genome Editing in Ornamental Plants: Current Findings and Future Perspectives 289

Kumaresan Kowsalya, Nandakumar Vidya, Packiaraj Gurusaravanan, Bashyam Ramya, Arumugam Vijaya Anand, and Muthukrishnan Arun

Chapter 18 A Convenient CRISPR/Cas9 Mediated Plant Multiple Gene Editing Protocol by In-Fusion Technology 301

Jun-Li Wang, Luo-Yu Liang, and Lei Wu

Chapter 19 The Application of CRISPR Technology for Functional Genomics in Oil Palm and Coconut 313

Siti Nor Akmar Abdullah and Muhammad Asyraf Md Hatta

Chapter 20 Advances and Perspectives in Genetic Engineering and the CRISPR/Cas-Based Technology for Oil Crop Enhancement 332

Kattilaparambil Roshna, Sathasivam Vinoth, Muthukrishnan Arun, Bashyam Ramya, Annamalai Sivaranjini, Arumugam Vijaya Anand, and Packiaraj Gurusaravanan

Chapter 21 CRISPR/Cas9-Based Technology for Functional Genomics and Crop Improvement in Soybean 348

Cuong Xuan Nguyen and Phat Tien Do

Index 357

Preface

For decades, functional genomics has been conducted intensively to explore plant biological networks and continues to identify critical components of regulatory and controlling machinery as well as their functions in sustaining growth and development. In the meantime, the resulting knowledge and technologies keep benefiting agricultural production through the creation of stress-tolerant, high-yield, and high-quality crops using multi-omics-assisted breeding strategies. In recent years, a goal that fits SDGs (Sustainable Development Goals) by the United Nations is to precisely breed climate-smart crops against global climate change, and therefore, definitely, functional genomics is the major technology for future sustainable agriculture.

The system of functional genomics evolves rapidly, and one of the game-changing tools is genome editing technology. Among them, an emerging one is based on CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and the associated Cas proteins. CRISPR-based genome editing technology can specifically introduce targeted sequence alterations, and thus, it leads to a new era of plant functional genomics and, meanwhile, a practical system of precision molecular plant breeding. This book summarizes and comprehensively discusses the introduction and integration of CRISPR-based technology into plant functional genomics and precision breeding. It consists of 21 chapters to refine current knowledge from the literature and is organized by scientists and experts in this field.

Since the post-genomics era, bioinformatics has become an essential tool for dealing with huge amounts of genomic datasets and for the research field of systems biology, contributing to an array of research fields, including the construction of CRISPR systems. In this book, a part of the content pays attention to the design of guide RNA for CRISPR technology, which is critical to ensure efficient and precisely targeted modification of interest genes, and it can be customized by some tools belonging to bioinformatics. Another essential component of the CRISPR system is the association of Cas proteins, and a chapter was organized to illustrate the structures and functions of their varieties, which provide available types for developing appropriate systems in epigenetic manipulation.

To deal with the growing human population, the UN sets SDG goals to achieve zero hunger around the world. The issue of food security majorly depends on agricultural production, and it can be enhanced by crop improvement. Without a doubt, in the future, CRISPR-assisted precision plant breeding will be a promising tool to edit bases with the aid of advanced genomics tools, such as NGS (next-generation sequencing), single-cell omics, spatial transcriptomics, and so on. In modern times, another global threat is climate change, and therefore, scientists attempt to mitigate the effect by gaining future crops with the capacity of "climate-smart" or "climate-resilient" through cutting-edge breeding strategies. One of the effective ways is to apply the emerging CRISPR/Cas technology, particularly using Cas9, and some achievements have been reported, chiefly focusing on environmental/abiotic stress tolerance under heat, drought, flood, and so on, and disease resistance when faced with a changing climate that probably leads to severe damage on yield and quality of crops. For developing future crops, a revolutionary system must be taken into consideration, namely pan-genomics which explores a collection of genomic sequences for the entire species or population and can provide a rich resource for identifying critical genetic variations to advance plant breeding. In the future, crop breeders can perform CRISPR more efficiently based on the information and knowledge of the research in pan-genomes to optimize their programs for "speed breeding".

In agricultural production, a serious negative factor that impacts food security is the huge loss of crop yield and quality caused by pathogens and pests. Fortunately, plants have evolved an innate system of defense against viruses, bacteria, fungi, herbivores, nematodes, and so on. Theoretically, plant immunity has the potential to be released or improved, and it can be achieved by CRISPR-based technology through the understanding of the interactions between plants and microbes or pests and

subsequently ensuring the editing of targeted genes for resistance. For plant pathologists, it's often challenging to monitor and control plant viral diseases, and some efforts have been made to use CRISPR-based technology against plant viruses. In this book, the current achievements using the emerging CRISPR/Cas13 system to successfully produce virus-resistant plants are systematically summarized.

Altogether, this book presents the current knowledge of the CRISPR technology based on refining literature with an emphasis on the fundamental technologies as well as upgraded tools for the exploration of functional genomics and then toward accelerated and precision plant breeding. The applications have been reported in some significant crops, such as rice, wheat, maize, soybean, oil palms, coconut, and ornamental plants. This book provides sufficient and updated knowledge of CRISPR technology and plant functional genomics and thus is an ideal reference for graduate students, teachers, researchers, and experts in the field of plant biology as well as agronomy and agriculture.

As the book editor, I'd like to thank all the authors of this book for their expertise and for their time and effort in organizing chapters. The friendly assistance and instructions from the staff of CRC Press/Taylor & Francis Group are very much appreciated.

Editor

Jen-Tsung Chen is a professor of cell biology at the National University of Kaohsiung in Taiwan. He also teaches genomics, proteomics, plant physiology, and plant biotechnology. His areas of research interest include bioactive compounds, chromatography techniques, plant molecular biology, plant biotechnology, bioinformatics, and systems pharmacology. He is an active editor of academic books and journals to advance the exploration of multidisciplinary knowledge involving plant physiology, plant biotechnology, nanotechnology, ethnopharmacology, and systems biology. He serves as an editorial board member in reputed journals, including *Plant Methods, GM Crops & Food, Plant Nano Biology, Biomolecules, International Journal of Molecular Sciences,* and a guest editor in *Frontiers in Plant Science, Frontiers in Pharmacology, Journal of Fungi*, and *Current Pharmaceutical Design*. Dr. Chen published books in collaboration with Springer Nature, CRC Press/Taylor & Francis Group, and In-TechOpen, and he is handling book projects for more international publishers on diverse topics such as drug discovery, herbal medicine, medicinal biotechnology, nanotechnology, bioengineering, plant functional genomics, plant speed breeding, and CRISPR-based plant genome editing.

Contributors

Malik Z. Abdin
Centre for Transgenic Plant Development, Department of Biotechnology
School of Chemical and Life Sciences
New Delhi, India

Siti Nor Akmar Abdullah
Department of Agriculture Technology, Faculty of Agriculture
Universiti Putra Malaysia
Selangor, Malaysia
and
Laboratory of Agronomy and Sustainable Crop Protection, Institute of Plantation Studies
Universiti Putra Malaysia
Selangor, Malaysia

Mahtab Ahmad
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Abdul Razak Ahmed
Molecular Mycology Laboratory, Plant Protection Department, Faculty of Agriculture
Akdeniz University
Antalya, Turkey

André Albuquerque
MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE - Global Change and Sustainability Institute, Institute for Advanced Studies and Research
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Abdel Rahman Al-Tawaha
Department of Biological Sciences
Al-Hussein Bin Talal University
Maan, Jordon

Arumugam Vijaya Anand
Department of Human Genetics and Molecular Genetics
Bharathiar University
Coimbatore, India

Kumari Anjani
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Sinto Antoo
Division of Plant Physiology
ICAR-Indian Agricultural Research Institute
New Delhi, India

Anuradha
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Arpitha S R
Division of Biochemistry
ICAR-Indian Agricultural Research Institute
New Delhi, India

Boro Arthi
Department of Human Genetics and Molecular Genetics
Bharathiar University
Coimbatore, India

Muthukrishnan Arun
Department of Biotechnology
Bharathiar University
Coimbatore, India

Balaji Balamurugan
ICAR-National Institute for Plant Biotechnology
New Delhi, India

Mamta Bhattacharjee
Department of Agricultural Biotechnology
Assam Agriculture University
Jorhat, India

Sayan Bhattacharya
School of Ecology and Environment Studies
Nalanda University
Rajgir, India

Karma Landup Bhutia
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Nangsol Dolma Bhutia
Department of Vegetable Sciences, College of Horticulture and Forestry
Central Agricultural University (Imphal)
Pasighat, India

Bhavna Borah
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Ercan Bursal
Department of Biochemistry
Mus Alparslan University
Merkez/Muş, Turkey

Ozer Calis
Molecular Virology Laboratory, Plant Protection Department, Faculty of Agriculture
Akdeniz University
Antalya, Turkey

Maria Doroteia Campos
MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE - Global Change and Sustainability Institute, Institute for Advanced Studies and Research
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Abira Chaudhuri
National Institute of Plant Genome Research
New Delhi, India

Channakeshavaiah Chikkaputtaiah
Biological Sciences and Technology Division
CSIR-North East Institute of Science and Technology (CSIR-NEIST)
Jorhat, India

Viswanathan Chinnusamy
Division of Plant Physiology
ICAR-Indian Agricultural Research Institute
New Delhi, India

Anamika Das
Department of Genetics and Plant Breeding
Bidhan Chandra Krishi Viswavidyalaya
Nadia, India
and
Department of Agriculture
Netaji Subhas University
Jamshedpur, India

Asis Datta
National Institute of Plant Genome Research
New Delhi, India

Johni Debbarma
Department of Botany
Assam Don Bosco University
Sonapur, India
and
Biological Sciences and Technology Division
CSIR-North East Institute of Science and Technology (CSIR-NEIST)
Jorhat, India

Sandip Debnath
Department of Genetics and Plant Breeding
Palli Siksha Bhavana (Institute of Agriculture), Visva-Bharati
Sriniketan, India

Barasha Rani Deka
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Abhijit Dey
Department of Life Sciences
Presidency University
Kolkata, India

Phat Tien Do
Institute of Biotechnology
Vietnam Academy of Science and Technology
Hanoi, Vietnam
and
Graduate University of Science and Technology
VietnamAcademy of Science and Technology
Hanoi, Vietnam

Maria do Rosário Félix
MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE - Global Change and Sustainability Institute, Departamento de Fitotecnia, Escola de Ciências e Tecnologia
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Hakan Fidan
Molecular Mycology Laboratory, Plant Protection Department, Faculty of Agriculture
Akdeniz University
Antalya, Turkey

Nicolás Garrido
MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE - Global Change and Sustainability Institute, Institute for Advanced Studies and Research
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Mimosa Ghorai
Department of Life Sciences
Presidency University
Kolkata, India

Gopika
Department of Human Genetics and Molecular Genetics
Bharathiar University
Coimbatore, India

Packiaraj Gurusaravanan
Plant Biotechnology Laboratory, Department of Botany
Bharathiar University
Coimbatore, India

Koushik Halder
National Institute of Plant Genome Research
New Delhi, India
and
Centre for Transgenic Plant Development, Department of Biotechnology
School of Chemical and Life Sciences
New Delhi, India

Muhammad Asyraf Md Hatta
Department of Agriculture Technology, Faculty of Agriculture
Universiti Putra Malaysia
Selangor, Malaysia

Akbar Hossain
Division of Soil Science
Bangladesh Wheat and Maize Research Institute
Dinajpur, Bangladesh

Hao Hu
Agriculture and Agri-Food Canada
Saskatoon Research and Development Centre
Saskatoon, SK, Canada

Ishfaq Majid Hurrah
Plant Biotechnology Division
CSIR-Indian Institute of Integrative Medicine
Srinagar, India

Tofazzal Islam
Institute of Biotechnology and Genetic Engineering (IBGE)
Bangabandhu Sheikh Mujibur Rahman Agricultural University
Gazipur, Bangladesh

M. James
School of Crop Improvement, College of Post Graduate Studies in Agricultural Sciences
Central Agricultural University (Imphal)
Imphal, India

Gayatri Jamwal
School of Biotechnology
Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu
Chatha, India

Senthil Kalaiselvi
Department of Biochemistry Biotechnology and Bioinformatics
Avinashilingam Institute for Home Science & Higher Education for Women
Coimbatore, India

Danish Mushtaq Khanday
Division of Plant Breeding and Genetics
Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu
Chatha, India

Anima Kisku
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Trishna Konwar
Department of Agricultural Biotechnology
Assam Agriculture University
Jorhat, India

Kumaresan Kowsalya
Department of Biotechnology
Bharathiar University
Coimbatore, India

Santhanu Krishnapriya
Department of Biochemistry Biotechnology and Bioinformatics
Avinashilingam Institute for Home Science & Higher Education for Women
Coimbatore, India

Sudhir Kumar
Division of Plant Physiology
ICAR-Indian Agricultural Research Institute
New Delhi, India

Sarita Kumari
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Bharti Lap
School of Crop Improvement, College of Post Graduate Studies in Agricultural Sciences
Central Agricultural University (Imphal)
Imphal, India

Luo-Yu Liang
Department of MOE Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences
Lanzhou University
Lanzhou, China
and
Gansu Province Key Laboratory of Gene Editing for Breeding, School of Life Sciences
Lanzhou University
Lanzhou, China

Limasunep Longkumer
Department of Agricultural Biotechnology
Assam Agricultural University
Jorhat, India

Sayanti Mandal
Department of Biotechnology, Dr. D. Y. Patil Arts
Commerce & Science College (affiliated to Savitribai Phule Pune University)
Pune, India
and
Institute of Bioinformatics and Biotechnology
Savitribai Phule Pune University
Pune, India

Patrick Materatski
MED - Mediterranean Institute for Agriculture, Environment and Development &
CHANGE - Global Change and Sustainability Institute, Institute for Advanced Studies and Research
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Kanshouwa Modunshim
Department of Agricultural Biotechnology
Assam Agricultural University
Jorhat, India

Tabasum Mohiuddin
Govt. Degree College for Women
Baramulla, India

Tomás Monteiro
MED - Mediterranean Institute for Agriculture, Environment and Development &
CHANGE - Global Change and Sustainability Institute, Institute for Advanced Studies and Research
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Ponnuchamy Mugudeshwari
Indian Institute of Rice Research
Hyderabad, India

Israt Nadia
Mawlana Bhashani Science and Technology University
Tangail, Bangladesh

Cuong Xuan Nguyen
Institute of Biotechnology
Vietnam Academy of Science and Technology
Hanoi, Vietnam

Prabhakar Nishi
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Potshangbam Nongdam
Department of Biotechnology
Manipur University
Imphal, India

Devendra Kumar Pandey
Department of Biotechnology
Lovely Professional University
Punjab, India

Mariana Patanita
MED - Mediterranean Institute for Agriculture, Environment and Development &
CHANGE - Global Change and Sustainability Institute, Institute for Advanced Studies and Research
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Ayyadurai Pavithra
Department of Human Genetics and Molecular Genetics
Bharathiar University
Coimbatore, India

Gadpayale Durgeshwari Prabhakar
Division of Biochemistry
ICAR-Indian Agricultural Research Institute
New Delhi, India

Biswajit Pramanik
Department of Genetics and Plant Breeding
Palli Siksha Bhavana (Institute of Agriculture), Visva-Bharati
Sriniketan, India

S. M. Hisam Al Rabbi
Biotechnology Division
Bangladesh Rice Research Institute
Gazipur, Bangladesh

Gyanendra Kumar Rai
School of Biotechnology
Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu
Chatha, India

Bashyam Ramya
Department of Biochemistry
Holy Cross College (Autonomous)
Tiruchirappalli, India

Reena Rani
National Institute for Biotechnology and Genetic Engineering
Faisalabad, Pakistan
and
Constituent College Pakistan Institute of Engineering and Applied Sciences
Faisalabad, Pakistan

Ghulam Raza
National Institute for Biotechnology and Genetic Engineering
Faisalabad, Pakistan
and
Constituent College Pakistan Institute of Engineering and Applied Sciences
Faisalabad, Pakistan

Muhammad Khuram Razzaq
Soybean Research Institute & MARA National Center for Soybean Improvement & MARA Key Laboratory of Biology and Genetic Improvement of Soybean & National Key Laboratory for Crop Genetics and Germplasm Enhancement & Jiangsu Collaborative Innovation Center for Modern Crop Production
Nanjing Agricultural University
Nanjing, China

Joana A. Ribeiro
MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE - Global Change and Sustainability Institute, Institute for Advanced Studies and Research
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Kattilaparambil Roshna
Plant Biotechnology Laboratory, Department of Botany
Bharathiar University
Coimbatore, India

Filipa Santos
MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE - Global Change and Sustainability Institute, Departamento de Fitotecnia, Escola de Ciências e Tecnologia
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Chinnasamy Sashtika
Department of Biochemistry Biotechnology and Bioinformatics
Avinashilingam Institute for Home Science & Higher Education for Women
Coimbatore, India

Lekshmy Sathee
Division of Plant Physiology
ICAR-Indian Agricultural Research Institute
New Delhi, India

Natchiappan Senthilkumar
Division of Bioprospecting
Institute of Forest Genetics and Tree Breeding (IFGTB)
Coimbatore, India

Vinay Kumar Sharma
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Muhammad Basit Shehzad
College of Plant Protection
Nanjing Agricultural University
Nanjing, China

Mahipal S Shekhawat
Plant Biotechnology Unit
KM Government Institute for Postgraduate Studies and Research
Puducherry, India

Monika Singh
Department of Applied Sciences & Humanities
GL Bajaj Institute of Technology and Management
Greater Noida, India

Dhanawantari L. Singha
Department of Botany
Rabindranath Tagore University
Hojai, Assam

Annamalai Sivaranjini
Department of Biotechnology
Dwaraka Doss Goverdhan Doss Vaishnav College
Chennai, India

Rajalingam Amutha Sudhan
Department of Agricultural Biotechnology and Molecular Biology, College of Basic Sciences & Humanities
Dr. Rajendra Prasad Central Agricultural University
Pusa (Samastipur), India

Mumin Ibrahim Tek
Molecular Virology Laboratory, Plant Protection Department, Faculty of Agriculture
Akdeniz University
Antalya, Turkey

Mireia Uranga
Department of Plant Biotechnology and Bioinformatics
Ghent University
Ghent, Belgium
and
VIB Center for Plant Systems Biology
Ghent, Belgium
and
KU Leuven Plant Institute (LPI)
Heverlee, Belgium

Asif Ali Vadakkethil
Centre for Agricultural Bioinformatics
ICAR-Indian Agricultural Statistics Research Institute
New Delhi, India

Carla M. R. Varanda
ESAS, Instituto Politécnico de Santarém
Santarém, Portugal
and
MED - Mediterranean Institute for Agriculture, Environment and Development & CHANGE - Global Change and Sustainability Institute, Institute for Advanced Studies and Research
Universidade de Évora, Pólo da Mitra
Évora, Portugal

Nandakumar Vidya
Department of Botany
Bharathiar University
Coimbatore, India

Sathasivam Vinoth
Department of Biotechnology
Sona College of Arts and Science
Salem, India

Jun-Li Wang
Department of MOE Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences
Lanzhou University
Lanzhou, China
and
Gansu Province Key Laboratory of Gene Editing for Breeding, School of Life Sciences
Lanzhou University
Lanzhou, China

Archana Watts
Division of Plant Physiology
ICAR-Indian Agricultural Research Institute
New Delhi, India

Lei Wu
Department of MOE Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences
Lanzhou University
Lanzhou, China
and
Gansu Province Key Laboratory of Gene Editing for Breeding, School of Life Sciences
Lanzhou University
Lanzhou, China

Guangnan Xing
Soybean Research Institute & MARA National Center for Soybean Improvement & MARA Key Laboratory of Biology and Genetic Improvement of Soybean & National Key Laboratory for Crop Genetics and Germplasm Enhancement & Jiangsu Collaborative Innovation Center for Modern Crop Production
Nanjing Agricultural University
Nanjing, China

Fengqun Yu
Agriculture and Agri-Food Canada, Saskatoon Research and Development Centre
Saskatoon, SK, Canada

1 Advances in CRISPR/Cas-based Genome Editing
Break New Ground for Plant Functional Genomics

Hao Hu and Fengqun Yu

1.1 INTRODUCTION

Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein (CRISPR/Cas) technology has revolutionized the field of genetic engineering and has become a prominent tool in genome editing. It is derived from the bacterial immune system and has been adapted for precise and efficient genome editing in a wide range of organisms, including plants. The discovery of CRISPR/Cas can be traced back to the early 1990s when unusual repetitive DNA sequences were identified in the genomes of bacteria and archaea.[1–4] However, it wasn't until 2007 that researchers recognized the potential role of these repetitive sequences as part of a prokaryotic immune system.[5] Further studies led to the understanding that CRISPR sequences serve as an adaptive immune defense mechanism against invading genetic elements such as viruses and plasmids.[6]

The CRISPR/Cas system consists of two main components: the guide RNA (gRNA) and the Cas protein. The gRNA is a synthetic RNA molecule that guides the Cas protein to the target DNA sequence of interest based on RNA-DNA recognition and binding. The Cas protein is an endonuclease that cleaves DNA at specific sites determined by the gRNA sequence. This cleavage, i.e., double-strand break (DSB), initiates DNA repair either by the error-prone non-homologous end joining (NHEJ) mechanism or by the donor-dependent homology-directed repair (HDR) mechanism, leading to various genetic modifications like sequence insertion/deletion (indel) and gene replacement.[6] The power and versatility of CRISPR/Cas technology lie in its flexibility to be easily reprogrammed by designing a specific gRNA sequence complementary to the target DNA region, which allows researchers to precisely edit genes and introduce designed genetic modifications. Compared to earlier genome editing techniques, such as Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), CRISPR/Cas is simpler, faster, and more cost-effective, making it widely adopted across various fields of research.[7]

Since its initial discovery, CRISPR/Cas technology has rapidly evolved and expanded, with different variants of Cas proteins, gRNA formats, and new effector proteins being discovered and engineered. Each Cas variant has unique characteristics, such as Protospacer Adjacent Motif (PAM) requirements, cleavage patterns, and target specificity. Together with other optimization strategies, especially by linking novel effectors (such as deaminase and reverse transcriptase) to Cas protein, current CRISPR/Cas genome editing in plants has been greatly improved in editing efficiency, specificity, targeting scopes, and the introduction of numerous types of precise editing. These advancements have deeply impacted fundamental biological research such as plant functional genomics and enabled the development of more sophisticated applications, such as multiplexed genome editing (simultaneously targeting multiple genes), base editing (precise nucleotide

DOI: 10.1201/9781003387060-1

substitutions), prime editing (precise search-and-replace genome editing to install indels and/or base substitutions), genome-wide screening, and epigenomic modifications. Since its first application in plants in 2013,[8–10] CRISPR/Cas has been extensively utilized in agriculture to improve yield, disease resistance, and other agronomically important traits.[11–13] Overall, the discovery and development of CRISPR/Cas technology have transformed the field of genetic engineering, providing plant scientists with an efficient and precise tool for understanding gene function, unraveling genetic networks, and driving innovations in various plant research areas including crop improvement.

1.2 PART I. ADVANCES IN CRISPR/CAS TECHNOLOGIES

1.2.1 Cas Variants

Cas proteins, the key components of the CRISPR/Cas system, exhibit remarkable diversity across different bacterial and archaeal species. CRISPR/Cas systems can be categorized into two classes based on their mechanism of action. In Class 1 systems, a multi-protein complex (multi-effector) is involved in the degradation of foreign nucleic acids. On the other hand, Class 2 systems accomplish the same function using a single, large Cas protein (single effector).[14–16] Due to its simplicity in application, Cas proteins from Class 2 systems are extensively studied and utilized. Cas9 is the most widely used Cas protein variant in Class 2. It was initially discovered in *Streptococcus pyogenes* and has become synonymous with CRISPR/Cas technology. Cas9 is an RNA-guided endonuclease that generates DSBs at target DNA sites. The two main domains, known as the RuvC and HNH nuclease domains, are responsible for cleaving the DNA strands.[17] These domains are guided to the target DNA sequence by a single guide RNA (sgRNA), which is a fusion of a CRISPR RNA (crRNA, carrying a sequence complementary to the target DNA) and a trans-activating crRNA (tracrRNA, helping in the processing and stability of the crRNA) and provides the necessary specificity by base-pairing with the complementary DNA sequence (namely protospacer). In addition, a short DNA sequence located immediately adjacent to the protospacer, i.e., PAM, is also essential for Cas9 binding and cleavage. The specific PAM sequence recognized by Cas9 varies depending on the Cas9 ortholog and species.[18–20] For the commonly used *S. pyogenes* Cas9 (SpCas9), the PAM sequence is NGG (where N can be any nucleotide). Over the years, researchers have identified, characterized, and even modified numerous Cas protein variants of Class 2[14,16] (Table 1.1). These Cas variants exhibit distinct features and functionalities, which greatly serve versatile applications and the ever-increasing need for editing specificity, efficiency, targeting range, etc.

1. **nCas9 (nickase Cas9):** nCas9 is a modified version of the Cas9 nuclease protein that retains its ability to bind to target DNA but has lost one of its two nuclease domain activities. Unlike the wild-type Cas9, which creates a DSB in the target DNA, nCas9 induces a single-strand break (SSB) or nick in one of the DNA strands within the protospacer region. The nicking activity of nCas9 is achieved through specific point mutations in the nuclease domains of the Cas9 protein. The most commonly used mutation is the D10A mutation, which disrupts the endonuclease activity of RuvC domain of Cas9. By using a pair of gRNAs, one targeting each DNA strand, nCas9 can be employed to create a pair of nicks on opposite DNA strands.[21] This generates a DSB at the desired genomic locus. The paired nicking strategy reduces off-target effects, as the nicks can be repaired by error-free mechanisms such as homologous recombination rather than relying on error-prone NHEJ repair pathways. The use of nCas9 offers several advantages over wild-type Cas9, including improved specificity and reduced potential for off-target effects. It allows for precise genome editing with minimal disruption to the DNA sequence, making it particularly useful in applications that require high accuracy and minimal impact on the target locus.[21]
2. **dCas9 (deactivated Cas9 or dead Cas9):** dCas9 is a modified version of the Cas9 protein that lacks both of its nuclease activities. Through point mutations, such as D10A and H840A,

TABLE 1.1
Popular Cas Variants in Class 2

Cas Type	Cas Variant	Target Type	PAM (5′-3′)	Characteristics	Applications	References
II	Cas9 (i.e., SpCas9 from *Streptococcus pyogenes*)	DNA	NGG	The activity of both RuvC and HNH nuclease domains create a DSB at the targeted location on dsDNA	Numerous applications involving dsDNA/genome editing such as gene knockout, replacement, etc.	14–17,23
	Cas9-VQR		NGA			19,27
	Cas9-EQR		NGAG			
	Cas9-VRER		NGCG			
	Cas9-NG		NGN			28,29
	Cas9-xCas9		NG/GAA/GAT			
	Cas9-SpG		NGN			30,31
	Cas9-SpRY		PAM-less			
	St1Cas9 (from *S. thermophilus*)		NNAGAA			18
	NmCas9 (from *Neisseria meningitidis*)		NNNNGATT			
	TdCas9 (from *Treponema denticola*)		NAAAAN			
	FnCas9 (from *Francisella novicida*)		NGG			32
	FnCas9-RHA		YG			
	nCas9		NGG	Lack one nuclease activity (usually RuvC) through mutation, creating a nick (single-strand break) at a specific location on dsDNA	Link with various effectors for precise editing (base editing, prime editing, etc.) or gene regulations	21,33,34
	dCas9		NGG	Lack both RuvC and HNH nuclease activities through mutations, no cleavage but can still bind to a specific location on dsDNA.	Link with various effectors for precise editing (prime editing, etc) or gene regulations	22,23,35,36
V	Cas12a	DNA	TTN, or TTTN	Staggered cutting	Auto-processing pre-crRNA activity for multiplex gene regulation	14–16,24,37,38
VI	Cas13	RNA	A, U, C (no G in PFS)	Non-specifically cleaves non-target RNA	mRNA knockdown and RNA editing	14–16,25,39
V	Cas14	DNA	None	ssDNA specific	ssDNA cutting and single nucleotide variant detection	14–16,26

which alter critical residues necessary for endonuclease activity, dCas9 is rendered inactive. It can still bind to target DNA sequences, guided by the associated sgRNA, but does not induce DNA cleavage. This binding alone is often sufficient to attenuate or block the transcription of the targeted gene, especially when the sgRNA positions dCas9 in a manner that obstructs the access of transcription factors and RNA polymerase to the DNA. Instead of introducing permanent genetic changes, dCas9 can be utilized for gene regulation purposes.[22] The modifiable regions of dCas9, typically located at the N- and C-terminus of the protein, can be utilized to attach transcriptional activators, repressors, or other effector domains. When dCas9 is fused with transcriptional activators, it is referred to as CRISPR activation (CRISPRa), enabling targeted upregulation of gene expression. Conversely, when dCas9 is fused with transcriptional repressors, it is known as CRISPR interference (CRISPRi), allowing targeted downregulation of gene expression. This approach offers a powerful tool for studying gene function, gene networks, and regulatory elements in plants.[22,23]

3. **Cas12a (previously known as Cpf1):** Cas12a is an alternative RNA-guided endonuclease that was first identified in the bacterium *Francisella novicida*. Cas12a exhibits a key distinction from Cas9 in its ability to create staggered breaks (i.e., sticky ends).[24] When Cas12a cleaves DNA, it produces overhangs of five base pairs, which proves advantageous for specific applications that necessitate the generation of single-stranded DNA, like HDR experiments. Moreover, Cas12a is well-suited for targeting genomic regions with a high adenine-thymine (A-T) content since its PAM does not rely on the presence of guanine-cytosine (G-C) pairs.[24]
4. **Cas13 (previously known as C2c2):** Cas13 is an RNA-guided nuclease that distinguishes itself from Cas9 by its ability to target RNA instead of DNA, and it has been found in different bacterial species. This unique characteristic makes Cas13 particularly valuable for generating transient modifications in signaling molecules, specifically RNA, as opposed to permanent alterations in the genome. Similar to Cas14, Cas13 exhibits non-specific cleavage of single-stranded RNA when bound to its RNA target sequence.[25] This RNA-targeting capability renders Cas13 highly effective in targeting mRNA and serves as a useful mechanism for temporarily suppressing gene expression. As a result, this system can be employed as an alternative to small interfering RNA (siRNA) or short hairpin RNA (shRNA) for efficient and multiplexable knockdown of RNA.
5. **Cas14 (or Cas12f):** In contrast to other Cas enzymes, Cas14 exhibits a preference for single-stranded DNA (ssDNA) rather than double-stranded DNA (dsDNA). It displays a remarkable ability to cleave ssDNA with exceptional accuracy, as even a single mismatch in the target sequence can be detected. Similar to Cas12, Cas14 is considerably smaller in size compared to the conventional Cas9 protein. Its small size offers advantages for packaging into viral vectors, potentially facilitating its delivery into cells for genome editing applications. In addition, akin to Cas12a, Cas14 demonstrates non-specific cleavage of non-complementary ssDNA upon engagement with its target sequence.[26]

1.2.2 Precise Editing Technologies

The efficacy of gene targeting technology largely depends on HDR for introducing desired sequence modifications, but the limited efficiency of HDR has hindered its application.[40,41] To address this constraint, alternative genome editing technologies have emerged, including base editing and prime editing. These CRISPR/Cas-based technologies offer precise sequence editing without requiring DSBs or donor DNA, making them more efficient than HDR in plants. Initially developed for human cells, cytosine base editors (CBEs) and adenine base editors (ABEs) laid the foundation for the advancement of dual base editors and precise DNA deletions specifically tailored for plants. In this section, we provide a brief overview of the recently developed CRISPR/Cas-based technologies used to achieve precise editing of plant genomes (Table 1.2).

TABLE 1.2
Summary of CRISPR/Cas-based Precise Editing Technologies in Plants

Editing Technology	Main Effectors	Cas Variant	Sequence Modification	Editing Window	Editing Efficiency (up to %)	References
APOBEC1-CBE2/ CBE3/CBE4	Rat APOBEC1	nCas9 (D10A), dCas9 (D10A and H840A), or nCas9-NG	C:G>T:A	C4-C8, or C3-C9	75.00	34,43,45,47,82,83
hAID-CBE3	Human AID	nCas9, nSpCas9-NG, nScCas9, or nCas9-SpRY		C3-C8	97.92	31,33,84,85
APOBEC3A-CBE3/ CBE4	Human APOBEC3A	nCas9		C1-C17	82.90	49
PmCDA1-CBE2/ CBE3/CBE4	Petromyzon marinus CDA1	nCas9, dCas9, xCas9, nSpCas9-NG, nCas9-NG, or nScCas9^{++}		C2-C5, or C1-C17	86.10	46,47,86–88
ABE7.10	TadA-TadA7.10	nCas9, nSpCas9-NG, nSaCas9, or nScCas9	A:T>G:C	A4-A8	94.12	50,53,54,85,89
ABE-P1S	TadA7.10	nCas9, or nSaCas9		A1-A12	96.30	55
ABE8e	TadA8e	nCas9, nCas9-NG, nCas9-SpG, or nCas9-SpRY		A4-A8, A3-A10, or A1-A14	100.00	31,90,91
ABE9	TadA9	nCas9, nSpCas9-NG, nCas9-SpRY, or nScCas9		A1-A12, A4-A10, A3-A10, or A4-A12	100.00	58
CGBE	Anc689(R33A)	nCas9	C:G>G:C	C4-C9	52.50	61
STEME	APOBEC3A-ecTadA-ecTad7.10	nCas9, or nCas9-NG	C:G>T:A and A:T>G:C	C1-C17, and A4-A8	15.10, 73.21	62
pDuBE1	eCDAL-TadA8e	nCas9	C:G>T:A and A:T>G:C	C2-C5, and A4-A8	87.60	92
AFID	APOBEC3A, APOBEC3Bctd	Cas9	Precise, predictable multinucleotide deletion	N/A	34.80	63
PE2	M-MLV-RT (H9Y/D200N/ T306K/W313F/T330P/L603W)	nCas9 (H840A)	all kinds of base substitutions, precise insertions, deletions, and combinations of these sequence modifications	1-80	59.90	66,67,69,93–96
PE3					70.30	66,70,75,79,94,95,97–100
PE4					2.10	66
PE5					18.30	66
PEmax		nCas9 (R221K/N394K/ H840A)			77.08	66,67

1. **CBE:** The initial version of CBE, known as CBE1, was created by linking a rat cytidine deaminase, rAPOBEC1, to a dCas9.[42] CBE1 induces the substitution of cytosine (C) to thymine (T) by deaminating C to uracil (U) on the non-target DNA strand, which is then converted to T during DNA repair and replication. However, the presence of uracil N-glycosylase (UNG) in the cellular base excision repair (BER) pathway leads to low editing efficiency of CBE1 due to the elimination of uracil.[42] To enhance efficiency, the second-generation CBE, CBE2, was developed by adding a uracil DNA glycosylase inhibitor (UGI) to the C-terminal of CBE1, preventing UNG activity.[42] CBE2 improves editing efficiency threefold and reduces unexpected indels.[43] To further enhance efficiency, the third-generation CBE, CBE3, was created by adding rAPOBEC1 and UGI to nCas9 (D10A).[42,44,45] Although CBE3 does not cleave dsDNA, it creates an incision in the target strand to initiate the repair process. The fourth-generation CBE, CBE4, improves deamination activity by linking two UGIs to nCas9, resulting in increased base editing efficiency and reduced incidents of undesired C to A or G transversions compared to CBE3.[46–48] In addition, the Mu Gam protein from bacteriophage was linked to CBE4, resulting in CBE4-Gam, which further enhances product purity and reduces indel occurrences.[48,49]
2. **ABE:** ABE7.10 was the first ABE created by linking nCas9 (D10A) to a dimer comprising a wild-type adenine deaminase called TadA and an evolved adenine deaminase called TadA7.10.[50] ABE7.10 allows the precise conversion of adenine (A) to inosine (I), which is then recognized as guanine (G) during DNA repair and replication processes.[51] The editing window of ABE7.10 is located at positions 4–8 nt in the protospacer region. To enhance editing efficiency, ABE7.10 underwent codon optimization to improve its performance. In addition, a nuclear localization sequence (NLS) was added to facilitate the transport of ABE7.10 into the cell nucleus.[52] ABEmax was developed by adding NLS sequences at both ends of ABE7.10. This modification further increased the editing efficiencies to less than 50% at most targets.[50,53,54] ABEmax demonstrated successful A-to-G conversions in various target genes in rice plants. A simplified version called ABE-P1S was developed (i.e., TadA7.10-nCas9 vs. commonly used TadA-TadA7.10-nCas9), and it showed significantly higher editing efficiency in rice, indicating its potential for precise editing in plants.[55] Later, by using another adenine deaminase variant TadA8e (V106W), the new ABE8e exhibited significantly improved A-to-G conversion and reduced off-target effects.[56,57] Further, TadA9, an improved adenosine deaminase by introducing V82S and Q154R mutations into TadA8e, was found to be compatible with various Cas variants. Thus, ABE9 with TadA9 should have quite relaxed PAM restrictions and demonstrate stronger editing capabilities with an expanded editing window.[58]
3. **CGBE (C-to-G base editor):** CBEs and ABEs are limited to inducing base transitions rather than base transversions. To overcome this limitation, a novel base editor known as CGBE has been developed. CGBE consists of a variant of the rAPOBEC1 cytidine deaminase (R33A), a nCas9 (D10A), and a UNG. Recent studies have demonstrated the successful use of CGBE in achieving efficient C-to-G transversion in bacteria and mammalian cells.[59,60] Further, a rice-specific base editor called OsCGBE03 was created through codon optimization on UNG, which showed efficient C-to-G editing in five rice genes.[61] The development of CGBE expands the repertoire of base editing tools, offering a powerful approach for generating diverse base substitution types in precise crop breeding and the creation of novel germplasm resources.
4. **Dual base editor:** A novel gene editing tool called the "saturated targeted endogenous mutagenesis editor" (STEME) has been developed for the simultaneous editing of cytosine and adenine bases in plants using a sgRNA.[62] This system consists of a cytidine deaminase (APOBEC3A), an adenosine deaminase (ecTadA–ecTadA7.10), a nCas9 (D10A), and a UGI. STEME enables the conversion of cytidines to uridines and adenosines to inosines within the editing window, which are then replicated and repaired by the plant's DNA

repair mechanisms, resulting in dual substitutions of C:G>T:A and A:T>G:C. To expand the editing capacity and target a wider range of sequences, an SpCas9–NG PAM variant is used in the STEME system, which recognizes NG PAM sequences.[28] This allows for the editing of a larger number of target sites. The ability to perform dual base editing with STEME opens up possibilities for the directed evolution of endogenous plant genes in their native context. In addition, STEME can be utilized for modifying cis-regulatory elements in gene regulatory regions and for high-throughput genome-wide screening in plants.

5. **Multinucleotide deletion:** APOBEC–Cas9 fusion-induced deletion systems (AFIDs) are a type of genome editing tool that combines the activity of APOBEC and Cas9 proteins to induce targeted deletions in DNA.[63] APOBEC proteins are naturally occurring enzymes that can induce DNA mutations, particularly cytosine-to-uracil changes. AFIDs utilize two cytidine deaminases, namely hAPOBEC3A and the C-terminal catalytic domain of hAPOBEC3B (hAPOBEC3Bctd), which could generate DNA deletions spanning from the targeted cytidine or a preferred TC motif to the DSB induced by Cas9, respectively.[63] These predictable deletions ensure more consistent editing outcomes. AFIDs could be applied to create predictable multinucleotide deletions and study DNA regulatory regions and protein domains.
6. **PE (prime editor):** PEs are a class of CRISPR/Cas-based genome editing tools that offer precise and versatile editing capabilities for targeted genetic modifications. They are designed to generate all 12 types of base substitutions, precise insertions, deletions, and combinations of these sequence modifications without the requirement for DSBs or the reliance on donor DNA templates. The prime editing system consists of a fusion protein composed of a nCas9 (H840A) (or dCas9), a Moloney murine leukemia virus reverse transcriptase (M-MLV-RT), and an engineered prime editing guide RNA (pegRNA). The pegRNA consists of a reverse transcriptase template (RTT, harboring designed edits) and a primer-binding site (PBS, initiating reverse transcription) at the 3′ end of a sgRNA (targeting the specific location). PBS pairs with the single-strand DNA nicked by nCas9, which allows reverse transcription and incorporation of the designed edits from the RTT into the genome. Subsequently, a series of processes, including equilibration, ligation, and repair, lead to the desired edit. Since its first introduction in 2019,[64] several generations of PEs (PE1, PE2, PE3, PE3b, and subsequent iterations) have been developed to expand their functionality and improve editing efficiency, and the engineering of core editor protein and the redesigning of pegRNA have played a crucial role in the gradual improvement. Initially, PE1 was created by adding a wild-type virus reverse transcriptase to nCas9 (H840A) to enable prime editing. To increase the editing efficiency, PE2 was developed by changing the wild-type reverse transcriptase in PE1 with a modified version containing six specific mutations. Subsequently, by incorporating an additional nicking sgRNA to induce another cut, PE3 was introduced to further increase editing efficiency. However, the PE3 system, inducing two SSBs on complementary DNA strands, could lead to higher indel frequencies through the NHEJ repair pathway. To mitigate undesired indels, PE3b employs a specific sgRNA to induce the second nick after the incorporation of the designed edit into the targeted genome locus is done. In addition, inhibiting critical components of the DNA mismatch repair (MMR) pathway, like MLH1, has been shown to effectively enhance prime editing efficiency.[65] PE4 and PE5 were generated by adding a dominant negative MMR effector (i.e., MLH1dn) to PE2 or PE3, respectively, which can help avoid MMR and thus enhance prime editing capacity and significantly increase editing efficiency by several folds. Furthermore, the ability to create nicks in the non-target strand is essential for efficient prime editing. By switching the nCas9 (H840A) of PE2 with the SpCas9max variant containing two additional mutations (i.e., R221K and N394K), PEmax has achieved significantly improved prime editing efficiency.[65–67] Prime editing has several advantages over conventional HDR strategies, including improved precise genome editing efficiency

and the ability to generate combinations of various sequence modifications at a relatively wide range of positions, which reduces constraints imposed by its PAM.[64,68–72] However, the editing efficiency of PEs in plants is still unsatisfying, even with the utilization of various strategies like alternative reverse transcriptase orthologs, ribozymes for precise pegRNA production, temperature optimization, enhanced sgRNA scaffold modifications, and selective markers for cell enrichment.[65,73–79] In addition, the capacity of PEs to induce larger genetic edits spanning hundreds of nucleotides and their level of specificity have yet to be verified in plants.[68,72,80,81]

1.2.3 Delivery Mechanisms

To effectively utilize CRISPR/Cas9 technology in plants, it is crucial to have a reliable and universally applicable method for delivering the necessary CRISPR/Cas components into plant cells. However, the two commonly used delivery strategies, i.e., *Agrobacterium*-mediated transformation and biolistics, are both inadequate to meet our needs. First of all, both strategies require tissue culture, which is time-consuming and laborious. The recipient plant species available for *Agrobacterium* infection is increasing, but the editing efficiency still varies significantly among different genotypes, especially in monocots.[101] Further, the intrinsic feature of random insertion of foreign DNA into the host genome stirs concerns over safety risks. Regarding biolistics, the mechanical force used for delivery may cause damage to the host genome, and the delivery efficiency is still unsatisfying. As a result, there is an urgent need for innovative delivery strategies to overcome these challenges and enhance the application of CRISPR/Cas9 in plant research.

De novo induction of meristem is a promising approach that utilizes morphogenetic regulators to facilitate CRISPR/Cas-mediated gene editing in plants. These regulators not only assist in transforming recalcitrant cultivars but can also trigger the formation of new meristems in plants, eliminating the need for tissue culture.[102] A recent study demonstrated the effectiveness of this approach by injecting morphogenetic regulators and sgRNA expression cassettes-loaded *Agrobacterium* into pruned sites of Cas9-overexpressing tobacco plants (*Nicotiana benthamiana*).[103] The resulting shoots directly gave rise to gene-edited plants with inheritable mutations. This methodology holds potential for various plant species and can greatly expedite plant research. Virus-assisted gene editing is another promising strategy that leverages the capabilities of plant viruses to generate gene-edited plants without relying on tissue culture. Certain plant viruses can efficiently deliver sgRNA for genome modifications. Several single-stranded RNA viruses, such as tobacco rattle virus and the single-stranded DNA cabbage leaf curl virus, have been utilized for this purpose, and the editing efficiencies could reach 80%.[104–109] Still, these viruses cannot deliver Cas9 and sgRNA expression cassettes together due to cargo limitations. To overcome this, researchers inserted Cas9 and sgRNA cassettes into the genomes of another two plant viruses with better capacity, which enabled systemic gene editing in tobacco plants.[110,111] To address the limitation of heritability, sgRNAs were fused with RNA mobile elements and incorporated into tobacco rattle virus RNA2. These mobile elements guided the sgRNAs into shoot apical meristem cells, resulting in high efficiencies of heritable mutations in the offsprings.[112]

1.3 PART II. APPLICATIONS OF CRISPR/CAS IN PLANT FUNCTIONAL GENOMICS

CRISPR/Cas technology has provided plant scientists with a precise, efficient, and versatile tool for studying gene function, unraveling genetic networks, and accelerating crop improvement efforts. It has significantly advanced our understanding of plant functional genomics and has the potential to revolutionize plant breeding and agriculture. Here are some examples of its vast applications in plant functional studies:

1. **Gene knockdown/activation/visualization:** Cas9 can effectively target and silence specific genes at the DNA level, a phenomenon observed in bacteria where the presence of Cas9 alone is sufficient to block transcription.[113,114] The use of dCas9 eliminates CRISPR's ability to cut DNA while retaining its capability to target specific sequences. When dCas9 is guided to the target gene by the sgRNA, it can effectively block gene transcription or interfere with RNA processing, leading to a decrease in the expression level of the target gene. This approach is known as CRISPRi or gene knockdown. CRISPRi operates similarly to RNA interference (RNAi) by targeting specific sites without cutting them, resulting in reversible gene silencing.[115] Further, various regulatory factors have been added to dCas9s by different research groups, enabling them to effectively control gene expression by turning genes on or off or adjusting their activity levels.[11,12,116,117] By fusing a repressor domain to dCas9, it becomes possible to enhance transcriptional repression by inducing heterochromatinization. One notable example is the fusion of the Krüppel-associated box (KRAB) domain with dCas9, which can effectively repress the transcription of the target gene by up to 99% in human cells.[114] On the other hand, CRISPRa enhances gene transcription. In this approach, dCas9 is fused with transcriptional activators or activation domains. When guided to the target gene's promoter by the sgRNA, dCas9 recruits transcriptional machinery to promote gene expression and enhance transcriptional activity.[116] CRISPRa enables researchers to study the effects of gene overexpression and gain-of-function phenotypes. Furthermore, CRISPR/Cas technology can be utilized for gene visualization or labeling within living cells. By fusing fluorescent proteins or other visual markers to dCas9, researchers can target specific genomic loci and visualize the dynamic behavior and spatial organization of genes in real time. This technique, known as CRISPR imaging or CRISPR Live-Cell Imaging, provides valuable insights into gene regulation, chromatin organization, and nuclear architecture.[118] Overall, these applications of CRISPR/Cas technology in gene regulations have greatly expanded our ability to investigate gene functions, regulatory networks, and cellular processes.
2. **RNA editing:** RNA editing using CRISPR/Cas technology has emerged as a powerful approach to precisely modify RNA sequences, opening up new possibilities for studying RNA functions at the RNA level without permanently changing the underlying DNA sequence. Several CRISPR/Cas systems targeting RNA, such as Cas13a, have recently been developed in plants.[12] These Cas proteins are engineered to be catalytically active and capable of performing targeted nucleotide conversions on the RNA molecule. This is achieved by incorporating an RNA-targeting domain into the Cas protein, enabling it to bind to the target RNA sequence with high specificity. Once bound, the Cas protein can introduce single-base changes, insertions, or deletions at specific positions within the RNA molecule. These systems can downregulate specific transcripts with higher specificity compared to RNAi.[119] Apart from direct RNA targeting, CRISPR/Cas systems can also modulate pre-mRNA splicing. By editing critical splicing motifs that adhere to the canonical GU-AG rule, splicing can be disrupted, resulting in changes to gene function.[89,120] Further, CRISPR/Cas systems can be utilized to modify micro-RNA and long-noncoding RNA in plants.[121] RNA editing allows for the investigation of RNA functions and regulatory mechanisms. By introducing specific modifications into RNA molecules, researchers can probe the consequences of these alterations on RNA stability, structure, splicing, translation, and protein interactions. This provides valuable insights into the roles of RNA in cellular processes and advances our understanding of RNA biology. However, it is important to note that RNA editing using CRISPR/Cas technology is still an evolving field, and there are challenges to overcome. Delivery of the editing components to specific tissues or cells remains a hurdle, as does the efficiency and specificity of RNA editing. Continued research and development in this area will further refine and expand the capabilities of RNA editing technologies.

3. **Epigenomic editing:** Epigenomic editing using CRISPR/Cas technology is a rapidly advancing field that allows precise modifications to be made to the epigenetic marks on DNA or chromatin, enabling the manipulation of gene expression and regulation. Epigenetic modifications, such as DNA methylation and histone modifications, play crucial roles in determining gene activity and cellular identity. Similar to the concept of utilizing the dCas9 fusion protein for gene knockdown/activation/visualization, the CRISPR/Cas system is adapted for epigenomic editing by fusing dCas9 with effector domains capable of modifying the epigenetic landscape (such as DNA methyltransferases, acetyltransferases, etc.).[35] By directing the fused protein to specific genomic loci using sgRNAs, targeted changes can be made to the epigenetic marks associated with those loci. For example, by fusing the catalytic domain of a DNA methyltransferase DNMT3a and a human acetyltransferase p300 with dCas9, dCas9-DNMT3a and dCas9-p300 have successfully accomplished DNA methylation and acetylation of the targeted region as specified by sgRNA, respectively.[122,123] However, challenges remain in optimizing the efficiency and specificity of epigenomic editing,[35] and the potential off-target effects and unintended alterations to the epigenome need to be carefully evaluated and minimized. To address this concern, a CRISPR/Cas-based epigenomic editing system, namely FIRE-Cas9, has been developed for rapid modification of targeted epigenomic marks, and it allows to reverse the changes made if something goes wrong.[124] Epigenomic editing using CRISPR/Cas technology provides a powerful means to investigate the functional impact of epigenetic modifications and offers potential therapeutic avenues for diseases linked to aberrant epigenetic regulation. Continued advances in this field will further refine and expand the scope of epigenomic editing, unlocking new insights into the complex interplay between the epigenome and gene expression.
4. **Multiplexed genome editing:** Multiplexed genome editing refers to the simultaneous targeting and modification of multiple genomic loci using CRISPR/Cas technology. It allows for the efficient and precise editing of multiple genes or DNA sequences within a single experiment. This approach is particularly useful for studying gene function, pathway engineering, and crop improvement. One of the main advantages of multiplexed genome editing is its efficiency and cost-effectiveness compared to traditional breeding or single-gene editing approaches, which could enable the generation of plant lines with multiple desired traits in a single generation, greatly accelerating the breeding process. Most studies of multiplexed genome editing in plants involve one kind of Cas protein and multiple sgRNAs. Various strategies have been developed to express and deliver multiple sgRNAs efficiently. These include using RNA polymerase III (Pol III)-driven systems, where multiple Pol III promoters (such as U3 and U6) are used to express multiple sgRNAs within a single construct.[125,126] Pol II-driven systems are also used, employing different strategies such as ribozyme sequences, polycistronic tRNA-sgRNA transcripts, or adding linkers to flank the sgRNAs.[127–129] These systems allow for the simultaneous expression of multiple sgRNAs, each targeting a specific genomic site. However, a single type of Cas protein cannot meet the needs of multiplexed orthogonal editing, which requires manipulation of the genome in a synthetic manner. By utilizing different Cas proteins or engineering the sgRNA sequences, each target site is modified independently without interfering with the editing activity at other sites. For example, one strategy utilizes a dCas9 combined with different single-chain RNAs (scRNAs) that carry RNA aptamers to recruit various transcription activators or repressors in mammalian cells.[130] Another strategy utilizes one sgRNA with a complete protospacer for gene knockout and another sgRNA with a partial protospacer for regulating another gene with Cas9, Cas12a-repressor, or Cas12a-activator systems.[131,132] These strategies have also been successfully implemented in plants. Furthermore, the development of a dual-function system, namely SWISS (simultaneous and wide editing induced by a single system), allows for concurrent base modifications and gene knockouts

in rice.[133] Another similar system combines a complete and a partial protospacer to modulate the activity of a modified CBE with improved specificity, enabling indels and C:G>T:A base transitions.[134] These multiplexed and/or orthogonal editing systems represent significant advances in genome manipulation, providing synthetic tools to precisely engineer the genome and explore diverse applications in plant research and biotechnology.

5. **Conditional genome editing:** Conditional CRISPR/Cas systems in plants refer to the use of regulatory elements and inducible promoters to precisely control the timing and location of gene editing events. These systems provide greater flexibility and specificity in targeting specific genes or tissues, allowing for more precise functional analysis and manipulation of plant genomes. One approach in conditional CRISPR/Cas systems is the use of tissue-specific promoters. By incorporating tissue-specific promoters into the CRISPR/Cas system, the Cas9 enzyme can only be expressed in certain types of cells or organs. This enables gene editing to be limited to particular tissues, preventing off-target effects and minimizing potential adverse effects in other parts of the plant. For example, a CRISPR/Cas system has been developed with tissue-specific promoters *CLV3* or *AP1* controlling the expression of Cas9, and inheritable editing results have been obtained as designed in *Arabidopsis thaliana* with superior editing efficiency.[135] Tissue-specific CRISPR/Cas systems have been employed to study gene function in various plant structures, such as stomata and lateral roots.[136] Inducible expression systems are another important component of conditional CRISPR/Cas systems. These systems allow researchers to control the timing and level of Cas9 expression by using exogenous inducers, such as chemical compounds or light. For example, researchers have developed CRISPR/Cas systems that are responsive to blue light repression or red light induction, as well as heat induction.[135,137] Furthermore, the combination of these elements allows for precise control over gene editing, limiting it to certain cells or organs and regulating it based on the timing of inducer application. This approach ensures that gene modifications occur only in the desired tissues and at the desired developmental stages. Overall, by utilizing tissue-specific promoters and/or inducible expression systems, conditional CRISPR/Cas systems provide researchers with the means to manipulate plant genomes with high specificity, minimal off-target effects, and temporal control. They enable a deeper understanding of gene functions, elucidation of gene regulatory networks, and potential applications in crop improvement and biotechnology.
6. **Functional genomics screening:** CRISPR screening is a powerful tool in functional genomics that allows for the systematic identification of genes involved in specific biological processes or phenotypes. It leverages the precision and efficiency of the CRISPR/Cas system to perturb the function of individual genes in a high-throughput manner, enabling the discovery of gene functions and genetic interactions on a genome-wide scale. The workflow of CRISPR screening typically involves the generation of a sgRNAs library targeting individual genes throughout the genome. These sgRNAs are then delivered to a population. Each sgRNA guides the Cas protein (e.g., Cas9) to a specific genomic locus, inducing targeted DNA cleavage or modulation of gene expression, depending on the experimental design. The pooled CRISPR library is subsequently subjected to selection or screening assays to identify individuals with specific phenotypes of interest. By analyzing the representation of each sgRNA in the selected population using high-throughput sequencing, the enrichment or depletion of specific sgRNAs provides insight into the functional relevance of the targeted genes. For example, over 14000 independent lines of rice with confirmed edits were developed using a library of 25604 pooled sgRNAs.[138] In maize, 1244 candidate loci were screened using high-throughput CRISPR/Cas editing, leading to the accurate mapping of genes related to agronomically important traits.[139] Similar approaches have also been applied to tomatoes and soybeans.[140,141] CRISPR screening enables the systematic interrogation of gene function and genetic interactions. It can identify essential

genes required for cell survival, genes involved in specific signaling pathways, or genes associated with disease phenotypes. Furthermore, CRISPR screens can uncover genetic interactions by assessing the effects of gene perturbations in combination, providing insights into complex biological networks and pathways. Despite its tremendous potential, CRISPR screening has certain challenges. Off-target effects of CRISPR/Cas systems and incomplete sgRNA representation in the library can introduce noise and false positives. Careful experimental design and rigorous bioinformatic analysis are essential to mitigate these issues and ensure reliable results.

7. **Directed evolution:** CRISPR/Cas-directed evolution is a cutting-edge technique that combines the precision of CRISPR/Cas genome editing with the power of directed evolution. Directed evolution aims to modify genes and proteins to acquire enhanced or novel properties through iterative rounds of mutagenesis and selection. In the context of CRISPR/Cas, this approach allows for the targeted and controlled evolution of specific genes or gene products. The traditional directed evolution methods, like error-prone PCR, generate random mutations throughout the entire gene or gene pool. In contrast, CRISPR/Cas-directed evolution offers a more targeted approach by utilizing the programmable nature of CRISPR/Cas systems to introduce precise mutations in specific regions of the genome.[12] The process of CRISPR/Cas-directed evolution typically involves the following steps: (1) Designing a library of sgRNAs to target the gene or genes of interest (GOIs), covering different regions or variations within the target sequences; (2) Introduction of mutations (indels, base substitutions, etc.) at the target sites within GOIs using CRISPR/Cas system guided by the sgRNA library; (3) Selection for desired traits using selection pressures (e.g., herbicides or antibiotics) or screening assays; (4) Iterative cycles of mutagenesis and selection to further enhance the desired traits. CRISPR/Cas-directed evolution has been successfully applied in various plant systems to modify genes and proteins with specific objectives. For example, it has been used to confer herbicide resistance in crops, enhance plant stress tolerance, improve enzyme efficiency, and modify metabolic pathways.[62,142,143] The ability to introduce precise mutations in a targeted manner greatly expands the possibilities of directed evolution in plants and enables the creation of customized genetic variants with desired properties. As the field progresses, ongoing research aims to develop more efficient and versatile selection methods, expand the range of target genes and traits that can be evolved, and improve the scalability and throughput of the process. CRISPR/Cas-directed evolution holds tremendous potential for applications in agriculture, biotechnology, and synthetic biology, as it allows for the rapid and controlled evolution of genes and proteins to meet specific needs and challenges.

1.4 CONCLUSION AND PROSPECTS

In this chapter, the topic of "CRISPR/Cas and plant functional genomics" was discussed by summarizing recent advancements in CRISPR/Cas technology and their applications in plant functional studies. CRISPR/Cas technology has revolutionized both basic and applied plant research by enabling precise genome manipulation. In addition to inducing indel mutations, numerous Cas variants have been developed to perform various precise modifications in the plant genome. Along with the development of Cas variants, various CRISPR/Cas-based plant biotechnologies have also been developed or improved, encompassing precise gene modulation techniques at different stages of gene expression, multiplexed and high-throughput methods for sequence modifications at multiple genomic sites, and novel delivery strategies for efficient gene editing in plants. The fast-evolving technology of CRISPR/Cas-based genome editing has enabled plant scientists, for the first time in history, to control the specific introduction of targeted sequence alterations in the plant, thus breaking new ground for studying gene functions in plants. Meanwhile, human society is facing unprecedented challenges in agriculture brought about by climate change and the booming population,

which requires constant crop improvement.[144] CRISPR/Cas-based genome editing technologies have significant potential for accelerating the breeding process and ensuring the development of sustainable agriculture.[11–13] The new Cas variants, CRISPR/Cas-based biotechnologies, and the various applications in plant functional studies introduced in this chapter could also be used for crop improvement. For example, the strategy and workflow of CRISPR/Cas-directed evolution could be directly utilized in crop improvement if the aimed phenotype of directed evolution is set to be important agronomic traits such as biotic/abiotic stress tolerance. In fact, many CRISPR/Cas-based techniques developed for plant functional studies have been extensively utilized in the development of novel crops and plant breeding technologies. For instance, BEs and PEs have demonstrated the ability to introduce a substantial proportion of causative mutations (35% and 85%, respectively) in 225 important agronomic trait genes in rice.[71] Further, by combining CRISPR/Cas technology and conventional breeding methods, several innovative breeding methods have recently been developed. These novel breeding methods have utilized CRISPR/Cas to specifically target reproduction-related genes and successfully achieved valuable goals like inducing haploid lines, generating male sterile lines, fixing hybrid vigor, and manipulating self-incompatibility.[12]

Despite these exciting advancements, there are still unmet needs in CRISPR/Cas-based plant genome manipulation. Some agricultural traits are controlled by multiple genes, necessitating the development of efficient CRISPR/Cas-based technologies to stack desired alleles. To minimize fitness penalties caused by gene disruptions, further progress is required to improve the specificity of precise editing. Further, understanding the underlying mechanisms that influence the genome editing results will greatly enhance the precise editing of target genes in plants. Lastly, improvements in fundamental genetic research are also needed for discovering genes associated with agronomically important traits. This knowledge will aid in developing tailored genome editing strategies for targeted improvements in crops. Nevertheless, with ongoing efforts, CRISPR/Cas-based technologies are expected to become commonplace and adaptable tools for precise gene editing in fundamental plant research and crop enhancement in the coming years.

REFERENCES

1. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of Bacteriology* 1987;169(12):5429–5433. DOI: 10.1128/jb.169.12.5429-5433
2. van Soolingen D, de Haas PE, Hermans PW, Groenen PM, van Embden JD. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* 1993;31(8):1987–1995. DOI: 10.1128/jcm.31.8.1987-1995
3. Groenen PM, Bunschoten AE, Soolingen Dv, Errtbden JD. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. *Molecular Microbiology* 1993;10(5):1057–1065. DOI: 10.1111/j.1365-2958.1993.tb00976.x
4. Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology* 2002;43(6):1565–1575. DOI: 10.1046/j.1365-2958.2002.02839.x
5. Barrangou R, Fremaux C, Deveau H, et al. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 2007;315(5819):1709–1712. DOI: 10.1126/science.1138140
6. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012;337(6096):816–821. DOI: 10.1126/science.1225829
7. Kumar K, Gambhir G, Dass A, et al. Genetically modified crops: Current status and future prospects. *Planta* 2020;251(4):91. DOI: 10.1007/s00425-020-03372-8
8. Shan Q, Wang Y, Li J, et al. Targeted genome modification of crop plants using a CRISPR/Cas system. *Nature Biotechnology* 2013;31(8):686–688. DOI: 10.1038/nbt.2650
9. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nature Biotechnology* 2013;31(8):691–693. DOI: 10.1038/nbt.2655

10. Li J-F, Norville JE, Aach J, et al. Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nature Biotechnology* 2013;31(8):688–691. DOI: 10.1038/nbt.2654
11. Li J, Zhang C, He Y, et al. Plant base editing and prime editing: The current status and future perspectives. *Journal of Integrative Plant Biology* 2023;65(2):444–467. DOI: 10.1111/jipb.13425
12. Zhu H, Li C, Gao C. Applications of CRISPR-Cas in agriculture and plant biotechnology. *Nature Reviews Molecular Cell Biology* 2020;21(11):661–677. DOI: 10.1038/s41580-020-00288-9.
13. Chen K, Wang Y, Zhang R, Zhang H, Gao C. CRISPR/Cas genome editing and precision plant breeding in agriculture. *Annual Review of Plant Biology* 2019;70:667–697. DOI: 10.1146/annurev-arplant-050718-100049
14. Makarova KS, Wolf YI, Iranzo J, et al. Evolutionary classification of CRISPR-Cas systems: A burst of class 2 and derived variants. *Nature Reviews Microbiology* 2020;18(2):67–83. DOI: 10.1038/s41579-019-0299-x
15. Shmakov S, Smargon A, Scott D, et al. Diversity and evolution of class 2 CRISPR-Cas systems. *Nature Reviews Microbiology* 2017;15(3):169–182. DOI: 10.1038/nrmicro.2016.184
16. Makarova KS, Zhang F, Koonin EV. SnapShot: Class 2 CRISPR/Cas systems. *Cell* 2017;168(1–2):328–328.e1. (In eng). DOI: 10.1016/j.cell.2016.12.038
17. Mali P, Esvelt KM, Church GM. Cas9 as a versatile tool for engineering biology. *Nature Methods* 2013;10(10):957–963. DOI: 10.1038/nmeth.2649
18. Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nature Methods* 2013;10(11):1116–1121. DOI: 10.1038/nmeth.2681
19. Kleinstiver BP, Prew MS, Tsai SQ, et al. Engineered CRISPR/Cas9 nucleases with altered PAM specificities. *Nature* 2015;523(7561):481–485. DOI: 10.1038/nature14592
20. Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* 2014;513(7519):569–573. DOI: 10.1038/nature13579
21. Mali P, Aach J, Stranges PB, et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nature Biotechnology* 2013;31(9):833–838. DOI: 10.1038/nbt.2675
22. Pinto BS, Saxena T, Oliveira R, et al. Impeding transcription of expanded microsatellite repeats by deactivated Cas9. *Molecular Cell* 2017;68(3):479–490.e5. DOI: 10.1016/j.molcel.2017.09.033
23. Barrangou R, Horvath P. A decade of discovery: CRISPR functions and applications. *Nature Microbiology* 2017;2(7):17092. DOI: 10.1038/nmicrobiol.2017.92
24. Paul B, Montoya G. CRISPR/Cas12a: Functional overview and applications. *Biomedical Journal* 2020;43(1):8–17. DOI: 10.1016/j.bj.2019.10.005
25. Cox DBT, Gootenberg JS, Abudayyeh OO, et al. RNA editing with CRISPR/Cas13. *Science* 2017;358(6366):1019–1027. DOI: 10.1126/science.aaq0180
26. Harrington LB, Burstein D, Chen JS, et al. Programmed DNA destruction by miniature CRISPR/Cas14 enzymes. *Science* 2018;362(6416):839–842. DOI: 10.1126/science.aav4294
27. Hirano S, Nishimasu H, Ishitani R, Nureki O. Structural basis for the altered PAM specificities of engineered CRISPR/Cas9. *Molecular Cell* 2016;61(6):886–894. DOI: 10.1016/j.molcel.2016.02.018
28. Nishimasu H, Shi X, Ishiguro S, et al. Engineered CRISPR/Cas9 nuclease with expanded targeting space. *Science* 2018;361(6408):1259–1262. DOI: 10.1126/science.aas9129
29. Kim N, Kim HK, Lee S, et al. Prediction of the sequence-specific cleavage activity of Cas9 variants. *Nature Biotechnology* 2020;38(11):1328–1336. DOI: 10.1038/s41587-020-0537-9
30. Liang F, Zhang Y, Li L, et al. SpG and SpRY variants expand the CRISPR toolbox for genome editing in zebrafish. *Nature Communications* 2022;13(1):3421. DOI: 10.1038/s41467-022-31034-8
31. Xu Z, Kuang Y, Ren B, et al. SpRY greatly expands the genome editing scope in rice with highly flexible PAM recognition. *Genome Biology* 2021;22(1):6. DOI: 10.1186/s13059-020-02231-9
32. Hirano H, Gootenberg Jonathan S, Horii T, et al. Structure and engineering of *Francisella novicida* Cas9. *Cell* 2016;164(5):950–961. DOI: 10.1016/j.cell.2016.01.039
33. Ren B, Yan F, Kuang Y, et al. Improved base editor for efficiently inducing genetic variations in rice with CRISPR/Cas9-guided hyperactive hAID mutant. *Molecular Plant* 2018;11(4):623–626. DOI: 10.1016/j.molp.2018.01.005
34. Lu Y, Zhu J-K. Precise editing of a target base in the rice genome using a modified CRISPR/Cas9 system. *Molecular Plant* 2017;10(3):523–525. DOI: 10.1016/j.molp.2016.11.013.
35. O'Geen H, Ren C, Nicolet CM, et al. dCas9-based epigenome editing suggests acquisition of histone methylation is not sufficient for target gene repression. *Nucleic Acids Research* 2017;45(17):9901–9916. DOI: 10.1093/nar/gkx578

36. Karlson CKS, Mohd-Noor SN, Nolte N, Tan BC. CRISPR/dCas9-based systems: Mechanisms and applications in plant sciences. *Plants* 2021;10(10) (In eng). DOI: 10.3390/plants10102055
37. Zetsche B, Gootenberg Jonathan S, Abudayyeh Omar O, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR/Cas system. *Cell* 2015;163(3):759–771. DOI: 10.1016/j.cell.2015.09.038
38. Fonfara I, Richter H, Bratovič M, Le Rhun A, Charpentier E. The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature* 2016;532(7600):517–521. DOI: 10.1038/nature17945
39. Abudayyeh OO, Gootenberg JS, Konermann S, et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* 2016;353(6299):aaf5573. DOI: 10.1126/science.aaf5573
40. Atkins PAP, Voytas DF. Overcoming bottlenecks in plant gene editing. *Current Opinion in Plant Biology* 2020;54:79–84. DOI: 10.1016/j.pbi.2020.01.002
41. Huang T-K, Puchta H. CRISPR/Cas-mediated gene targeting in plants: Finally a turn for the better for homologous recombination. *Plant Cell Reports* 2019;38(4):443–453. DOI: 10.1007/s00299-019-02379-0
42. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 2016;533(7603):420–424. DOI: 10.1038/nature17946
43. Zong Y, Wang Y, Li C, et al. Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nature Biotechnology* 2017;35(5):438–440. DOI: 10.1038/nbt.3811
44. Qin L, Li J, Wang Q, et al. High-efficient and precise base editing of CG to T•A in the allotetraploid cotton (*Gossypium hirsutum*) genome using a modified CRISPR/Cas9 system. *Plant Biotechnology Journal* 2020;18(1):45–56. DOI: 10.1111/pbi.13168
45. Zhang R, Liu J, Chai Z, et al. Generation of herbicide tolerance traits and a new selectable marker in wheat using base editing. *Nature Plants* 2019;5(5):480–485. DOI: 10.1038/s41477-019-0405-0
46. Liu T, Zeng D, Zheng Z, et al. The ScCas9++ variant expands the CRISPR toolbox for genome editing in plants. *Journal of Integrative Plant Biology* 2021;63(9):1611–1619. DOI: 10.1111/jipb.13164
47. Zeng D, Liu T, Tan J, et al. PhieCBEs: Plant high-efficiency cytidine base editors with expanded target range. *Molecular Plant* 2020;13(12):1666–1669. DOI: 10.1016/j.molp.2020.11.001
48. Komor AC, Zhao KT, Packer MS, et al. Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T:A base editors with higher efficiency and product purity. *Science Advances* 2017;3(8):eaao4774. DOI:10.1126/sciadv.aao4774
49. Zong Y, Song Q, Li C, et al. Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. *Nature Biotechnology* 2018;36(10):950–953. DOI: 10.1038/nbt.4261
50. Li C, Zong Y, Wang Y, et al. Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. *Genome Biology* 2018;19(1):59. DOI: 10.1186/s13059-018-1443-z
51. Gaudelli NM, Komor AC, Rees HA, et al. Programmable base editing of AT to G•C in genomic DNA without DNA cleavage. *Nature* 2017;551(7681):464–471. DOI: 10.1038/nature24644
52. Koblan LW, Doman JL, Wilson C, et al. Improving cytidine and adenine base editors by expression optimization and ancestral reconstruction. *Nature Biotechnology* 2018;36(9):843–846. (In eng). DOI: 10.1038/nbt.4172
53. Yan F, Kuang Y, Ren B, et al. Highly efficient AT to G•C base editing by Cas9n-guided tRNA adenosine deaminase in rice. *Molecular Plant* 2018;11(4):631–634. DOI: 10.1016/j.molp.2018.02.008
54. Hua K, Tao X, Yuan F, Wang D, Zhu J-K. Precise AT to G•C base editing in the rice genome. *Molecular Plant* 2018;11(4):627–630. DOI: 10.1016/j.molp.2018.02.007
55. Hua K, Tao X, Liang W, Zhang Z, Gou R, Zhu J-K. Simplified adenine base editors improve adenine base editing efficiency in rice. *Plant Biotechnology Journal* 2020;18(3):770–778. DOI: 10.1111/pbi.13244
56. Gaudelli NM, Lam DK, Rees HA, et al. Directed evolution of adenine base editors with increased activity and therapeutic application. *Nature Biotechnology* 2020;38(7):892–900. DOI: 10.1038/s41587-020-0491-6
57. Richter MF, Zhao KT, Eton E, et al. Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. *Nature Biotechnology* 2020;38(7):883–891. DOI: 10.1038/s41587-020-0453-z
58. Yan D, Ren B, Liu L, et al. High-efficiency and multiplex adenine base editing in plants using new TadA variants. *Molecular Plant* 2021;14(5):722–731. DOI: 10.1016/j.molp.2021.02.007
59. Zhao D, Li J, Li S, et al. Glycosylase base editors enable C-to-A and C-to-G base changes. *Nature Biotechnology* 2021;39(1):35–40. DOI: 10.1038/s41587-020-0592-2
60. Kurt IC, Zhou R, Iyer S, et al. CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. *Nature Biotechnology* 2021;39(1):41–46. DOI: 10.1038/s41587-020-0609-x

61. Tian Y, Shen R, Li Z, et al. Efficient C-to-G editing in rice using an optimized base editor. *Plant Biotechnology Journal* 2022;20(7):1238–1240. DOI: 10.1111/pbi.13841
62. Li C, Zhang R, Meng X, et al. Targeted, random mutagenesis of plant genes with dual cytosine and adenine base editors. *Nature Biotechnology* 2020;38(7):875–882. (In eng). DOI: 10.1038/s41587-019-0393-7
63. Wang S, Zong Y, Lin Q, et al. Precise, predictable multi-nucleotide deletions in rice and wheat using APOBEC-Cas9. *Nature Biotechnology* 2020;38(12):1460–1465. DOI: 10.1038/s41587-020-0566-4
64. Anzalone AV, Randolph PB, Davis JR, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 2019;576(7785):149–157. DOI: 10.1038/s41586-019-1711-4
65. Chen PJ, Hussmann JA, Yan J, et al. Enhanced prime editing systems by manipulating cellular determinants of editing outcomes. *Cell* 2021;184(22):5635–5652.e29. DOI: 10.1016/j.cell.2021.09.018
66. Jiang Y, Chai Y, Qiao D, et al. Optimized prime editing efficiently generates glyphosate-resistant rice plants carrying homozygous TAP-IVS mutation in EPSPS. *Molecular Plant* 2022;15(11):1646–1649. DOI: 10.1016/j.molp.2022.09.006
67. Li J, Chen L, Liang J, et al. Development of a highly efficient prime editor 2 system in plants. *Genome Biology* 2022;23(1):161. DOI: 10.1186/s13059-022-02730-x
68. Anzalone AV, Gao XD, Podracky CJ, et al. Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing. *Nature Biotechnology* 2022;40(5):731–740. DOI: 10.1038/s41587-021-01133-w
69. Jiang Y-Y, Chai Y-P, Lu M-H, et al. Prime editing efficiently generates W542L and S621I double mutations in two ALS genes in maize. *Genome Biology* 2020;21(1):257. DOI: 10.1186/s13059-020-02170-5
70. Li H, Li J, Chen J, Yan L, Xia L. Precise modifications of both exogenous and endogenous genes in rice by prime editing. *Molecular Plant* 2020;13(5):671–674. DOI: 10.1016/j.molp.2020.03.011
71. Hua K, Han P, Zhu J-K. Improvement of base editors and prime editors advances precision genome engineering in plants. *Plant Physiology* 2021;188(4):1795–1810. DOI: 10.1093/plphys/kiab591
72. Wang J, He Z, Wang G, et al. Efficient targeted insertion of large DNA fragments without DNA donors. *Nature Methods* 2022;19(3):331–340. DOI: 10.1038/s41592-022-01399-1
73. Lin Q, Jin S, Zong Y, et al. High-efficiency prime editing with optimized, paired pegRNAs in plants. *Nature Biotechnology* 2021;39(8):923–927. DOI: 10.1038/s41587-021-00868-w
74. Li X, Zhou L, Gao B-Q, et al. Highly efficient prime editing by introducing same-sense mutations in pegRNA or stabilizing its structure. *Nature Communications* 2022;13(1):1669. DOI: 10.1038/s41467-022-29339-9
75. Xu W, Yang Y, Yang B, et al. A design optimized prime editor with expanded scope and capability in plants. *Nature Plants* 2022;8(1):45–52. DOI: 10.1038/s41477-021-01043-4
76. Kim HK, Yu G, Park J, et al. Predicting the efficiency of prime editing guide RNAs in human cells. *Nature Biotechnology* 2021;39(2):198–206. DOI: 10.1038/s41587-020-0677-y
77. Liu Y, Yang G, Huang S, et al. Enhancing prime editing by Csy4-mediated processing of pegRNA. *Cell Research* 2021;31(10):1134–1136. DOI: 10.1038/s41422-021-00520-x
78. Nelson JW, Randolph PB, Shen SP, et al. Engineered pegRNAs improve prime editing efficiency. *Nature Biotechnology* 2022;40(3):402–410. DOI: 10.1038/s41587-021-01039-7
79. Zou J, Meng X, Liu Q, et al. Improving the efficiency of prime editing with epegRNAs and high-temperature treatment in rice. *Science China Life Sciences* 2022;65(11):2328–2331. DOI: 10.1007/s11427-022-2147-2
80. Jiang T, Zhang X-O, Weng Z, Xue W. Deletion and replacement of long genomic sequences using prime editing. *Nature Biotechnology* 2022;40(2):227–234. DOI: 10.1038/s41587-021-01026-y
81. Choi J, Chen W, Suiter CC, et al. Precise genomic deletions using paired prime editing. *Nature Biotechnology* 2022;40(2):218–226. DOI: 10.1038/s41587-021-01025-z
82. Li J, Sun Y, Du J, Zhao Y, Xia L. Generation of targeted point mutations in rice by a modified CRISPR/Cas9 system. *Molecular Plant* 2017;10(3):526–529. DOI: 10.1016/j.molp.2016.12.001
83. Ren B, Yan F, Kuang Y, et al. A CRISPR/Cas9 toolkit for efficient targeted base editing to induce genetic variations in rice. *Science China Life Science*s 2017;60(5):516–519. DOI: 10.1007/s11427-016-0406-x
84. Ren B, Liu L, Li S, et al. Cas9-NG greatly expands the targeting scope of the genome-editing toolkit by recognizing NG and other atypical PAMs in rice. *Molecular Plant* 2019;12(7):1015–1026. DOI: 10.1016/j.molp.2019.03.010
85. Wang M, Xu Z, Gosavi G, et al. Targeted base editing in rice with CRISPR/ScCas9 system. *Plant Biotechnology Journal* 2020;18(8):1645–1647. DOI: 10.1111/pbi.13330
86. Shimatani Z, Kashojiya S, Takayama M, et al. Targeted base editing in rice and tomato using a CRISPR/Cas9 cytidine deaminase fusion. *Nature Biotechnology* 2017;35(5):441–443. DOI: 10.1038/nbt.3833

87. Zhong Z, Sretenovic S, Ren Q, et al. Improving plant genome editing with high-fidelity xCas9 and non-canonical PAM-targeting Cas9-NG. *Molecular Plant* 2019;12(7):1027–1036. DOI: 10.1016/j.molp.2019.03.011
88. Veillet F, Perrot L, Guyon-Debast A, et al. Expanding the CRISPR toolbox in *P. patens* using SpCas9-NG variant and application for gene and base editing in solanaceae crops. *International Journal of Molecular Sciences* 2020;21(3):1024. https://www.mdpi.com/1422-0067/21/3/1024
89. Kang B-C, Yun J-Y, Kim S-T, et al. Precision genome engineering through adenine base editing in plants. *Nature Plants* 2018;4(7):427–431. DOI: 10.1038/s41477-018-0178-x
90. Wei C, Wang C, Jia M, et al. Efficient generation of homozygous substitutions in rice in one generation utilizing an rABE8e base editor. *Journal of Integrative Plant Biology* 2021;63(9):1595–1599. DOI: 10.1111/jipb.13089
91. Tan J, Zeng D, Zhao Y, et al. PhieABEs: A PAM-less/free high-efficiency adenine base editor toolbox with wide target scope in plants. *Plant Biotechnology Journal* 2022;20(5):934–943. DOI: 10.1111/pbi.13774.
92. Xu R, Kong F, Qin R, Li J, Liu X, Wei P. Development of an efficient plant dual cytosine and adenine editor. *Journal of Integrative Plant Biology* 2021;63(9):1600–1605. DOI: 10.1111/jipb.13146.
93. Butt H, Rao GS, Sedeek K, Aman R, Kamel R, Mahfouz M. Engineering herbicide resistance via prime editing in rice. *Plant Biotechnology Journal* 2020;18(12):2370–2372. DOI: 10.1111/pbi.13399
94. Hua K, Jiang Y, Tao X, Zhu J-K. Precision genome engineering in rice using prime editing system. *Plant Biotechnology Journal* 2020;18(11):2167–2169. DOI: 10.1111/pbi.13395
95. Xu R, Li J, Liu X, Shan T, Qin R, Wei P. Development of plant prime-editing systems for precise genome editing. *Plant Communications* 2020;1(3):100043. DOI: 10.1016/j.xplc.2020.100043
96. Zong Y, Liu Y, Xue C, et al. An engineered prime editor with enhanced editing efficiency in plants. *Nature Biotechnology* 2022;40(9):1394–1402. DOI: 10.1038/s41587-022-01254-w
97. Lin Q, Zong Y, Xue C, et al. Prime genome editing in rice and wheat. *Nature Biotechnology* 2020;38(5):582–585. DOI: 10.1038/s41587-020-0455-x
98. Xu W, Zhang C, Yang Y, et al. Versatile nucleotides substitution in plant using an improved prime editing system. *Molecular Plant* 2020;13(5):675–678. DOI: 10.1016/j.molp.2020.03.012
99. Li H, Zhu Z, Li S, et al. Multiplex precision gene editing by a surrogate prime editor in rice. *Molecular Plant* 2022;15(7):1077–1080. DOI: 10.1016/j.molp.2022.05.009
100. Lu Y, Tian Y, Shen R, et al. Precise genome modification in tomato using an improved prime editing system. *Plant Biotechnology Journal* 2021;19(3):415–417. DOI: 10.1111/pbi.13497
101. Ran Y, Liang Z, Gao C. Current and future editing reagent delivery systems for plant genome editing. *Science China Life Sciences* 2017;60(5):490–505. DOI: 10.1007/s11427-017-9022-1
102. Lowe K, Wu E, Wang N, et al. Morphogenic regulators baby boom and wuschel improve monocot transformation. *The Plant Cell* 2016;28(9):1998–2015. DOI: 10.1105/tpc.16.00124
103. Maher MF, Nasti RA, Vollbrecht M, Starker CG, Clark MD, Voytas DF. Plant gene editing through de novo induction of meristems. *Nature Biotechnology* 2020;38(1):84–89. DOI: 10.1038/s41587-019-0337-2
104. Ali Z, Abul-Faraj A, Li L, et al. Efficient virus-mediated genome editing in plants using the CRISPR/Cas9 system. *Molecular Plant* 2015;8(8):1288–1291. DOI: 10.1016/j.molp.2015.02.011
105. Ali Z, Eid A, Ali S, Mahfouz MM. *Pea early-browning virus*-mediated genome editing via the CRISPR/Cas9 system in *Nicotiana benthamiana* and Arabidopsis. *Virus Research* 2018;244:333–337. DOI: 10.1016/j.virusres.2017.10.009
106. Cody WB, Scholthof HB, Mirkov TE. Multiplexed gene editing and protein overexpression using a *Tobacco mosaic virus* viral vector. *Plant Physiology* 2017;175(1):23–35. DOI: 10.1104/pp.17.00411
107. Hu J, Li S, Li Z, et al. A barley stripe mosaic virus-based guide RNA delivery system for targeted mutagenesis in wheat and maize. *Molecular Plant Pathology* 2019;20(10):1463–1474. DOI: 10.1111/mpp.12849
108. Jiang N, Zhang C, Liu J-Y, et al. Development of *Beet necrotic yellow vein virus*-based vectors for multiple-gene expression and guide RNA delivery in plant genome editing. *Plant Biotechnology Journal* 2019;17(7):1302–1315. DOI: 10.1111/pbi.13055
109. Yin K, Han T, Liu G, et al. A geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. *Scientific Reports* 2015;5(1):14926. DOI: 10.1038/srep14926
110. Gao Q, Xu W-Y, Yan T, et al. Rescue of a plant cytorhabdovirus as versatile expression platforms for planthopper and cereal genomic studies. *New Phytologist* 2019;223(4):2120–2133. DOI: 10.1111/nph.15889
111. Ma X, Zhang X, Liu H, Li Z. Highly efficient DNA-free plant genome editing using virally delivered CRISPR-Cas9. *Nature Plants* 2020;6(7):773–779. DOI: 10.1038/s41477-020-0704-5

112. Ellison EE, Nagalakshmi U, Gamo ME, Huang P-J, Dinesh-Kumar S, Voytas DF. Multiplexed heritable gene editing using RNA viruses and mobile single guide RNAs. *Nature Plants* 2020;6(6):620–624. DOI: 10.1038/s41477-020-0670-y
113. Shariati SA, Dominguez A, Xie S, Wernig M, Qi LS, Skotheim JM. Reversible disruption of specific transcription factor-DNA interactions using CRISPR/Cas9. *Molecular Cell* 2019;74(3):622–633.e4. DOI: 10.1016/j.molcel.2019.04.011
114. Gilbert Luke A, Larson Matthew H, Morsut L, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013;154(2):442–451. DOI: 10.1016/j.cell.2013.06.044
115. Dhamad AE, Lessner DJ. A CRISPRi-dCas9 system for archaea and its use to examine gene function during nitrogen fixation by *Methanosarcina acetivorans*. *Applied and Environmental Microbiology* 2020;86(21):e01402–20. DOI: 10.1128/AEM.01402-20
116. Dominguez AA, Lim WA, Qi LS. Beyond editing: Repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nature Reviews Molecular Cell Biology* 2016;17(1):5–15. DOI: 10.1038/nrm.2015.2
117. Qi LS, Larson Matthew H, Gilbert Luke A, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 2013;152(5):1173–1183. DOI: 10.1016/j.cell.2013.02.022
118. Chen B, Gilbert Luke A, Cimini Beth A, et al. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 2013;155(7):1479–1491. DOI: 10.1016/j.cell.2013.12.001
119. Abudayyeh OO, Gootenberg JS, Essletzbichler P, et al. RNA targeting with CRISPR-Cas13. *Nature* 2017;550(7675):280–284. DOI: 10.1038/nature24049
120. Li Z, Xiong X, Wang F, Liang J, Li J-F. Gene disruption through base editing-induced messenger RNA missplicing in plants. *New Phytologist* 2019;222(2):1139–1148. DOI: 10.1111/nph.15647
121. Basak J, Nithin C. Targeting non-coding RNAs in plants with the CRISPR/Cas technology is a challenge yet worth accepting. *Frontiers in Plant Science* 2015;6 (Mini Review) (In eng). DOI: 10.3389/fpls.2015.01001
122. McDonald JI, Celik H, Rois LE, et al. Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation. *Biology Open* 2016;5(6):866–874. DOI: 10.1242/bio.019067
123. Hilton IB, D'Ippolito AM, Vockley CM, et al. Epigenome editing by a CRISPR/Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nature Biotechnology* 2015;33(5):510–517. DOI: 10.1038/nbt.3199
124. Braun SMG, Kirkland JG, Chory EJ, Husmann D, Calarco JP, Crabtree GR. Rapid and reversible epigenome editing by endogenous chromatin regulators. *Nature Communications* 2017;8(1):560. DOI: 10.1038/s41467-017-00644-y
125. Xing H-L, Dong L, Wang Z-P, et al. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology* 2014;14(1):327. DOI: 10.1186/s12870-014-0327-y
126. Ma X, Liu Y-G. CRISPR/Cas9-based multiplex genome editing in monocot and dicot plants. *Current Protocols in Molecular Biology* 2016;115(1):31.6.1–31.6.21. DOI: 10.1002/cpmb.10
127. Gao Y, Zhao Y. Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing. *Journal of Integrative Plant Biology* 2014;56(4):343–349. DOI: 10.1111/jipb.12152
128. Ding D, Chen K, Chen Y, Li H, Xie K. Engineering introns to express RNA guides for Cas9- and Cpf1-mediated multiplex genome editing. *Molecular Plant* 2018;11(4):542–552. DOI: 10.1016/j.molp.2018.02.005
129. Mikami M, Toki S, Endo M. In planta processing of the SpCas9-gRNA complex. *Plant and Cell Physiology* 2017;58(11):1857–1867. DOI: 10.1093/pcp/pcx154
130. Zalatan JG, Lee ME, Almeida R, et al. engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell* 2015;160(1):339–350. DOI: 10.1016/j.cell.2014.11.052
131. Dahlman JE, Abudayyeh OO, Joung J, Gootenberg JS, Zhang F, Konermann S. Orthogonal gene knockout and activation with a catalytically active Cas9 nuclease. *Nature Biotechnology* 2015;33(11):1159–1161. DOI: 10.1038/nbt.3390
132. Campa CC, Weisbach NR, Santinha AJ, Incarnato D, Platt RJ. Multiplexed genome engineering by Cas12a and CRISPR arrays encoded on single transcripts. *Nature Methods* 2019;16(9):887–893. DOI: 10.1038/s41592-019-0508-6
133. Li C, Zong Y, Jin S, et al. SWISS: Multiplexed orthogonal genome editing in plants with a Cas9 nickase and engineered CRISPR RNA scaffolds. *Genome Biology* 2020;21(1):141. DOI: 10.1186/s13059-020-02051-x