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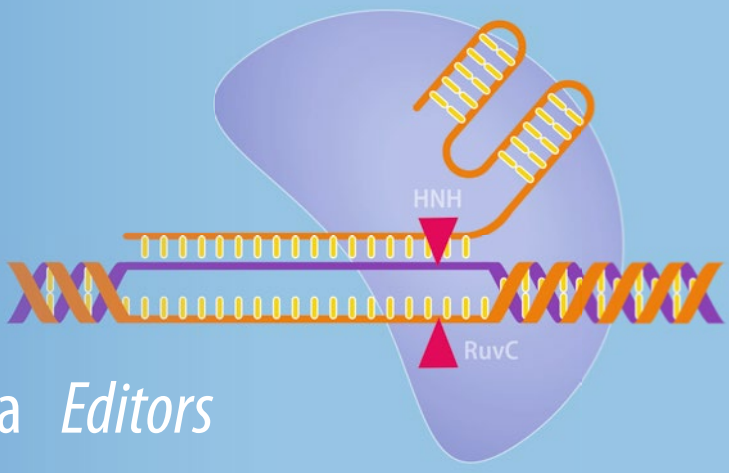

M. Tofazzal Islam
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CRISPR-Cas Methods

Humana Press

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CRISPR-Cas Methods

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Foreword

Sometimes in February 2016, I got in touch with Prof. Tofazzal Islam—an editor of this book—about applying pathogenomics to a disease epidemic that just broke out in his native country of Bangladesh. This story has been told over and over, and our perspective is documented in an article we wrote a few months ago after an emotional visit to affected farms in Bangladesh (Kamoun, S., Talbot, N.J., and Islam, M.T. 2019. PLOS Biology, 17: e3000302). However, what I doubt is that either one of us expected at the time the chain of events that have transformed our career paths and ultimately led to this book. What happened is that the blast fungus—the culprit of that dreadful outbreak—not only infected the wheat crop but also ended up redirecting our research activities towards investigating this formidable foe. That journey took us to view CRISPR-Cas9 gene editing of wheat for blast disease resistance as a particularly promising approach to deliver solutions to this problem.

How to manage destructive plant pathogens such as the wheat blast fungus? Genetics has to be part of the answer. Crop germplasm screens and breeding towards disease resistance is valuable but can be slow with limited potential. Biotechnology is a promising alternative that has yet to deliver its full potential to plant health problems like wheat blast. One such technology is gene editing or bioediting. Geneticists have long dreamed about editing their organisms' genomes as one would edit text on a computer with a word processor. The dream has started to become a reality when CRISPR-Cas9 took the biological world by storm just a few years ago in 2013. Other gene editing methods, notably zinc-finger nucleases and TALENs, were available at the time but CRISPR-Cas9 democratized gene editing in the biological sciences. It has made gene editing relatively easy and accessible to pretty much any laboratory with basic molecular biology skills. The breadth of topics and authors that Prof. Islam and his co-editors Dr. Pankaj K. Bhowmik and Dr. Kutubuddin A. Molla have lined up in this book is a great example of the range of developments and applications of this technology and its potential to impact plant health and many other areas of biology.

Why another book on CRISPR? The series of chapters reflect the unique perspective of the authors and their aspiration to see CRISPR-Cas9 bioediting applied to the improvement of crops and the human condition in general. My hope is that the book would guide a new generation of plant gene editors from all over the world. I hope it would inspire early career scientists, particularly from developing countries, to embrace gene editing technology and forge their own path in this rapidly expanding area of genetics.

The Sainsbury Laboratory, Norwich, UK ***Sophien Kamoun***

Preface

Precisely altering genetic sequences—in almost any kind of living cells including those of humans, at much higher accuracy and efficiency than ever before became possible only after the recent discovery of genome editing technologies like **z**inc-**f**inger **n**ucleases (ZFNs), **t**ranscriptional **a**ctivator-**l**ike **e**ffector **n**ucleases (TALENs), and **c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats (CRISPR)-associated nucleases (Cas). Those technologies allow us to make small changes to a DNA sequences at a known location. Compared to ZFN and TALEN, the CRISPR-Cas technology is faster, cheaper, and more user-friendly. Although CRISPR-Cas system was originally identified as an adaptive immune system from bacteria, its repurposing into versatile RNA-guided, DNA-targeting platforms revolutionized basic biology, agriculture, and medicine.

Since discovery, the CRISPR-Cas9 technology has attracted widespread attention of biologists, and its application has expanded dramatically in many areas including plant, microbial, and animal sciences. Over the last 6 years, the CRISPR-Cas systems have become a powerful toolbox for genetic manipulation on the basis of simpler RNA-guided DNA recognition. A small guide RNA (gRNA) directs the protein, Cas, to a targeted locus to bind with and make a double-strand break. Due to tremendous improvement in the methodologies, the CRISPR-Cas toolbox is now a useful technology for crop improvement, curing genetic diseases, and engineering desirable genetic traits, as well as new applications like live-cell imaging, high-throughput functional genomic screens, transcriptional regulation, epigenome modification, gene-drive, and point-of-care diagnostic. The diversity, modularity, and efficacy of CRISPR-Cas systems are driving a biotechnological revolution.

One of the unique features of the CRISPR-Cas system is the creation of DNA double-strand breaks (DSBs) at target loci. This feature can be used to introduce a variety of modifications in the genome of any target organism, including crop plants. Immediately after the DSBs, two main DNA repair pathways naturally become functional—(1) non-homologous end joining (NHEJ) and (2) homology-directed repair (HDR). In higher eukaryotes, NHEJ is predominant and error-prone. During NHEJ-mediated DSB repair, the cellular machinery erroneously incorporates small **in**sertion or **del**etions (indel) at the cut point. As a result, the open reading frame is disrupted, leading to the loss of function of the gene. So, knocking out of a gene is easily achievable using conventional CRISPR-Cas technique. Furthermore, with the help of an array of gRNA, the system can be conveniently used to target multiple genes at a time, a system is known as multiplex genome editing. HDR method is exploited for precise editing with the delivery of a donor template containing the desired changes, but it is inherently low in efficiency in higher eukaryotes.

An efficient and convenient genetic transformation protocol is a prerequisite for the success in genome editing by the CRISPR technology. There is no doubt that the rapid development of the CRISPR-Cas tools has tremendously assisted biologists to adopt the system for their specific purpose. However, the availability of a huge number of tools may sometimes be confusing for a new user to choose the most suitable for his/her purpose. We hope that the 'CRISPR-Cas Methods' book would assist anybody who is new in this field and need a direction to adopt this technology for a particular organism.

This book details various CRISPR-Cas technical protocols for 11 different organisms starting from fungi, algae, plant to human cell lines. As already mentioned, HDR-mediated gene targeting in plant system is extremely difficult. In the very **first chapter**, to give an overview to the readers, Molla and co-workers succinctly described the principles of different CRISPR-Cas-derived technologies for basic biology, agriculture, and medicine. In the Chapter 2, Holger Puchta and colleagues describe an efficient protocol for HDR-mediated gene targeting in *Arabidopsis* using *in planta* method of transformation. This *Staphylococcus aureus* Cas9 (SaCas9)-mediated "in planta gene targeting" (ipGT) approach in *A. thaliana* should enable stable heritable genome modifications, including integration of a tag or *cis*-element to a desired locus, or precise sequence modifications such as amino acid substitutions.

Rice is core to the nutrition and livelihood of more than half of the world population and plays a significant role in world food security. In Chapter 3, Ludwig and Slamet-Loedin from International Rice Research Institute (IRRI) describe a detailed stepwise protocol for sgRNA design, cloning and assembly of CRISPR constructs, and analysis of mutants for successful gene knockout. Base editing is a recently developed CRISPR-Cas-mediated genome editing technique, which enables precise point mutation in the genome without the need for a donor template. It enhances the efficiency of precise editing than that of HDR. In Chapter 4, Molla and Yang described the step-by-step procedure of multiplex adenine base editing experiments in rice. This protocol chapter would be useful for simultaneous CRISPR-Cas-mediated single base editing in rice and other crop plants at more than one locus.

Deborah Petrick elaborately described a detailed protocol of how to use CRISPR-Cas9 editing tools to generate knockout mutant of the model grass species *Brachypodium distachyon* in Chapter 5. Availability of whole genome sequence, brief life cycle, simple growth requirement, and its close relationship with most of the cereal crops and biofuel crops switchgrass, make *Brachypodium* as a valuable model organism. Deb's protocol would significantly expedite basic research, especially for functional genomics.

Oomycete phytopathogens under the genus *Phytophthora* are destructive to many economically important plant species. Molecular mechanisms underlying the pathogenicity and broad host range of these fungi are poorly understood. CRISPR-mediated genome editing has been found as an effective tool for oomycete functional genomics for accelerated dissection of gene functions. In Chapter 6, Tian and co-workers described a novel protocol for generating *P. palmivora* mutants via *Agrobacterium*-mediated transformation.

Chapter 7 by Cheng Dai and colleagues describes an efficient method for visual screening of mutants. This fluorescence screening method is validated in several dicot species like *Arabidopsis*, *Brassica napus*, strawberry, and soybean. The vector developed in their study could be applied in many other dicot plant species.

Similarly, Gasparis and Przyborowski in Chapter 8 and Adhikary and colleagues in Chapter 8 outlined protocols for applying the techniques in Barley and Flax, respectively.

Two detailed protocols for carrying out gene knockout experiments in human cell lines are described. In Chapter 10, Uddin and co-workers described a comprehensive protocol for gene deletion (knockout) and tagging (knockin) in mammalian cells, while in Chapter 11, Semiech et al. described a protocol of cloning-free (DNA-free) CRISPR-Cas9-mediated gene knockout in human liver cell line.

Recently, DNA-free genome editing with direct delivery of pre-assembled CRISPR-Cas9 ribonucleoprotein complex was successfully used to achieve targeted mutagenesis. Genome-editing using DNA-free systems could circumvent restrictive GMO regulations, thus paving the way for widespread use of this innovative technology in trait discovery and

crop improvement. Wheat (*Triticum aestivum* L.) is one of the most important cereal food crops in the world. This allohexaploid (AABBDD, $2n = 6x = 42$) is recalcitrant to genetic modification (GM). Chapter 12 by Bilchak and colleagues described the use of cell penetrating peptides to deliver Cas9 ribonuclear protein complex to wheat microspore. The chapter also detailed on transcriptional gene regulation by using dCas9-VP64. On the other hand, Bhowmik and Islam described a novel method for CRISPR-mediated wheat gene editing and trait improvement in Chapter 13. They provided a step-by-step procedure that begin with gRNA designing followed by assembling CRISPR constructs or ribonucleoprotein complex and in vitro and in vivo gRNA validation using wheat mesophyll protoplasts, transformation and regeneration, mutation detection, and phenotyping for the desired traits.

In Chapter 14, Tripathi et al. described the procedure for generating genome-edited banana by *Agrobacterium*-mediated delivery of the reagents to embryogenic cell suspension. The *Chlamydomonas reinhardtii* is a microalgal model organism, for which a suite of molecular and genetic techniques are available. In Chapter 15, Ferenczi and Molnar demonstrated the use of CRISPR-Cpf1 in conjunction with single-stranded DNA (ssODN) repair templates to achieve nuclear gene editing efficiencies as high as 30%. It produced edits with predictable outcomes in a transgene- and selection marker-free manner.

Although there are many recent reports of successful CRISPR-Cas9-mediated gene editing, the experimental tools required to implement this powerful technology is yet to be embraced by most of the biological science laboratories as routine protocols. There are several factors which affect the success and efficiency of CRISPR-mediated gene editing; efficient gRNA designing and assembling multiple gRNA cassettes, selection of suitable nuclease and base editors, efficient delivery of Cas9 and gRNA vectors, selection and regeneration of edited organisms, efficient detection of the gene editing event, and so on. The frequency of gene editing will depend on whether or not these conditions are optimal. This volume of CRISPR methods and protocols is aimed to provide the fundamentals of CRISPR-Cas system and the advances in the protocols of CRISPR-Cas genome editing for efficient genome editing. Contributors to this unique volume described their reproducible experimental methods and protocols in 15 chapters and suggested many troubleshooting ideas to avoid common CRISPR mistakes. Thus, this book will serve as a laboratory manual providing readers a holistic view of CRISPR methodologies and their practical application for modifying crop plants, microorganisms, and animals.

This book is the outcome of a cooperative effort from all the editors and contributors representing many different countries. The editors gratefully acknowledge the authors who contributed to this book of CRISPR-Cas methods. Prof. Sophien Kamoun, FRS, The Sainsbury Laboratory, Norwich, UK, deserves our sincere thanks for kindly writing the Foreword of the book. Two coordinators from the Springer Jayashree Dhakshnamoorthy and Sanjana Sundaram also deserve credits for their excellent coordination with authors and editors. Our thanks are also due to other editorial staff for their precious help in formatting and incorporating editorial changes in the manuscripts. We believe researchers who work or would work on gene editing through CRISPR technology will find this volume as an essential guidebook for implementing their research projects.

Gazipur, Bangladesh — ***M. Tofazzal Islam***
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Chapter 1

Wide Horizons of CRISPR-Cas-Derived Technologies for Basic Biology, Agriculture, and Medicine

Kutubuddin A. Molla, Subhasis Karmakar, and M. Tofazzal Islam

Abstract

The discovery and subsequent repurposing of the CRISPR (clustered regularly interspaced short palindromic repeats) and Cas proteins (CRISPR-associated proteins) has revolutionized our ability to manipulate DNA and RNA sequences in vitro, ex vivo, and in vivo. In this introductory chapter, we present a brief overview of basics of CRISPR-Cas-mediated genome editing and different orthologues and engineered versions of Cas protein with altered specificity and expanded targetability. More importantly, we comprehensively portray the advances made by developing diverse CRISPR-Cas-based genome modification tools and their application in basic biology, agriculture, and medicine.

Key words Orthologous and engineered Cas proteins, CRISPR activation, CRISPR interference, CRISPR screening, Epigenome editing, Live cell imaging, Base editing, RNA editing, Prime editing, Molecular diagnostic

1 Basics of CRISPR-Cas Immune System

The **c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats (CRISPR)-**C**RISPR-**a**ssociated **p**rotein (Cas), abbreviated as CRISPR-Cas, is a prokaryotic adaptive immune system. The organism having this immune system acquires a snippet of invading bacteriophage/plasmid DNA to store in their CRISPR locus as a memory. On successful transcription, the CRISPR locus produces precursor CRISPR RNA (pre-crRNA). To simplify, this system has three components, such as (i) a Cas9 nuclease protein, (ii) a precursor CRISPR RNA (pre-crRNA), and (iii) a partially complementary *trans*-activating crRNA (tracrRNA) [1]. The independently transcribed tracrRNA binds to pre-crRNA to form a hybrid RNA. Further cleavage and processing of pre-crRNA produce the mature CRISPR RNA (crRNA). Then the Cas9 nuclease protein binds to the assembled structure of crRNA-tracrRNA. This RNA structure guides Cas9 to specifically bind and cut a complementary DNA

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sequence that precedes the PAM sequence [1]. Protospacer adjacent motif (PAM) is "a short sequence (2–6 bp) of nucleotides situated immediately adjacent to the target DNA sequence and which is essential for target recognition by Cas nucleases" [2]. For *Streptococcus pyogenes* Cas9 (SpCas9), the PAM is a three-nucleotide sequence, 5′-NGG-3′. In the bacterial immune system, the active Cas9-crRNA-tracrRNA complex can cut the invading viral DNA if it has complementarity and contains the PAM sequence. Since the crRNA contains small portion of transcribed viral DNA, it finds complementarity with the invading DNA sequence if the same virus infects in the future. Using this system bacteria inhibit viral infection. The naturally derived CRISPR-Cas immune system in prokaryotes is recently reviewed [3].

According to current classification, the CRISPR-Cas systems are categorized into two distinct classes [4]. Class 1 comprises type I and type III that are abundant in archaea but are rare in bacteria. Type I and type III have complex organization and consist of multiple Cas protein subunits. Class 2 includes the most popular type II that is abundantly found in bacteria. Type II has simpler organization and consists of a single Cas protein. *Streptococcus pyogenes* Cas9 (SpCas9), the principal tool for genome engineering, is an example of type II effector nuclease [5]. A group of scientists in 2012 independently reported the applicability of CRISPR-Cas system as a precise tool for editing the genome of living organisms [5–8]. This chapter concisely presents the fundamentals of CRISPR-Cas-derived technologies for studies on basic biology and applications in agriculture and medicine.

2 Development of Genome Editing Tools from the Prokaryotic Immune System

Experimental results evidenced that a chimeric single-guide RNA (sgRNA) (containing the features of crRNA and tracrRNA) can program the Cas9 to a specific locus [5]. A small (~20 nucleotide) portion of the sgRNA directs or guides the binding of sgRNA-Cas9 complex to a complementary DNA sequence and subsequent DNA break by Cas9 [5]. So, customizing the 20 nucleotides from the 5′ end of the sgRNA would enable the sgRNA to bind with the sequence of interest. It allows researchers to retarget Cas9 to virtually any genomic sequence that precedes a PAM sequence (5′-NGG-3′). Cas9 makes a double-strand break (DSB) in the target DNA mostly 3 bp upstream of the PAM sequence [1].

It is clear that we can make a targeted and precise DSB in the DNA using the CRISPR-Cas9 system. But, how does it help in genome editing? The answer lies in the cellular repair mechanism. Since DSBs are the most toxic class of DNA damage, cells immediately deploy its various DSB repair pathways. In higher eukaryotes, nonhomologous end-joining (NHEJ) is a predominant repair

pathway, which is error-prone. Due to the error during NHEJ-mediated repair, random small insertions or deletions (indels) or nucleotide substitutions are incorporated at the cut point while rejoining the two ends (Fig. 1). Those incorporated errors frequently result in the disruption of the function of the target gene. By simply designing a 20-nucleotide guide RNA, researcher can develop a mutant organism with a targeted gene knocked out with high efficiency.

3 Orthologous and Engineered Cas Proteins for Diverse Genome Editing Platform

Presence of "NGG" protospacer adjacent motif (PAM) in the target site is a prerequisite for SpCas9 binding and cleavage. Therefore, a genomic site lacking an NGG sequence cannot be targeted by SpCas9. For those sites to be targeted, orthologues of SpCas9 which has a distinct PAM requirement could be utilized. Many Cas9 orthologues have been discovered and successfully demonstrated as genome editing tools. Another advantage of using SpCas9 orthologues is to avoid the large size of the protein which is a constraint in successful cellular delivery. SpCas9 is 1368 amino acid (AA) long (4104 bp), while the orthologue from *Streptococcus aureus* (SaCas9) is only 1053 AA in length [9]. SaCas9 can efficiently cut the target sites having "NNGRRT" (R = A/G) PAM sequence [9]. Similarly, Nme2Cas9 has a compact size (1082 AA) and alternative PAM (NNNNCC) requirement [10]. Cas12a (previously termed as cpf1) recognizes a T-rich PAM, 5′-TTTV-3′ [11]. The tool is expanded further to RNA targeting by the discovery of Cas13 (previously termed as C2c2) that binds and cut RNA [12]. For a further detail on different orthologues of Cas9 proteins discovered, *see* Table 1.

SpCas9 and other Cas proteins have been engineered to alter PAM compatibility, impair the cleavage activity, and increase specificity. SpCas9 has two nuclease domains, HNH and RuvC. HNH domain cuts the complementary or target strand, while RuvC cleaves the noncomplementary or nontarget strand (Fig. 1A) (adopted from [34]). Fully active Cas9 generates double-strand break in the target DNA. However, engineered Cas9 containing a mutation in the catalytic residues of either RuvC or HNH domains generates single-strand nick [5, 35]. This type of engineered Cas9 is known as Cas9 nickase (nCas9). D10A mutation in the RuvC domain converts Cas9 into nCas9 that nicks in the target strand. Similarly, H840A mutation in the HNH domain of Cas9 generates a nCas9 that nicks in the nontarget strand. Incorporation of both the mutations turns an active Cas9 to a dead Cas9 (dCas9), which can bind to the target locus but cannot cleave.

Table 1
A summary of orthologous CRISPR-associated (Cas) nucleases discovered and their respective PAM sequences

Class	Type	Nuclease	PAM (5′-3′)	Species	Size (aa)	References
II	II	SpCas9	NGG	*Streptococcus pyogenes*	1368	Jinek et al. [5]
II	II	NmCas9	NNNNGATT	*Neisseria meningitidis*	1082	Hou et al. [13]
II	II	St1Cas9	NNAGAAW	*Streptococcus thermophilus*	1121	Muller et al. [14]
II	II	SaCas9	NNGRRT or NNGRR(N)	*Staphylococcus aureus*	1053	Ran et al. [9]
II	II	FnCas9	NGG	*Francisella novicida*	1629	Fonfara et al. [15]; Hirano et al. [16]
II	II	TdCas9	NAAAAN	*Treponema denticola*	1395	Esvelt et al. [17]
II	II	St3Cas9	NGGNG	*Streptococcus thermophilus*	1409	Glemzaite et al. [18]
II	II	CjCas9	NNNNACAC or NNNNRYAC	*Campylobacter jejuni*	984	Kim et al. [19]
II	II	GeoCas9	NNNNCRAA	*Geobacillus stearothermophilus*	1087	Harrington et al. [20]
II	II	BlatCas9	NNNNCNDD	*Brevibacillus laterosporus*	1092	Karvelis et al. [21]
II	II	Nme2Cas9	NNNNCC	*Neisseria meningitidis*	1082	Edraki et al. [10]
II	II	Nme3Cas9	NNNNCAAA	*Neisseria meningitidis*	1082	Edraki et al. [10]
II	II	ScCas9	NNG	*Streptococcus canis*	1375	Chatterjee et al. [22]
II	II	SmacCas9	NAA	*Streptococcus macacae*	1130	Jakimo et al. [23]
II	V	MbCas12a (a.k.a. MbCpf1)	TTN	*Moraxella bovoculi*	1418	Zetsche et al. [11]
II	V	FnCas12a (a.k.a. FnCpf1)	TTN, KYTV	*Francisella novicida*	1300	Zetsche et al. [11]; Tu et al. [24]
II	V	AsCas12a (a.k.a. AsCpf1)	TTTV	*Acidaminococcus* sp.	1307	Zetsche et al. [11]
II	V	LbCas12a (a.k.a. LbCpf1)	TTTV	*Lachnospiraceae bacterium*	1228	Zetsche et al. [11]

(continued)

Table 1
(continued)

Class	Type	Nuclease	PAM (5′-3′)	Species	Size (aa)	References
II	V	PdCas12a (a.k.a. PdCpf1)	TTTV	*Prevotella disiens*	1323	Zetsche et al. [11]
II	V	AaCas12b (a.k.a. C2c1)	TTN	*Alicyclobacillus acidiphilus*	1129	Teng et al. [25]
II	V	AkCas12b (a.k.a. C2c1)	TTTN	*Alicyclobacillus kakegawensis*	1147	Teng et al. [25]
II	V	BhCas12b (a.k.a. C2c1)	ATTN	*Bacillus hisashii*	1108	Strecker et al. [26, 27]
II	V	DpbCasX (a.k.a. Cas12e)	TTCN	*Deltaproteobacteria*	986	Liu et al. [28]
II	V	Cas14	Not required	Uncultivated archaea	400–700	Harrington et al. [29]
II	VI	LbCas13a (a.k.a. C2c2)	**	*Leptotrichia buccalis*	1159	East-Seletsky et al. [30]
II	VI	LshCas13a (a.k.a. C2c2)	**	*Leptotrichia shahii*	1389	Abudayyeh et al. [12]
II	VI	LwaCas13a	**	*Leptotrichia wadei*	564	Abudayyeh et al., [31]
II	VI	PbuCas13b	**	*Prevotella buccae*	1127	Smargon et al., [32] Slaymaker et al. [33]

(Nucleotide abbreviation, N, A/T/G/C; W, A/T; R, A/G; Y, C/T; V, A/C/G; D, A/G/T; K, G/T) **, Cas13, a RNA-guided RNA nucleases, does not require a PAM but require protospacer flanking site (PFS); a.k.a., also known as

PAM specificities have been altered by engineering the native Cas9 in a plethora of earlier studies [36]. For example, SaCas9-KKH variant has NNNRRT PAM requirement [37]. Instead of NGG PAM, engineered variants like SpCas9-NG and xCas9 are able to recognize a two-nucleotide PAM, 5′-NG-3′ [38, 39]. For increasing specificity, SpCas9-HF1, a high-fidelity variant, has been developed by altering four amino acids [40]. A comprehensive list of engineered variants of Cas nucleases and their utilities is presented in Table 2.

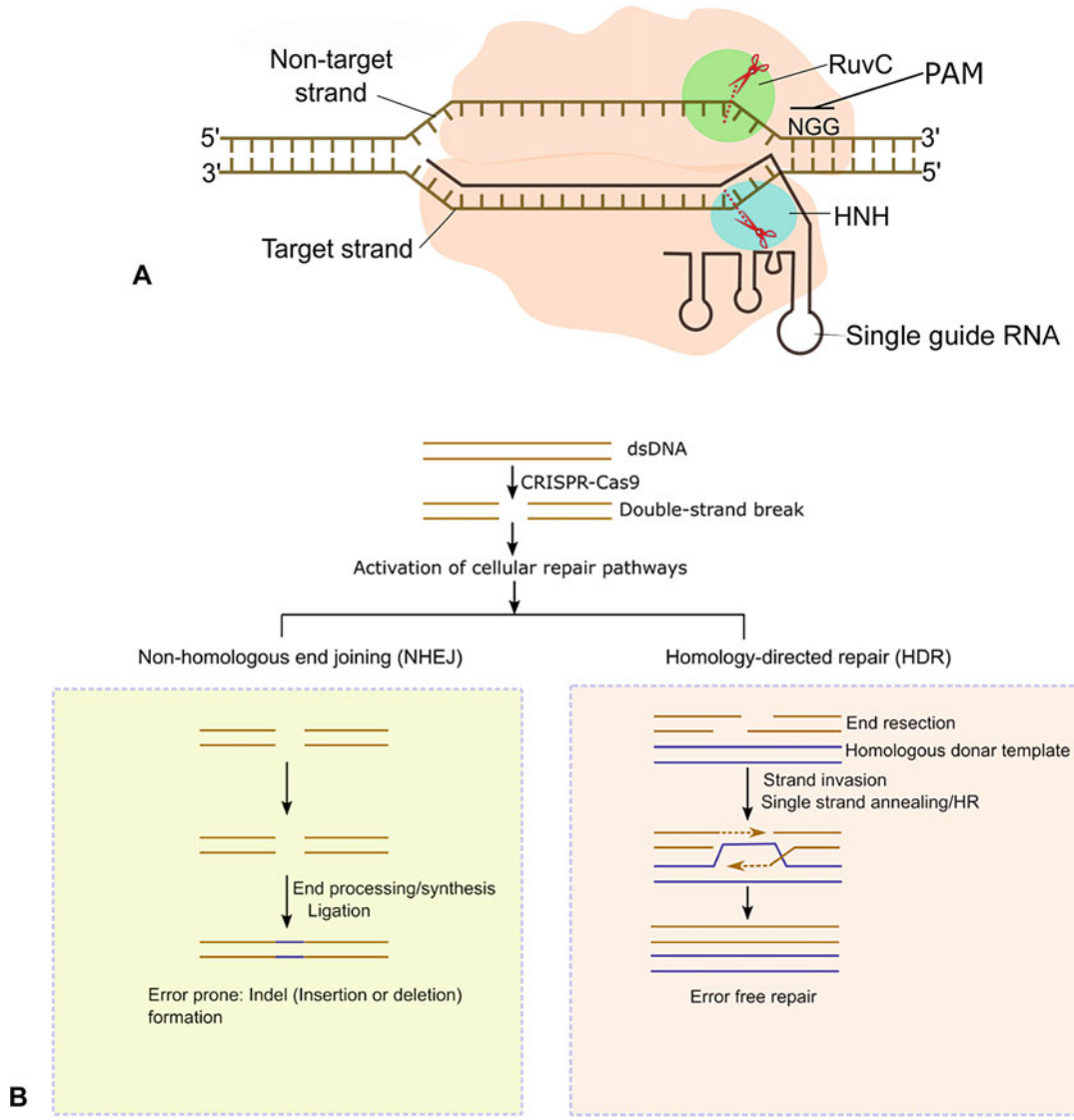

Fig. 1 (**A**) Active Cas9 complexed with single-guide RNA (sgRNA) and target DNA. RuvC and HNH are the two nuclease domains that cleave the DNA. NGG is the protospacer adjacent motif (PAM) for *Streptococcus pyogenes* Cas9. (**B**) Cellular NHEJ- and HDR-mediated repair after the generation of double-strand break by Cas9 (modified from [34])

4 CRISPR-Cas Tool Box Has Too Much to Consider

The CRISPR-Cas-derived technologies are the most rapidly adopted technologies in the history of life science. CRISPR-based tools are being developed and adopted in an unprecedented pace. In this section, we briefly overview different tools and their applications for different types of genome editing, novel allele generation, gene expression manipulation, genetic loci imaging, epigenome modification, and many others.

Table 2
Engineered Cas variants developed and their respective PAM sequences

Nuclease variant	Modified from	Modification	Size (AA)	PAM	Feature	References
Cas9 nickase (a.k.a. nCas9)	SpCas9	D10A	1368	NGG	Nicks the complementary strand/target strand	Jinek et al. [5]
Cas9 nickase (a.k.a. nCas9)	SpCas9	H840A	1368	NGG	Nicks the noncomplementary strand/nontarget strand	Jinek et al. [5]
Dead Cas9 (a.k.a. dCas9)	SpCas9	D10A/H840A	1368	NGG	Catalytically dead. Binds to targeted DNA but not cleave	Qi et al. [41]
Cas9-VQR Cas9-EQR Cas9-VRER Cas9-D1135E	SpCas9 SpCas9 SpCas9 SpCas9	D1135V/R1335Q/T1337R D1135E/R1335Q/T1337R D1135V/G1218R/R1335E/T1337R D1135E	1368 1368 1368 1368	NGA NGAG NGCG NGG	Altered PAM specificity Altered PAM specificity Altered PAM specificity More specific to "NGG" PAM than SpCas9. Reduced affinity for noncanonical "NAG" PAM	Kleinstiver et al. [42]
SaCas9-KKH	SaCas9	E782K/N968K/R1015H	1053	NNNRRT	Altered PAM specificity	Kleinstiver et al. [37]
Cas9-HF1	SpCas9	N497A/R661A/Q695A/Q926A	1368	NGG	High-fidelity variant to reduce nonspecific DNA contacts	Kleinstiver et al. [40]
Cas9-HF2	SpCas9	N497A/R661A/Q695A/Q926A/ D1135E	1368	NGG	High-fidelity variant to reduce nonspecific DNA contacts	
Cas9-HF3	SpCas9	N497A/R661A/Q695A/Q926A /L169A	1368	NGG	High-fidelity variant to reduce nonspecific DNA contacts	
Cas9-HF4	SpCas9	N497A/R661A/Q695A/Q926A/Y450A	1368	NGG	High-fidelity variant to reduce nonspecific DNA contacts	
Cas9-QQR1	SpCas9	G1218R/N1286Q/I1331F/D1332K/ R1333Q/R1335Q/T1337R	1368	NAAG	Altered PAM specificity	Anders et al. [43]

(continued)

Table 2
(continued)

Nuclease variant	Modified from	Modification	Size (AA)	PAM	Feature	References
eSpCas9–1.0	SpCas9	K810A/K1003A/R1060A	1368	NGG	Reduced off-target effects	Slaymaker et al. [44]
eSpCas9–1.1	SpCas9	K848A/K1003A/R1060A	1368	NGG	Reduced off-target effects	Slaymaker et al. [44]
xCas9–3.7	SpCas9	A262T/R324L/S409I/E480K/E543D/M694I/E1219V	1368	NG, GAA, and GAT	Altered PAM specificity	Hu et al. [38]
SpCas9-NG	SpCas9	R1335V/L1111R/D1135V/G1218R/E1219F/A1322R/T1337R	1368	NG	Altered PAM specificity	Nishimasu et al. [39]
SpG	SpCas9	D1135L/S1136W/G1218K/E1219Q/R1335Q/T1337R	1368	NGN	ALtered PAM specificity	Walton et al. [45]
SpRY	SpCas9	SpG with L1111R/A1322R/A61R/N1317R/R1333P	1368	NRN>NYN	ALtered PAM specificity	
FnCas9-RHA	FnCas9	E1369R/E1449H/R1556A	1632	YG	Altered PAM specificity	Hirano et al. [16]
iSpyMacCas9	SpCas9 and SmacCas9	Recombined PAM-interacting domain of SpCas9 and SmacCas9. With two additional mutations, R221K and N394K	-	NAA	Altered PAM specificity	Jakimo et al. [23]
ScCas9ΔLoop ScCas9ΔKQ variants	ScCas9	ΔLoop, AA367–376 removed; ΔKQ, AA1337–1338 removed	1043 1051	NNGA, NGG	Altered PAM specificity	Chatterjee et al. [22]
HiFi Cas9	SpCas9	R691A	1368	NGG	Enhanced specificity for ribonucleoprotein delivery	Vakulskas et al. [46]
HypaCas9	SpCas9	N692A/M694A/Q695A/H698A	1368	NGG	Enhanced specificity	Chen et al. [47]
evoCas9	SpCas9	M495V/Y515N/K526E/R661Q	1368	NGG	Enhanced specificity	Casini et al. [48]

LbCas12a-RVR	LbCas12a	G548R/K554V/Y558R	1228	TATG	Altered PAM recognition	Zhong et al. [49]
LbCas12a-RR	LaCas12a	G548R/K611R	1228	TYCV, CCCC	Altered PAM recognition	Zhong et al. [49]
FnCas12a-RVR	FnCas12a	N623R/K629R/N633R	1300	TATG	Altered PAM recognition	Gao et al. [50]
AsCas12a RR	AsCas12a	S542R/K607R	1307	TWTV, TYCV	Altered PAM recognition	Gao et al. [50]
FnCas12a RR	FnCas9	N623R/K687R	1300	TWTV, TYCV	Altered PAM recognition	Gao et al. [50]
MbCas12a RR	MbCas12a	N576R/K637R	1418	TWTV, TYCV	Altered PAM recognition	Toth et al. [51]
AsCas12a RVR	AsCas12a	S542R/K548V/N552R	1307	TATV	Altered PAM recognition	Gao et al. [50]
MbCas12a RVR	MbCas12a	N576R/K582V/N586R	1418	TATV	Altered PAM recognition	Toth et al. [51]

(Nucleotide abbreviation, N, A/T/G/C; W, A/T; R, A/G; Y, C/T; V, A/C/G; D, A/G/T; K, G/T) Sp/Spy, *Streptococcus pyogenes*; Sa, *Streptococcus aureus*; Fn, *Francisella novicida*; Smac, *Streptococcus macacae*; Sc, *Streptococcus canis*; AA, amino acid

4.1 Gene Knockout

Guided by sgRNA, Cas9 makes a double-strand cleavage in the target gene. The double-strand break is predominantly repaired by the error-prone NHEJ pathway, which often leads to accumulation of small insertions or deletions (indels) (Fig. 1b). Those indels are most likely to cause frameshift mutation causing effective knockout of the gene. After the development of CRISPR-Cas9 tool, the gene knockout has become easier than ever before [5, 6, 52]. Therefore, the system has been rapidly adopted by different laboratories working on a broad range of organisms ranging from bacteria, alga, fungus to higher eukaryotes, including plants and humans. For targeting each new gene, only the 20-nucleotide guide RNA needs to be redesigned. The system also allows efficient multiplex gene editing in a single experiment by designing an array of sgRNAs targeting multiple genes [6, 53].

If Cas9 could not be targeted to a locus due to the absence of an NGG PAM, a number of other nuclease variants could be used for the same purpose (Tables 1 and 2). CRISPR-Cas-mediated knockout tools have immensely impacted the fundamental biological studies as well as applications in agriculture and human therapeutics [3, 54–56].

4.2 Gene Regulation

Although catalytically dead Cas9 (dCas9) does not have the ability to cleave DNA, it can bind to a target locus guided by sgRNA. This feature has been utilized to regulate or manipulate the expression level of a gene of interest.

1. *CRISPRa*: CRISPR activation (CRISPRa) is a technique which utilizes the fusion of dCas9 with transcription activation domain to increase the expression of genes of interest [57, 58]. Transcription activator VP64 (tetramer of VP16) has been fused with dCas9 and targeted to the promoter region to increase the expression of target genes (Fig. 2A) [57, 58]. Multiple sgRNAs targeted to the promoter of a single gene have been proved to be more efficient in increasing expression than a single sgRNA [57, 58]. Since the activation depends on the VP64 domain, presence of multiple VP64 in the promoter region has a synergistic effect on enhancing expression.
2. *CRISPRi*: CRISPR interference (CRISPRi) is a technique which utilizes the programmable binding ability of dCas9 to repress the expression of gene(s) by blocking or interfering RNA polymerase binding, transcription factor binding, and transcriptional elongation [41]. sgRNA specific to the upstream regulatory region (e.g., promoter) or the transcription initiation site of a gene sequence could guide the dCas9 to bind and block the transcription initiation or elongation and effectively silence the gene expression. Interestingly, dCas9 was fused with transcription repression domain of Krüppel-

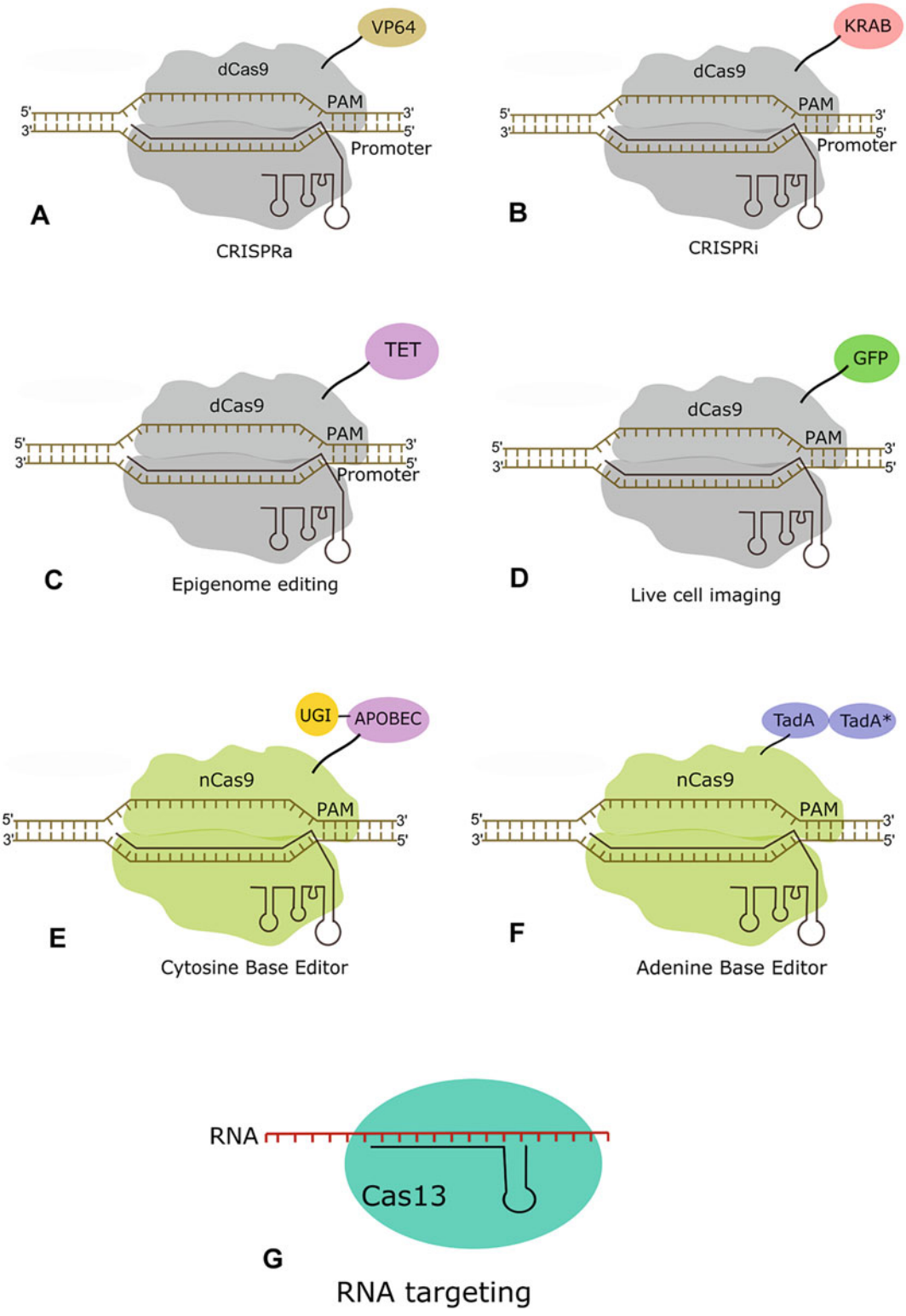

Fig. 2 Diverse CRISPR-Cas-derived tools. (**A**) CRISPR activator (CRISPRa) for activating a target gene. VP64, Viral protein 64, is a known transcription activator. (**B**) CRISPR interference (CRISPRi) for inhibiting gene expression. *KRAB,* Krüppel-associated box, is a known transcription inhibitor. (**C**) Epigenome editor to

associated box (KRAB) to silence target gene expression (Fig. 2B) [59]. The dCas9 alone or the fused dCas9-KRAB are efficient tools to knock down one or multiple genes.

4.3 Large-Scale CRISPR Screening

This technique allows to achieve large-scale genome-wide functional screening. A library of thousands of sgRNAs are prepared and delivered to a population of cells constitutively expressing effector proteins (Cas9, dCas9, dCas9-KRAB, dCas9-VP64, etc.). A low multiplicity of viral infection (MOI = 0.3 to 0.4) is used to ensure that each cell receives one or less sgRNA [60]. In this type of screening, each gene is targeted by 6–8 sgRNAs. This technique is utilized to identify a gene or a mutation responsible for a particular phenotype. CRISPR screening enables us to functionally screen or validate thousands of genes in one go. Identification and validation of novel drug targets along with analysis of effect of genetic mutations on drug activity are the sectors where CRISPR screening are being applied heavily. CRISPR screens are of three types, viz., (i) CRISPR-knockout screens which allow to screen for complete loss of function of the genes, (ii) CRISPRi screens which repress the expression of the genes instead of completely disrupting the function, and (iii) CRISPRa screens which enhance the level of expression of genes relative to their endogenous level. A detailed and in-depth review on CRISPR-mediated genetic screening is available [61].

4.4 Epigenome Editing

Modifications in the DNA that do not alter the sequence of the DNA but can regulate their activity are known as epigenetic marks. These epigenetic modifications may be present on DNA and histone proteins and play a significant role in gene expression. CRISPR-mediated epigenome editing tools enable us to unravel the role of those epigenetic marks in the genome of an organism. Once the role of an epigenetic mark is known, the tool can be used to alter the expression of the gene. Histone acetyltransferase (HAT) acetylates histone protein and makes the chromatin structure loose. Loose chromatin becomes more accessible to transcription machinery and thus more active transcriptionally. The fusion of catalytic histone acetyltransferase (HAT) with dCas9 has been demonstrated to increase the expression of target genes. sgRNAs were designed to target the dCas9-HAT fusion protein to the promoters of the target genes [62].

Fig. 2 (continued) demethylate promoter. *TET,* methylcytosine dioxygenase, catalyzes demethylation. (**D**) Genomic loci could be imaged by dCas9 fused with reporter GFP. (**E**) Cytosine base editor for precise C → T editing. APOBEC, rat cytidine deaminase, deaminates cytosine; *UGI,* uracil glycosylase inhibitor. (**F**) Adenine base editor for precise A → G editing. *TadA,* wild-type tRNA adenosine deaminase; *TadA*∗*,* laboratory-evolved DNA adenosine deaminase. (**G**) Cas13 for targeting RNA

Promoter hypermethylation is another type of epigenetic mark that usually suppresses gene expression. Methylcytosine dioxygenase (TET1) is a known enzyme for cytosine demethylation. dCas9-TET1 fusion, guided by sgRNA specific to the promoter region of *FMR1*, activates the expression of the *FMR1* gene and subsequently rescues the neurons from fragile X syndrome [63]. The dCas9-TET1 (Fig. 2C) helped in switching the heterochromatin state of the FMR1 promoter to an active chromatin state. Similarly, the fusion of a methyl transferase (Dnmt3a) with dCas9 has been used for targeted de novo methylation of the CTCF motif [64]. Like the CRISPRi and CRISPRa, for epigenome editing, dCas9 is also used as a precise vehicle to carry other effector proteins to a target locus.

4.5 3D Genome Organization

The three-dimensional organization of the genome or the spatial position of a genomic locus within the nucleus plays an important role in gene regulation. Genomic loci present in the interior of the nucleus tend to show higher activity than the loci present at the periphery [65]. In a recent study, Wang et al. [66] developed a CRISPR-genome organization (CRISPR-GO) system to control the dynamic positioning of specific genomic loci to specific nuclear compartments like Cajal bodies, nuclear periphery, and PML bodies.

Ligand-mediated dimerization has been utilized to bring a target locus to a specific location. The fusion of nuclear compartment-specific proteins with the CRISPR-dCas9 and subsequent dimerization with the protein present at the compartment do the job of bringing a genomic locus to the particular location.

4.6 Chromatin Topology

The formation of a chromatin loop brings two regulatory DNA elements in close physical proximity to each other. Many regulatory elements like the locus control region (LCR) and enhancer lie far from the promoter of a gene. In order to activate or enhance the expression of the gene, the enhancer needs to be in close proximity to the promoter region. dCas9-based tools have been developed to engineer artificial chromatin loop formation to induce the expression of a developmentally silent gene [67]. Hao et al. [68] fused heterodimerizing leucine zippers protein (Zip) with each of the dCas9 from *Streptococcus pyogenes* (Sp) and *S. thermophilus* (St). Sp-dCas9-Zip and St-dCas9-Zip were targeted to two DNA sites located 1.4 Kb apart from each other. In vivo dimerization of the Zips brought those two sites in close proximity. Morgan et al. [67] used the dCas9 from *S. pyogenes* (Sp) and *Staphylococcus aureus* (Sa). They fused ABA-inducible protein to force chromatin loop formation between a distantly placed enhancer and promoter region of the β-globin gene. Manipulating

DNA loop formation enables us to understand their function better and to modulate gene expression artificially.

4.7 Live Cell Imaging: Chromatin and RNA

Fluorescent in situ hybridization (FISH) has been in use to image specific DNA sequences. Rigorous cell fixation procedure, including heating, is the major limitation of the technique to image chromatin in a live cell. The green fluorescence protein (GFP) tagged dCas9 when targeted with optimized sgRNAs to repetitive elements or coding regions, a specific target genomic locus could be fluorescently imaged (Fig. 2D) [69]. This imaging method allowed them to follow the telomere length change and their movements and dynamics of gene loci throughout the cell cycle. For multiple genomic loci imaging, orthogonal dCas9 proteins, each labelled with different fluorescent proteins has been used [70]. Since targeting a non-repetitive region is more challenging due to the background fluorescent signal from free-floating fluorescent protein, 20–30 unique sgRNAs are needed to image a coding DNA (non-repetitive) region [60]. The use of an MS2 signal amplification system with an engineered sgRNA has been demonstrated to efficiently image non-repetitive regions with a minimum of 4 unique sgRNAs [71].

Similarly, real-time RNA imaging was made possible by the development of dCas13-fluorescent protein-based tools [72]. dCas13a has been successfully used in programmable tracking of transcripts in live cells [31]. dCas13 is an RNA-guided RNA binding protein [12]. These CRISPR-dCas-based imaging tools greatly enhance our ability to long-term image and dynamics of genomic loci or transcripts of interest. Different CRISPR-based live-cell chromatin imaging platforms and their advantages and limitations are detailed in an earlier review [73].

4.8 Base Editing

Conventional CRISPR-Cas tools generate targeted knockout mutants with very high efficiency. The mutations are generated due to the incorporation of random indels in the gene. However, those tools cannot create precise single nucleotide change in the genome. Many human and plant genetic diseases are caused by single nucleotide mutation. Supplying a donor template containing the desired changes could install the mutation via homology-directed repair (HDR). However, correcting through HDR has been incredibly difficult because of its extremely low efficiency.

Base editing is a technique that allows us to install precise single nucleotide changes in the genome. All four types of transition point mutations (A → G, T → C, G → A, and C → T) can be installed with the available base editors [74–76]. Base editors are developed by fusing a nCas9 (D10A) with a nucleotide deaminase.

Unlike HDR, base editing is efficient in both dividing and nondividing cells [2, 34]. The target base can be edited if they are located in a short window. The window is called the activity window

of a base editor, and it usually ranges from fourth to the tenth nucleotide of the target 20 bp seq in the genome (counting PAM as 21–23). The tools can be applied to correct 61% of human pathogenic mutations listed in the ClinVar database. There are three main types of base editors available, which are briefly mentioned below:

1. *Cytosine base editor (CBE)*: CBEs are constructed by fusing nCas9 with cytidine deaminase (Fig. 2E) [75, 76]. They can convert a C nucleotide to a T nucleotide (G to A in the opposite strand). The cytidine deaminase deaminates a target C into U. C-G base pair becomes U-G. The resultant U-G base pair is a mismatch. nCas9 makes a nick in the G-containing strand to trick the repair machinery that it considers the G as a mismatch. As a result, G is removed and an A is incorporated opposite to U. In subsequent steps, the pair becomes T-A. In this way, a C-G base pair is converted to a T-A base pair. Achieving this outcome is not easy since the cellular base (uracil) excision repair is very active. The incorporation of uracil in the genomic DNA is recognized as an error by the base excision repair pathway and is removed by uracil glycosylase. In order to increase the likelihood of the expected outcome (T-A base pair), CBE contains a uracil glycosylase inhibitor (UGI) protein [75, 76]. So, CBE is a fusion of Cyt deaminase-nCas9-UGI.
2. *Adenine base editor (ABE)*: ABEs are made up of a laboratory-evolved DNA adenosine deaminase fused with nCas9 (D10A) (Fig. 2F) [74]. ABEs can convert a target A nucleotide to a G nucleotide (T to C in the opposite strand). Once the adenosine deaminase deaminates the target A into inosine (I), the A-T base pair becomes I-T base pair. nCas9 generates a nick in the T-containing DNA strand so that the cellular mismatch repair system removes the T as a mismatched base. Since the I is treated as G by cellular repair machinery, a C is incorporated opposite to I. Subsequently, the I-C base pair is converted to G-C. In this way, ABEs convert an A-T base pair to a G-C base pair. Since inosine excision repair is not as active as uracil excision, no inhibitor protein was fused to construct ABE.
3. *RNA base editor (RBE)*: RBEs have been developed by utilizing the programmable RNA binding ability of deactivated Cas13b protein. Since RBEs can alter single nucleotides in the transcript, they do not create any change in the genome. Two types of RBEs, "RNA Editing for Programmable A to I Replacement" (REPAIR) system and "RNA Editing for Specific C to U Exchange" (RESCUE), have been developed [77, 78]. REPAIR has been constructed by fusing dCas13b with the adenine deaminase domain of ADAR2 [78], while RESCUE has been made by fusing a laboratory-evolved RNA

cytidine deaminase with dCas13b [77]. Unlike the 20-nucleotide-long guide RNA for CBEs and ABEs, RBE needs a 50-nucleotide-long guide RNA. A single nucleotide in the guide RNA needs to be mismatched opposite the target A or C.

The application of base editors not only includes alteration of functional single nucleotide mutations for developing disease models, human therapeutics, and superior agricultural crops but also as various basic biological tools. Base editors have been applied for molecular diversification, creating or correcting early stop codons, modulating splicing events, and molecular recording [2, 34]. Readers are suggested to go through the earlier reviews for comprehensive and in-depth technical details and a broad range of application of base editing tools [2, 79].

4.9 RNA Editing

Although CRISPR-Cas tools have been extensively used in DNA targeting for various purposes, targeting RNA was limited to antisense and RNAi technologies. However, recent discovery and repurposing of Cas orthologues have made targeting and manipulating RNA easier than before. Cas13 protein is an RNA-guided RNA nuclease and has been recently demonstrated as a potent tool for RNA targeting (Fig. 2G) [30–33]. Cas13 and its deactivated form dCas13 are being used for various purposes like knocking down of endogenous transcripts, live-cell RNA imaging, RNA interference, RNA base editing, and diagnostics. While PAM is an absolute necessity for Cas9 nucleases, Cas13 does not need a PAM. However, some of the Cas13 nucleases (e.g., LshCas13a) need the presence of a protospacer flanking site (PFS) at the target RNA [12], and some (e.g., PbuCas13b) do not need any PFS [31]. LshCas13 prefers the target sites that are flanked by an H (A or T or C) nucleotide. This feature is known as the protospacer flanking site or PFS.

Although SpCas9 is widely known to cleave only DNA substrates, if PAM is supplied in *trans* as a separate oligonucleotide, Cas9 could be repurposed to bind and cleave single-stranded RNA targets [80]. These complementary PAM-presenting oligonucleotides are known as PAMmers. Using a matching guide (sgRNA) and specifically designed PAMmer, SpCas9 can be targeted to bind or cleave specific ssRNA while avoiding corresponding DNA sequences [80].

Campylobacter jejuni Cas9 (CjCas9) and SaCas9 have been shown to cleave target ssRNA without the need of a PAM [81]. Interestingly, using an engineered RNA targeting sgRNA, *Francisella novicida* (FnCas9) has been utilized to target the hepatitis C virus RNA in eukaryotic cells [82].

4.10 CRISPR-Associated Transposase (CAST)

Earlier studies identified many CRISPR-Cas systems that lack active nuclease domains [83, 84]. Interestingly, few of those systems have been characterized recently and found to be associated with transposons [26, 27, 85, 86]. The functional relationship between RNA-guided DNA targeting and transposition has been established in those studies.

CAST systems are able to catalyze RNA-guided DNA transposition at 60–66 base pairs downstream of the protospacer [26, 27]. CAST from the cyanobacteria *Scytonema hofmanni*, ShCAST, integrates DNA unidirectionally [26, 27]. CAST from *Vibrio cholerae* Tn6677 has been shown to integrate DNA in the genome bidirectionally at around 49 bp downstream of the protospacer [85]. Both the CAST systems have been repurposed for targeted DNA knock-in in the *E. coli* genome [26, 27, 85]. CAST system could be used to integrate transgene in a predefined position in the genome and to insert corrected exons into the intron before the mutated exon [26, 27]. The system could also be utilized to place an exogenous promoter upstream of an endogenous gene or insert a transgene downstream of an endogenous promoter.

4.11 CRISPR-Mediated Homology-Directed Repair (HDR)

Although CRISPR-Cas base editors (*see* Subheading 4.8) can perform precise editing in the form of single nucleotide alteration, they cannot install transversion mutation and other types of precise editing like insertion or deletion of few bases in the genome. Researchers mostly depend on utilizing cellular homology-directed repair (HDR) mechanism for performing precise genome modification. Once Cas9 cuts the genome at a targeted locus, it is repaired mostly through either the NHEJ pathway or the HDR pathway. Cells perform error-free repair through HDR utilizing the sister chromatids as a donor template (Fig. 1B). If an abundant exogenous donor template with the desired nucleotide changes flanked by homologous arm is supplied into the cell, precise modification can be achieved. However, HDR-mediated precise genome modification has several practical limitations in higher eukaryotes. Although HDR is predominant in many fungal species and lower group of organisms, it is not the method of choice in higher eukaryotes. Besides the innate low efficiency of HDR, another limitation is that it occurs mostly in the G2 and S phase of the cell cycle. Furthermore, supplying donor repair template and their availability in their vicinity of the Cas9-generated double-strand break (DSB) are limiting factors. Many strategies have been studied to improve HDR efficiency in higher eukaryotes, including human cell line and plants without much success [87, 88]. Recent reports indicate the possibility of predicting the NHEJ repair outcome, which could potentially be utilized for precise editing without any donor template [34].

4.12 Prime Editing

Other than installing transition mutation in the genome through base editing, scientists are mostly dependent on employing HDR for precise editing. A state-of-the-art technique, named as prime editing, gives us much hope for high-efficiency precise editing, which does not require to make a double-strand DNA break or to supply donor template [89]. Starting from replacement of individual nucleotides to deletion or insertion of blocks of nucleotides into the genome have been achieved in cultured cells using prime editing with much higher efficiency than HDR.

Prime editing uses Cas9 nickase (nCas9-H840A) fused with a reverse transcriptase (RT) enzyme. The editing method is guided by pegRNA (prime editing guide RNA) that both specifies the target and encodes the desired edits [89]. Two extra elements, 10–13 nt primer binding site (PBS) and 10–16 nt long RT template with the desired changes, are added with the traditional sgRNA to construct pegRNA. Once the nick is made at the PAM-containing strand, the primer binding site hybridizes with the resulting 3′ end. This hybridization initiates direct polymerization of the PAM-containing strand by reverse transcription using the RT template with edits. Subsequent flap equilibration, 5′ flap removal, ligation, and DNA repair would result in the edited genomic DNA. The causal genetic mutations for sickle cell and Tay-Sachs diseases have been corrected employing prime editing. Theoretically, 89% of the known human pathogenic mutations could be attempted to cure using the prime editing tool.

4.13 Molecular Diagnostic

Pathogen detection and disease monitoring may be significantly improved with the availability of nucleic acid detection methods that are highly sensitive, specific, rapid, and inexpensive. Cas12a and Cas13 are able to indiscriminately cleave single-stranded DNA (ssDNA) and single-stranded-RNA (ssRNA), respectively, after binding to a target nucleic acid. This feature, known as collateral cleavage activity, has been repurposed for developing highly sensitive nucleic acid detection kits for diagnostic purposes [90–94]. Collateral cleavage activities of Cas12a and Cas13a have been utilized to develop an ssDNA detection system, DETECTR (DNA endonuclease-targeted CRISPR trans reporter), and an ssRNA detection system, SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing), respectively, with attomolar sensitivity [90, 91].

In these detection systems, a guide RNA is designed to target the specific nucleic acid to be detected, and an unrelated ssDNA or ssRNA labelled with reporter-quencher is supplied. Once the nuclease (Cas12a or Cas13a) finds and binds successfully to the target nucleic acid (ssDNA or ssRNA), it collaterally cleaves the unrelated nucleic acid and releases the quencher to produce the signal. Detection of the signal significantly higher than the control indicates the presence of the target nucleic acid. DETECTR platform have been

successfully used to develop lateral flow assay to diagnose the COVID19 disease.

4.14 CRISPR Gene Drive

Following Mendelian inheritance a specific allele inherits to the next generation with a probability of 50%. Gene drive is a genetic manipulation technique which enables to alter the probability to a higher level and allows biased inheritance of a specific allele or genetic element to the offspring. CRISPR-Cas endonuclease gene drive has been used to insert and spread the desired modification at a much higher rate than the usual rate of Mendelian inheritance [95]. In CRISPR-Cas gene drive, once the drive cassette integrates into one of the chromosomes, the offspring will have one chromosome with the drive and the other one normal inherited from wild-type parent. In an early stage of development, the CRISPR-Cas portion will make a cut in the normal chromosome, and the cut will be repaired using the drive cassette as a template. Therefore, the offspring will harbor both the chromosome and the drive containing the modification. It allows a nearly 100% inheritance of the modification.

CRISPR gene drive has been used to induce sterility in female *Anopheles gambiae* mosquito and successfully wiped out the entire caged population within 7–11 generations [96]. CRISPR gene drive has recently been shown to bias the inheritance of a desired allele in mice [97]. CRISPR gene drive could be applied to wipe out the population of disease vector species like mosquitoes and to control invasive species.

5 Concluding Remarks

The CRISPR-Cas and associated technologies derived from the naturally occurring prokaryotic CRISPR immune system are indeed revolutionary in studying basic biology and manipulating the genomes of diverse organisms. These robust, reproducible, and easy-to-use technologies allow the manipulation or modification of genome in several ways, including, but not limited to (i) by simply incorporating random mutation (insertion or deletion) through nonhomologous end joining to disrupt gene(s); (ii) by generating targeted point mutations in genes using precise base editors; and (iii) by a whole gene insertion employing the cell's homology-directed repair pathway. This chapter briefs the principles, diversity, and application of CRISPR-Cas technologies including gene knockout, gene regulation, CRISPR-screening, epigenome editing, 3D genome organization, chromatin topology, live cell imaging, disease diagnosis, base editing, RNA editing, CRISPR-associated transposase, prime editing, and CRISPR gene drive. Improvement in protocols, higher access to CRISPR-Cas tools, and necessary changes in the global regulatory environments are

needed for the broader application of this frontier technology in diverse areas like production of energy, health, environment, medicine, and sustainable food production in changing climate.

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Chapter 2

Efficient Homologous Recombination-Mediated in Planta Gene Targeting by Egg-Cell-Specific Expression of *Staphylococcus aureus* Cas9 from Arabidopsis

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Abstract

Site-specific genome engineering approaches were greatly facilitated by the recent emergence of the CRISPR-Cas system, enabling precise induction of DNA double-strand breaks. However, up to now its application was mostly restricted to nonhomologous end-joining-mediated targeted mutagenesis. In contrast, precise genome modifications using a suitable donor sequence for homologous still pose a particular challenge in plants, as NHEJ is the dominant repair mechanism for DSBs in somatic cells. To achieve efficient HR-mediated genome modifications in plants, we recently developed the in planta gene targeting (ipGT) system, which works via the induction of DSBs by Cas9 to activate the target and the targeting vector at the same time, making it independent of high transformation efficiencies. Here, we describe an updated protocol of ipGT for the model plant Arabidopsis, taking into account our recent improvements based on egg-cell-specific expression of *Staphylococcus aureus* Cas9.

Key words CRISPR-Cas, Double-strand break, Gene targeting, Homologous recombination, Non-homologous end joining

Abbreviations

CRISPR-Cas	Clustered regularly interspaced short palindromic repeats-CRISPR associated
DSB	Double-strand break
GT	Gene targeting
ipGT	In planta GT
NHEJ	Nonhomologous end joining
PPT	Phosphinothricin

1 Introduction

Gene targeting (GT) is a genome engineering method that achieves desired genetic modifications through an endogenous DNA repair

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system—homologous recombination. However, due to its low efficiency, GT was mostly demonstrated as a proof of concept in plants up to now. To stimulate HR-based GT in the target locus, the most significant and well-known method is the induction of a double-strand break (DSB) in the target locus [1]. Mainly, there are two distinctive goals in genome modification via induced DSBs: targeted mutagenesis and GT. DSBs used in genome editing result in mutagenesis mainly through the error-prone nonhomologous end-joining (NHEJ) repair pathway to obtain small deletions or insertions [2]. Differently, GT requires an additional homologous sequence to serve as template for homologous recombination, termed HR donor. It contains the desired modification in the center region and the homologous sequences in the flanking regions. Successful GT leads to transfer of sequence information from the DNA donor to the target locus in a predefined manner, enabling precise insertion, replacement, or substitution of a DNA fragment, which is not feasible through general NHEJ-based genome editing. For a more detail discussion of the development of the GT in plants, *see* [3, 4].

The development of programmable nucleases such as CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated), which enable precise DSB induction at desired loci, has become a routine method for mutagenesis purpose in plants [5, 6]. However, despite DSB induction, GT efficiency remains too low for routine usage. The main limitations are the lack of efficient transformation systems for most crops and the general low frequency of HR in plants. To address this problem, we developed the in planta GT (ipGT) approach that is independent on high transformation efficiencies, since the HR donor is stably integrated into the genome. By inducing DSBs at the flanks of the HR donor simultaneously to DSB induction in the target, the donor can be released from the genome to activate it for HR (*see* Fig. 1). This way, GT efficiency could be enhanced to percentile range [7, 8]. Later on, it was demonstrated in Arabidopsis that *Staphylococcus aureus* Cas9 (SaCas9) possesses higher DSB-based mutagenesis activity than *Streptococcus pyogenes* Cas9 (SpCas9) [9]. Therefore, SaCas9 became a more promising molecular tool for the induction of DSB in Arabidopsis.

The application of an egg-cell-specific promoter-driven SaCas9 was first demonstrated in genome editing, showing the enhancement of NHEJ-mediated mutagenesis [10–12]. More recently, two studies demonstrated that using an egg-cell promoter for Cas9 expression resulted in higher GT efficiency compared to other promoters tested, which included constitutive, meristem-specific, and pollen-specific promoters [13, 14]. By combining our ipGT approach with the high-efficient SaCas9 driven by an egg-cell-specific promoter, we obtained heritable GT events with high efficiencies up to 6% of T2 Arabidopsis seeds [13]. These studies

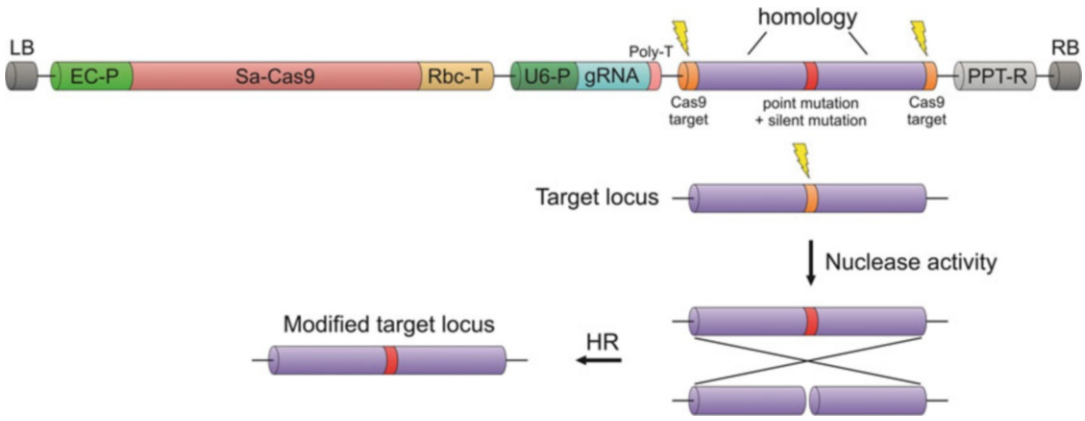

Fig. 1 Outline of the ipGT approach. The T-DNA is stably integrated into the plant genome and contains four elements: an egg-cell-specific *Staphylococcus aureus* Cas9 cassette, a gRNA cassette, a HR donor sequence flanked by gRNA recognition sites, and a plant selection marker. Upon expression of the nuclease, three DSBs are induced simultaneously, one in the target locus and two flanking the donor leading to its release. If the cells use the donor for HR-mediated repair, the desired genome modification is achieved

suggest that the egg cell of plants might have a more active HR DNA repair system and can be a valuable cell type to achieve successful GT. This was also confirmed by a recent study showing efficient in planta gene targeting using Cas12a from *Lachnospiraceae bacterium ND2006* (LbCas12a) instead of SaCas9 [15]. In principle, our egg-cell-specific ipGT protocol is not only applicable to the model plant Arabidopsis but can also be utilized in other transformable crop species.

Here, we describe the procedure to perform *Staphylococcus aureus* Cas9-mediated ipGT approach in *Arabidopsis thaliana*, enabling stable heritable genome modifications, including integration of a tag or *cis*-element to a desired locus, or precise sequence modifications such as amino acid substitutions.

2 Materials

2.1 Plasmids

All plasmids are available directly from the authors and full sequence information is available at www.botanik.kit.edu/crispr. Some plasmids have also been deposited at the Arabidopsis Biological Resource Center (ABRC).

2.1.1 pDe-SaCas9-EC

Binary vector for stable plant transformation via *A. tumefaciens*, conferring plant resistance to phosphinothricin (PPT). It contains an egg-cell-specific expression system (*EC1.1/1.2* promoter) for Cas9 from *Staphylococcus aureus*. It is also a Gateway destination vector containing the respective attachment sites flanking a *ccdB* gene for insertion of the gRNA expression cassette. Confers plant

resistance to PPT. The vector's multiple cloning site also contains restriction sites suitable for insertion of the HR donor sequence.

2.1.2 pDe-Sa-Cas9

Identical to pDe-Sa-Cas9-EC with the exception that the Cas9 expression system is constitutive (*PcUBI 4-2* promoter) and confers plant resistance to kanamycin.

2.1.3 pEn-Sa-Chimera

Gateway entry vector designed to take up annealed oligos to create a programmed gRNA. The respective Gateway attachment sites enable transfer of the programmed gRNA into the destination vector.

2.2 Organisms

1. *Escherichia coli*, standard cloning strain for all cloning steps; *ccdB*-resistant strain for propagation of pDe-Cas9 (e.g., DB3.1 [16]; *see* **Note 1**).
2. *A. thaliana* plants, either wild-type (e.g., Col-0) or any transformable mutant.
3. *Agrobacterium tumefaciens*, any conventional transformation strain, e.g., GV3101 [17].

2.3 Reagents

1. Restriction enzyme BbsI, additional restriction enzymes as required (*see* Fig. 1 and **Note 2**).
2. T4 Ligase for conventional cloning steps.
3. Proofreading DNA polymerase for the generation of the donor sequence.
4. A robust Taq polymerase for *E. coli* colony PCRs and for screening of putative GT plants.
5. Gateway LR Clonase II (only available from Thermo Fisher Scientific, supplied with proteinase K).
6. LB medium (for *E. coli*): 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl. Solid media: Add additionally 7.5 g/L agar. For selection media, add ampicillin (100 mg/L) or spectinomycin (100 mg/L).
7. YEB medium (for *A. tumefaciens*): 5 g/L beef extract and 5 g/L peptone. 1 g/L yeast extract, 5 g/L sucrose, 439 mg/L $MgSO_4$, and 7.5 g/L agar for solid media.
8. Germination medium (GM): 4.9 g/L Murashige & Skoog, 10 g/L sucrose, pH 5.7, and 8 g/L agar. For selection conditions, add PPT (6 mg/L) or kanamycin (30 mg/L).
9. TE buffer: 10 mM Tris–HCl and 1 mM EDTA at pH 8.

3 Methods

3.1 Experimental Design Considerations

1. Identify potential target sites for *Staphylococcus aureus* Cas9 in your target locus close to the desired modification containing the required PAM (*see* **Note 4**). Exclude sequences containing a stretch of five or more Ts as this terminates transcription by RNA polymerase III.
2. Design the HR donor sequence containing the desired modification. This is the most crucial step and should be given thorough planning. We recommend to use 600–800 bp homology on both flanks of the cleavage site (*see* **Note 5**). For excision of the donor sequence, add the gRNA target sequence including the PAM to both ends of the donor sequence (*see* **Note 6**). To exclude Cas9 activity on the donor, introduce silent mutations at the corresponding target site on the donor (*see* **Note 7**). In order to facilitate the later screening procedure, your modification or silent mutations should allow distinction between WT and GT events by PCR (*see* **Note 8**). Finally, suitable restriction sites on the very end of the HR donor are required for insertion of the HR donor into your T-DNA.
3. Check the feasibility of generating your HR donor sequence. Possible methods to generate the HR donor are overlap extension PCR, Gibson assembly, or gene synthesis (*see* **Note 9**).

3.2 Generating the Required T-DNAs

1. Order the required oligonucleotides for your target sequence. For SaCas9, the fw oligo should contain 20–21 nt upstream of the PAM with ATTG added at the 5′ end. The second oligo should contain the reverse complement of the target sequence with AAAC added at the 5′ end.
2. Dilute and mix your oligos in ddH_2O to a final concentration of 2 pmol/μL for each oligo in a total volume of 50 μL. Incubate for 5 min at 95 °C and put at room temperature for an additional 10 min for annealing.
3. Digest pEn-Sa-Chimera to completion with BbsI. Extract the 3 kb band by gel purification (*see* **Note 10**) and dilute the final concentration to 5 ng/μL.
4. Set up a ligation reaction containing 2 μL digested vector, 3 μL annealed oligos, 1 μL T4 ligase buffer, 1 μL T4 ligase, and 3 μL ddH_2O, and incubate as recommended by the vendor. Transform into *E. coli* and select for colonies on ampicillin-containing LB plates. Colonies can be screened for insert by colony PCR using your fw oligo and M13-rev as primers.
5. Purify plasmids from a small number (2–4) of correct clones and validate by sequencing with primer SS42.

6. Transfer the correct gRNA expression cassette to both pDe-Sa-Cas9 and pDe-Sa-Cas9-EC by Gateway cloning. pDe-Sa-Cas9-EC is for the actual GT construct. pDe-Sa-Cas9 is only required to check the cleavage activity at the chosen target sequence. Set up a reaction with 100 ng entry vector, 300 ng destination vector, 4 μL TE buffer, and 1 μL LR Clonase II in a total volume of 10 μL, and incubate for 2–3 h at room temperature. Before transformation into *E. coli*, stop the reaction by adding 1 μL proteinase K and incubation at 37 °C for 10 min. Select on LB plates containing spectinomycin. Screening for correct insert can be achieved by colony PCR with primers SS42/SS102 (for both Gateway reactions).
7. Isolate plasmids from 2–4 PCR-positive colonies each. A control restriction digestion is recommended to check for the integrity of the plasmid. For Gateway reactions involving pDe-Sa-Cas9, the enzymes AflII and NheI can be used generating bands at approximately 5.9, 5.0, and 3.8 kb. For Gateway reactions involving pDe-Sa-Cas9-EC, the enzymes HindIII and NheI can be used generating bands at approximately 5.9, 3.3, 1.9, 1.8, and 1.2 kb.
8. Add your GT donor sequence to the plasmid obtained after insertion of the gRNA cassette into pDe-Sa-Cas9-EC using the restriction sites included on the donor sequence. Identify correct clones by a suitable colony PCR and restriction digestion depending on your donor sequence (*see* **Note 10**).
9. Verify the correctness of both of your final constructs by full sequencing of the Cas9 cassette, gRNA cassette, and donor. The final constructs are the GT construct (pDe-Sa-Cas9-EC + gRNA+donor) and the construct to check cleavage efficiency (pDe-Sa-Cas9 + gRNA).
10. Transform your final verified plasmids into *A. thaliana* (e.g., [18]).

3.3 Obtaining Heritable GT Plants (See also Fig. 2)

1. To generate primary transformants, sow seeds of the transformation on germination medium containing PPT for your GT construct and kanamycin for your construct to check cleavage efficiency (*see* **Note 12**).
2. For the GT construct, pick at least 40 T1 plants for further cultivation in soil (*see* **Note 13**). Extract DNA from a leaf and verify the presence of the correct construct by PCR.
3. To check for cleavage activity at your chosen target site, extract DNA from 20 primary transformants carrying the cleavage efficiency construct after 3 weeks. Analyze mutagenesis efficiency by, e.g., TIDE, RE assay, T7E1, HRM, or NGS (described in detail elsewhere: [19, 20]).

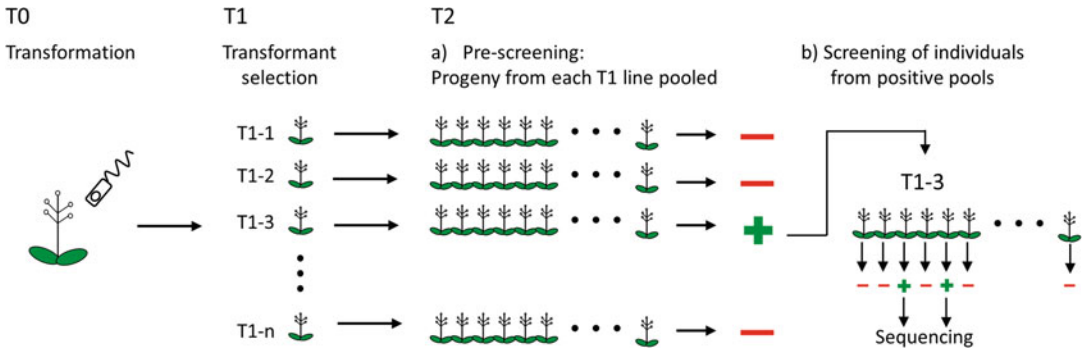

Fig. 2 Overview of the two-step screening procedure to identify GT-positive plants

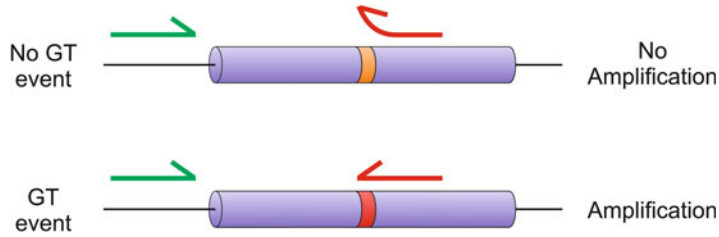

Fig. 3 PCR-based identification of GT events. One primer should be placed outside the homologous sequence region, the other primer inside the homology in such a way that the specific sequence modification is recognized (*see* also **Note 14**)

4. Harvest the seeds for each of your T1 plants carrying the GT construct separately, and sow a small amount of seeds (~100 per line) on GM medium.
5. After 14 days of growth, isolate DNA from leaf material of 100 plants per line as a pool (one big leaf per plant). Screen for a positive GT signal via PCR (*see* Fig. 3 and **Note 14**).
6. From those T1 pools identified in **step 5**, isolate DNA from each plant separately after another week of growth. Now, analyze individual T2 plants for heritable GT by PCR. Transfer GT-positive T2 plants to soil and cultivate to maturity.
7. Harvest seeds from each GT-positive T2 line separately and screen for loss of the T-DNA in the T3 generation. Confirm successful GT again to assure stable inheritance and screen for loss of the transgene.

4 Notes

1. All cloning steps require a conventional *E. coli* strain, e.g., DH5α. The *ccdB*-resistant strain is only required to propagate pDe-CAS9 before removing the *ccdB* gene in a gateway reaction.

2. Best choose a restriction site in the multiple cloning site, and take care that the restriction site is not present in your HR donor sequence.
3. We routinely use Q5 DNA polymerase (NEB) for proofreading purposes and DreamTaq polymerase (Thermo Fisher Scientific) for colony and plant screening PCRs.
4. We highly recommend to use NNGRRT as PAM of SaCas9, even though NNGRRV can also be cleaved. Furthermore, avoid target sites containing too low GC content. There are many software tools available to aid in target choice, reviewed in detail in [21].
5. The actual size can vary greatly depending on your experiment. Generally, longer homologies (0.8–1 kb) should improve HR frequencies. However, if the homology contains parts of a promoter region, one has to be aware of potential expression from the T-DNA itself.
6. Using a vector set which is capable of expressing more than one sgRNA [8], it is also possible to have different Cas9 target sites for the target locus and for releasing the donor sequence.
7. A silent mutation disrupting the PAM is ideal. Consult Ran et al. [22] which PAMs are still cleavable by *Sa*Cas9. If disrupting the PAM is not possible, two to three silent mutations in the seed region are sufficient. If you require a confirmation of your GT events via Southern blot, include a restriction site in the silent mutations that is not present in the genomic sequence or vice versa.
8. Three mismatches immediately at the 3′end should be sufficient to prevent amplification from WT alleles.
9. Having the donor sequence synthesized is the easiest, yet most expensive, method. However, overlap PCR or Gibson assembly may be challenging and time-consuming due to reoccurring sequence elements flanking the construct.
10. To save time and effort, it is also possible to purify the BbsI digest by a PCR purification kit, as the excised fragment is very small. However, cloning efficiency is higher using gel purification, because all of the unwanted fragments are removed.
11. The orientation of the donor construct within the T-DNA is irrelevant. Thus, a single restriction enzyme can be used for this cloning step. However, this requires dephosphorylation which is not required when two enzymes are used.

12. When floral dipping is used to transform Arabidopsis, cefotaxime should be added to the germination medium to limit *Agrobacterium* growth.
13. All numbers given in this paragraph are based on experience. It might be necessary to scale them up to detect a GT event.
14. Proper functionality of the PCR assay is crucial. Thus, optimization of primers and PCR conditions will be necessary. For details on primer design and PCR conditions, *see* also [23]. Note that when analyzing the pools, the GT signal is strongly diluted so a large number of cycles (at least 40) is required.

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Chapter 3

Rice Gene Knockout or Downregulation through CRISPR-Cas9

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Abstract

Precise genome modification via clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein (Cas)9 in rice plants, is a powerful tool for improving abiotic and biotic stress tolerance and increasing yield in rice. Valuable agronomical traits and functional study of a novel gene can be obtained by targeting single or multiple mutations in the rice genome. Here we describe the sgRNA design, cloning, and assembly of CRISPR constructs for specific gene knockout and the variant analysis in the rice plant.

Key words CRISPR-Cas9, sgRNA design, Knockout, Mutagenesis, Rice

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
Cas9	CRISPR-associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
dATP	Deoxyadenosine triphosphate
DNA	Deoxyribonucleic acid
GMO	Genetically modified organism
HPT	Hygromycin phosphotransferase gene
IE	Immature embryo
Indels	Insertions and deletions
LB	Luria broth
MES	2-(N-Morpholino)ethanesulfonic acid
MS medium	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid.
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
PNK	Polynucleotide kinase
RT	Room temperature
sgRNA	Single-guide ribonucleic acid

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SOC medium	Super optimal broth
T7E1	T7 Endonuclease 1
YEP	Yeast extract peptone dextrose

1 Introduction

In the recent years, genome editing has become an essential molecular biotechnology tool for advancing and improving food crops. Rice is an important staple food and a major food source for over three billion people worldwide [1]. Soon, the demand for higher-yielding rice plants will increase as well as the development of rice cultivars tolerant to different biotic and abiotic stresses due to climate change. Through the application of the genome editing tool clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein (Cas)9, researchers have been able to improve different traits of the rice plants [1, 2]. The system is based on a single-sequence DNA recognition mechanism, which will produce specific genomic double-strand breaks [3]. Precise modification within the genome is one of the significant advantages using CRISPR-Cas9 system, and in some countries, the null segregant containing the mutation has been considered as a breeding product or non-GMO (genetically modified organism) product, reducing the requirement of a complex risk assessment on the safety of the product. It is regarded as one of the breeding methods to produce mutation [4]. Starting in 2013, several articles have been published regarding the specific modifications of the rice genome. These articles have not only discussed the application of CRISPR-Cas9 tools for trait improvement or gene function validation, but also development of improved tools for precise modification. Different tools have also been developed for the investigation of off-targets [5], and a precise sgRNA design that will reduce the presence of off-targets [6, 7], besides if there are any, it can be eliminated through crossing and selection. Successful modification using CRISPR-Cas9 has been shown in several plants species [2]; unfortunately, the delivery of the CRISPR construct is often still challenging. *Agrobacterium*-mediated transformation is the chosen approach for the majority. Different vector systems have been developed to deliver the sgRNA and Cas9 nuclease. Generally, the nuclease and sgRNA are being expressed in different vectors; optionally we can use the "compact" system, which includes all relevant CRISPR components [8–10].

Here we are using different modular vectors (provided by Daniel Voytas Laboratory [10]) to assemble the final CRISPR construct in a "compact system" (Fig. 1) for *Agrobacterium*-mediated transformation into Indica rice.

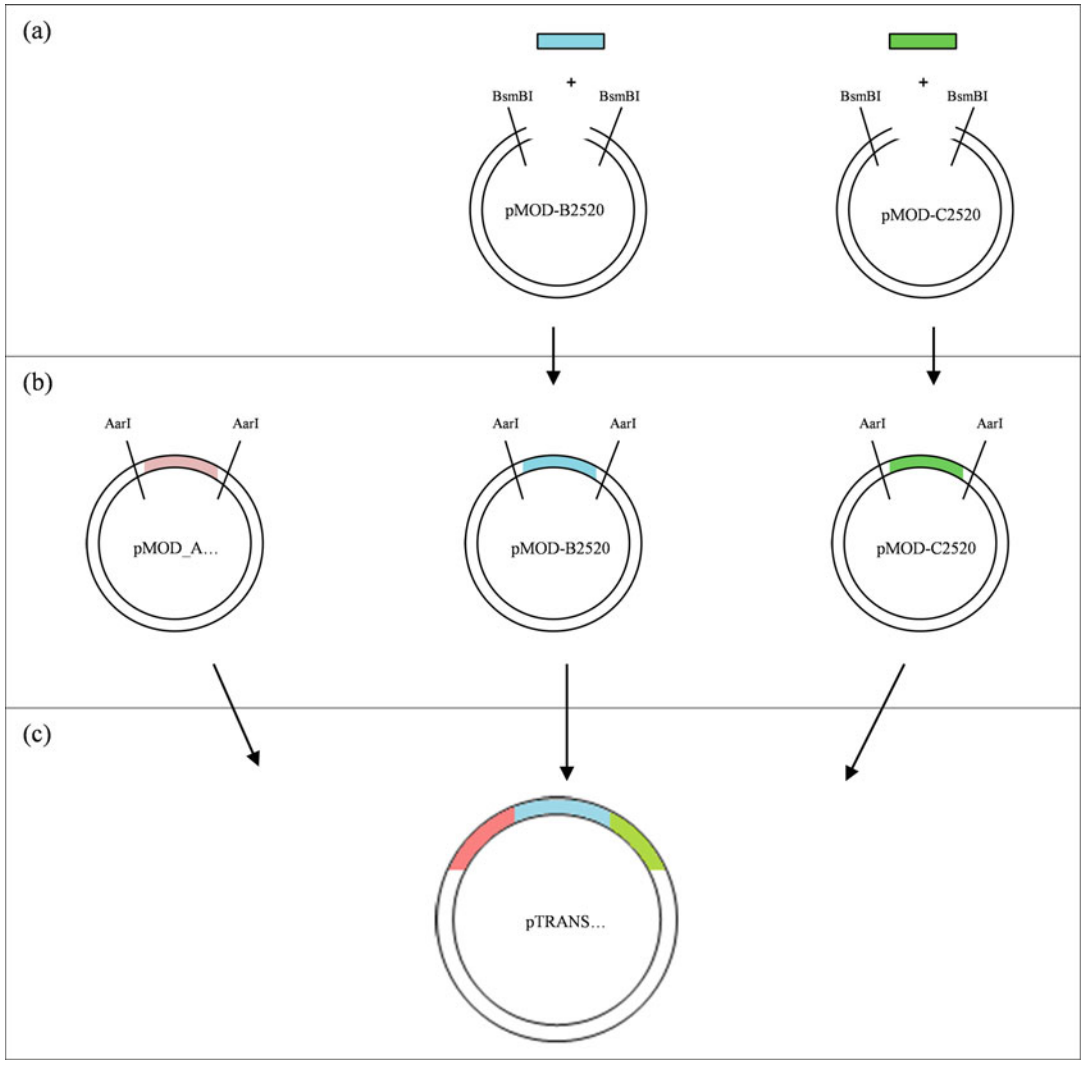

Fig. 1 Schematic overview of cloning CRISPR vector. Cloning the CRISPR vector involves two major steps: (**a**) insertion of sgRNA into the modular vector pMOD_B2520 and/or pMOD_C2520 and (**b**) combining all CRISPR components (nuclease, sgRNA) into destination vector pTRANS. . . via Golden Gate reaction (**c**)

2 Materials

2.1 Web Tools/ Databases

2.1.1 Sequence Information Acquisition

1. MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).
2. Rice Annotation Project Database(https://rapdb.dna.affrc.go.jp/).
3. Gramene (http://www.gramene.org/)when.
4. National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/).

5. *sgRNA design (specifically for rice)*.
6. CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/).
7. CCTop—CRISPR-Cas9 target online predictor (https://crispr.cos.uni-heidelberg.de/index.html).
8. GT-Scan (https://gt-scan.csiro.au/home).
9. CRISPR MultiTargeter (http://www.multicrispr.net/).
10. Deskgen (https://www.deskgen.com/landing/cloud.html).
11. Cas-OFFinder (http://www.rgenome.net/cas-offinder/).

2.1.2 Analysis of PCR Sequencing Results

1. Indigo (https://gear.embl.de/indigo/).
2. Poly Peak Parser (http://yosttools.genetics.utah.edu/PolyPeakParser/).
3. CRISP-ID (http://crispid.gbiomed.kuleuven.be/).

2.2 Chemicals/Reagents/Buffers/Kits

2.2.1 Cloning sgRNA, Construct of CRISPR End Vector (Specific Example Used in GTL Lab, IRRI), Analysis of the Transgenic Plant Material

1. Desired oligonucleotides and PCR primers.
2. T4 ligase buffer.
3. T4 PNK (polynucleotide kinase).
4. T4 ligase.
5. Specific enzymes (EspI or BsmBI, AarI).
6. dATP (25 mM).
7. Plasmid-Safe enzyme.
8. *E. coli* DH5α.
9. SOC medium.
10. LB plates with antibiotic (ampicillin or kanamycin).
11. PCR kit (for screening colonies).
12. Plasmid extraction kit.
13. Vectors (Addgene: nuclease vector [different available: pMOD_A...], sgRNA vector [pMOD_B2520, pMOD_C2520], and destination vector pTC200 [different available: pTRANS...]. Source: Daniel Voytas Laboratory).
14. T7EI assay kit.
15. PCR clean-up kit.

2.2.2 Protoplast Assay

Seeds of desired rice cultivar:

1. Ethanol (75%).
2. Bleach (50%).
3. ½ MS medium
4. Cellulase RS (1.5%).
5. Macerozyme R10 (0.75%).

6. Mannitol (0.6 M).
7. MES (10 mM).
8. CaCl2 (10 mM).
9. BSA (0.1%).
10. W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES, pH 5.7, filter-sterilized).
11. MMG solution (0.4 M mannitol, 15 mM MgCl2, 4 mM MES, pH 5.7, filter-sterilized).
12. Sucrose solution (21%).
13. PEG solution (40% (w/v) PEG4000, 0.2 M mannitol, 0.1 M CaCl2)—freshly prepared.
14. WI solution (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7, filter-sterilized).

2.2.3 Indica Rice Transformation [11]

1. YEP plates (with respective antibiotics).
2. Stock solutions:
   (a) N6 major 1, N6 major 2, N6 major 3, N6 major 4.
   (b) B5 minor 1, B5 minor 2, B5 minor 3, B5 minor 4.
   (c) B5 vitamins.
   (d) AA macro salts.
   (e) AA micro salts.
   (f) AA iron stock = B5 minor 1.
   (g) MS1, MS2, MS3, MS4.
   (h) MS vitamins.
   (i) Yoshida solution [12].
3. Chemicals:
   (a) L-glutamine.
   (b) Aspartic acid.
   (c) Arginine.
   (d) Casamino acid.
   (e) L-proline.
   (f) Sucrose.
   (g) D-glucose monohydrate.
   (h) Mannitol.
   (i) Maltose.
   (j) Sorbitol.
   (k) Agarose type1.
   (l) Gelrite.

4. Growth regulator:
   (a) 2,4-D, NAA, BAP, kinetin
5. Antibiotic:
   (a) Claforan, carbenicillin, hygromycin.

3 Methods

3.1 Vector Design

To create a knockout or knockdown mutation, several systems are known, which can be acquired. These systems consist either of (i) modular vector assembly (focus of this chapter; *see* **Note 1**) or (ii) single-vector approach.

3.1.1 Selection of the CRISPR System

3.1.2 sgRNA Design/ off-Target Identification

1. Acquire the genomic DNA sequence from database.
2. Place the sequence in the web tool to generate single-guide RNAs (*see* **Note 2**).
3. Select sgRNA with highest on-target scores and less off-target hits (*see* **Note 3**).
4. Some web tools for sgRNA design do not display the off-targets. You can check your sgRNA with the web tool Cas-OFFinder.
5. Before ordering, make sure the specific overhang for cloning is adjusted (*see* **Note 4**).

3.1.3 Cloning

1. Perform the phosphorylation and annealing of the sgRNA oligonucleotides with T4 PNK; incubate at 37 °C for 1.5 h (*see* **Note 5**).
2. Add 2 μL 0.5 M NaCl to mixture and place it in boiling water bath for 2 min.
3. Remove the water bath from the heat source and allow it to cool down to RT.
4. Dilute oligo mixture (1:200) with water.
5. Perform Golden Gate reaction to insert the sgRNA oligos into the modular vector pMOD_B2520 or pMOD_C2520 (Fig. 2) (*see* **Note 6**).
6. Add 1 μL 25 mM ATP and 1 μL Plasmid-Safe enzyme; incubate for 1 h at 37 °C.
7. Perform a heat-shock transformation of restriction-ligation mixture into *E. coli* DH5α.
8. PCR and digest screening for positive colonies, followed by Sanger sequencing (*see* **Note 7**).
9. Perform final Golden Gate reaction using module vectors and destination vector to create final construct (*see* **Note 8**).
10. Add 1 μL 25 mM ATP and 1 μL Plasmid-Safe enzyme; incubate for 1 h at 37 °C.

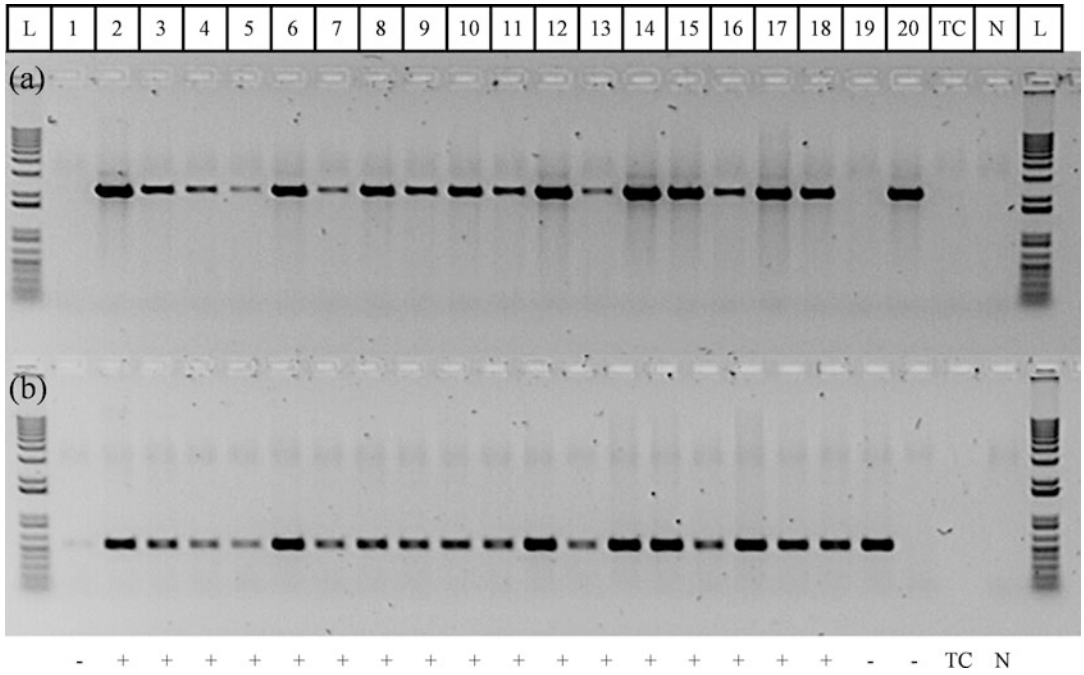

Fig. 2 PCR screening for nuclease and biomarker. Pre-screening of 20 plantlets for the presence of PCR amplicon of Cas9 (**a**) and HPT (**b**) genes. Only plants displaying both bands are kept and analyzed further. Abbreviations: *TC* tissue culture control, *N* negative control, *L* Ladder; "+," positive; "−," negative

11. Transform final Golden Gate mixture into *E. coli* DH5α.
12. PCR screening for positive colonies, followed by Sanger sequencing to verify correct assembly of the CRISPR construct (*see* **Note 9**).

3.2 Functionality Test of sgRNA

*3.2.1 Protoplast Isolation [13, 14] (See **Note 10**)*

1. Sterilize rice seeds with 75% ethanol, followed by 50% bleach for 30 min.
2. Dry seeds and place on ½ MS medium for 7–10 days at 28 °C.
3. Collect tissue from stem and sheath for seedling and cut in 0.5 mm small strips.
4. Place small leaf and stem pieces in freshly prepared enzyme solution and incubate for 4–5 h at 37 °C at 80 rpm (*see* **Note 11**).
5. After incubation add equal amount of W5 solution and shake well.
6. Filter protoplast through nylon mesh (101 and 40 μm) into falcon tube and wash with W5 solution.
7. Centrifuge with swinging buckets and collect protoplast pellet (*see* **Note 12**).
8. Remove supernatant and add MMG solution.

9. Load the MMG-protoplast solution on top of 21% sucrose solution and centrifuge for 10 min (*see* **Note 13**).
10. Collect the intact protoplast and wash with 7 mL MMG solution (*see* **Note 14**).
11. After centrifugation, discard supernatant, and resuspend protoplast pellet with MMG solution for counting, using hemocytometer (*see* **Note 15**).

Adjust concentration to 2×10^6 protoplast/mL for transformation.

3.2.2 Protoplast Transformation

1. Prepare 5–10 μg pDNA for protoplast transformation.
2. Mix 100 μL protoplast with pDNA in 14 mL falcon tube (*see* **Note 16**).
3. Add 110 μL freshly prepared PEG solution and incubate for 20–30 min at RT (*see* **Note 17**).
4. Wash mixture with 440 μL W5 solution, by gently swirling it.
5. Centrifuge and resuspend pellet with 1 mL WI solution (*see* **Note 18**).
6. Incubate at 26 °C (darkness) for 16 h.

3.2.3 PCR-Enzyme Assay

1. Collect protoplast for genomic DNA extraction [15] or perform direct PCR on pellet (*see* **Note 19**).
2. Amplify target region via PCR and run an agarose gel to confirm single band.
3. Screen PCR product for mutation, either using T7E1 assay or via restriction enzyme digest, if you target disrupt recognition site of enzyme (*see* **Note 20**).
4. Check digest on agarose gel for positive result.

3.3 Indica Rice Transformation ([11], See Note 21)

3.3.1 Panicle Harvest, Immature Embryo Isolation, and Sterilization

1. Streak *Agrobacterium* containing CRISPR construct 3 days before harvest and isolation of immature embryo (IE).
2. Sterilize dehulled IEs with 70% ethanol followed by bleach treatment for 5 min. Use plenty of water to rinse IEs.

3.3.2 Co-cultivation: Immature Embryo Transformation

1. About 1 h before infection of IEs, scoop one full loop of *Agrobacterium* culture, and resuspend it in A200 solution (B5 vitamins, AA macro salts stock, AA micro salts stock, AA iron stock, glycine, L-glutamine, aspartic acid, arginine, casamino acid, sucrose, and D-glucose monohydrate). Adjust bacterial density to OD0.3 and incubate suspension at 25 °C for 1 h in darkness.

2. Place IEs on A201 media plates (N6 major 1–4, B5 minor 1–4, B5 vitamins, casamino acid, L-proline, sucrose, D-glucose monohydrate, agarose type 1, 2,4-D, NAA, and BAP). Add 5 μL of *Agrobacterium* solution to each IE and incubate at 25 °C for 7 days in darkness.

3.3.3 Resting and Selection

1. Remove elongated shoot and transfer dried IEs to resting medium A202 (N6 major 1–4, B5 minor 1–4, B5 vitamins, L-glutamine, casamino acid, L-proline, mannitol, maltose, gelrite, 2,4-D, NAA, BAP, claforan, and carbenicillin). Incubate at 30 °C for 5 days.
2. Transfer IEs onto A203 selection medium (N6 major 1–4, B5 minor 1–4, B5 vitamins, L-glutamine, casamino acid, L-proline, mannitol, maltose, 2,4-D, NAA, BAP, claforan, carbenicillin, and hygromycin), and incubate at 30 °C for 10 days.
3. Second selection: transfer IEs onto fresh A203 and incubate at 30 °C for 10 days.
4. Transfer embryogenic callus to fresh A203 and incubate at 30 °C for 10 days.

3.3.4 Plant Regeneration

1. Transfer resistant calli to A204 pre-regeneration medium (B5 minor 1, MS1–4, MS vitamins, maltose, sorbitol, agarose type 1, NAA, kinetin, claforan, and hygromycin), and incubate at 30 °C for 10 days.
2. Transfer proliferating calli to A205 regeneration medium (B5 minor 1, MS1–4, MS vitamins, sucrose, gelrite, NAA, kinetin, claforan, and hygromycin).
3. Take one plantlet from each callus and transfer it into MSO rooting medium (B5 minor 1, MS1–4, MS vitamins, sucrose, and gelrite). Incubate plantlet at 25 °C for 14 days.
4. Transfer plants into trays containing Yoshida culture solution [12] and grow them for 2 weeks in growth chamber.
5. Transfer plants to pots.

3.4 Plant Analysis

3.4.1 Screening for Biomarker and Nuclease

1. Collect leaf sample for genomic DNA [15] extraction or for direct leaf PCR of transgenic plantlets.
2. For validation of positive transformation, perform PCR for selective biomarker and nuclease (*see* **Note 22**).
3. Plants displaying both bands will be used for further analysis (Fig. 2).

3.4.2 Screening and Validation of Variation in Genomic Region of Interest

1. Evaluate preselected plants for variation in target region.
2. The specific region of interest will be amplified via PCR with genomic region-specific primers.

3. PCR product will be further analyzed via T7E1 assay, for the detection of INDELS (*see* **Note 23**).
4. Plants revealing negative results will be discarded.
5. PCR product or a sequencing vector containing target region will be sent for sequencing to analyze mutation variation.
6. Sequencing results can be analyzed using different web tools like CRISP-ID or alignment programs like Clustal Omega or MEGA6 (Fig. 3).

3.4.3 Off-Target Analysis

1. In parallel to designing the sgRNA for cutting the target site, the web tools will also display potential off-targets.
2. Design specific primers to amplify all potential off-target sites.
3. Perform PCR and send its product for sequencing.
4. Results can be analyzed with sequence alignment programs, to check for variation within the off-target region.

4 Notes

1. At Genetic Transformation Laboratory, IRRI, we are using the modular vectors provided by Daniel Voytas Laboratory, which are also available at Addgene.
2. Keep in mind not all single-guide RNA design tools provide sequence information for Indica, since the design is mostly based on Japonica reference genome. Try to add/modify the required parameters based on your vector system like sgRNA length or PAM type.
3. Use different sgRNA design tools and compare the output. The generated guides will differ, as well as the on- and off-target scores. Choose several sgRNAs for functionality test.
4. By using modular vector, pMOD_B2520- and pMOD_C2520-specific overhangs need to be added for cloning oligos into the modular vector. Oligonucleotide structure is the following (Sequence 5′-3′):

   Oligo I: ACTT (G) NNNNNNNNNNNNNNNNNNN G.

   Oligo II: AAAA C NNNNNNNNNNNNNNNNNNN (C).

   Additional G (indicated in brackets) is required, if guide RNA does not start with G.

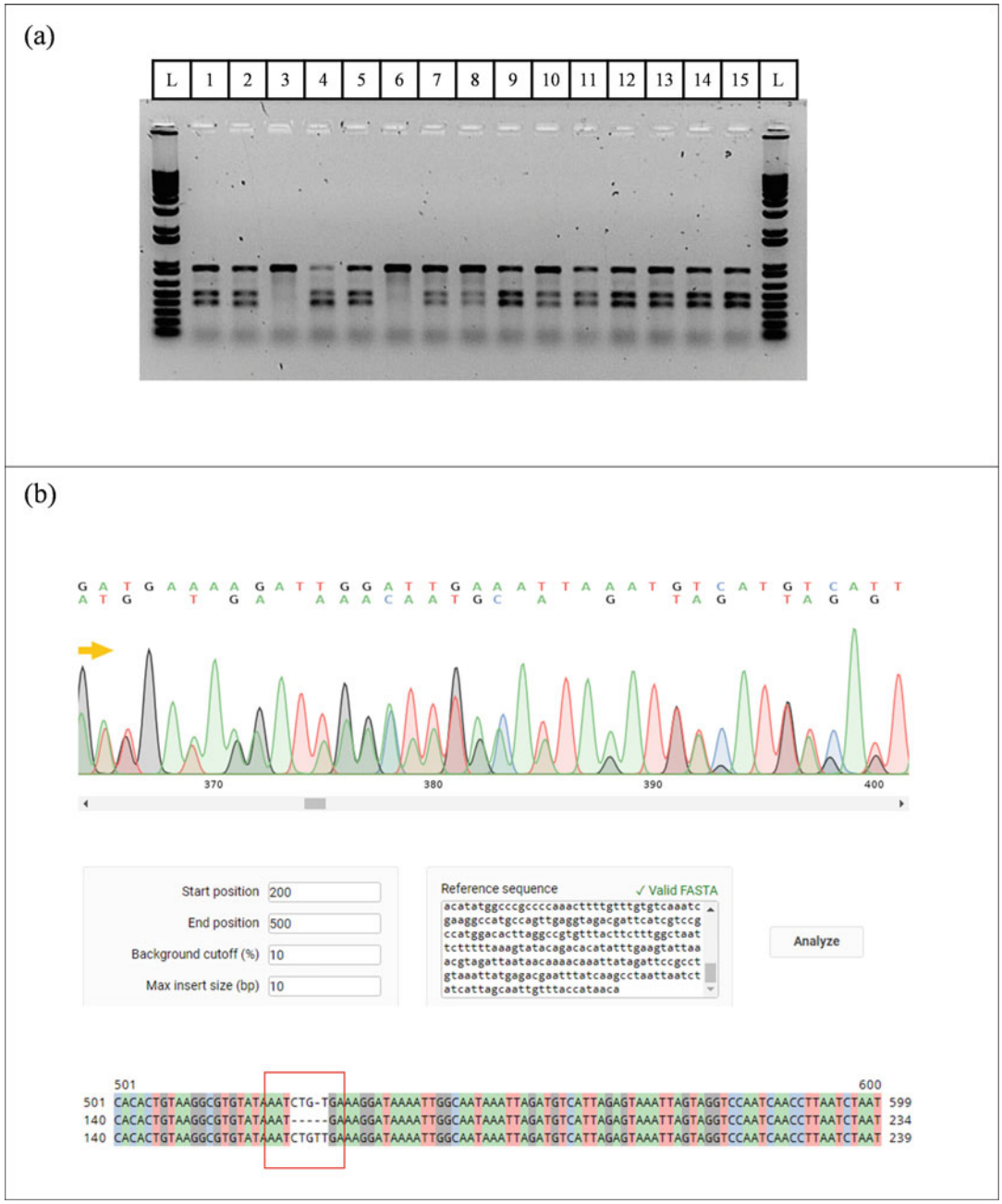

Fig. 3 T7E1 assay. Amplified PCR product was digested using T7 Endonuclease I and run on a 2% gel. Positive samples display two extra bands after digest (**a**), and negative only show one band (samples 3 and 6). Further sequencing analysis is required to determine the genomic modification. Samples (PCR product) were sent for sequencing and analyzed via CRISP-ID (**b**) to identify the variation within the target region, framed in red. Abbreviation: *L* ladder

5. Set up for annealing and phosphorylation step.

Amount [μL]	Component
1.5	100 μM sense oligo
1.5	100 μM antisense oligo
1.5	10× T4 ligase buffer
1	T4 PNK
9.5	Water

Incubate mixture for 1.5 h at 37 °C in the water bath.

6. Golden Gate Reaction to insert sgRNA in modular vector

Amount [μL]	Component
X	150 ng modular vector
2	1:200 diluted sgRNA oligos
1	BsmBI
1	T4 ligase
2	10× T4 DNA ligase buffer
X	Water

Mix all components into a total volume of 20 μL and place in the thermocycler.

Thermocycler protocol

Temperature [C]	Time [min]
37	10
16	15
37	15
80	5
12	∞

7. Perform colony PCR with specific primers, amplifying sgRNA insertion region. Following amplification digest PCR product with BsmBI restriction enzyme to identify positive colonies. A single band after digest indicates sgRNA was inserted, and double band means negative result. Send positive colonies for sequencing to confirm correct insertion of sgRNA into modular vector.

8. Final Golden Gate reaction to assemble CRISPR construct.

Amount [μL]	Component
X	150 ng destination vector (pTRANS…)
X	150 ng of each modular vector
1	AarI
0.4	AarI oligos
1	T4 ligase
2	10× T4 DNA ligase buffer
X	Water

Mix all components into a total volume of 20 μL and place in thermocycler.

Thermocycler protocol

Temperature [C]	Time [min]
37	10
16	15
37	15
80	5
12	∞

9. Perform colony PCR with specific primers, amplifying the region covering nuclease to sgRNA and sgRNA to destination vector. Positive colonies displaying band will be sent for sequencing to verify proper assembly of all modular vector elements.
10. *General note*: It is best to use cut tips or Pasteur pipettes for handling protoplast to avoid damage.
11. Enzyme solution for protoplast isolation.

Components
1.5% cellulase RS
0.75% macerozyme R10
0.6 M mannitol
10 mM MES
10 mM $CaCl_2$
0.1% BSA

Prepare enzyme solution fresh. Mix first four components together and adjust pH to 5.7. Then heat the mixture for 10 min at 55 °C in a water bath. Let it cool down to room temperature (RT) and add $CaCl_2$ and BSA. Filter the mixture with 0.45 um pore membrane.

12. Centrifuge protoplast in swinging bucket at 800 rpm for 5 min. Make sure deceleration of centrifuge is set on "low" or "off": otherwise, the protoplast pellet will be disturbed.
13. Sucrose solution acts as gradient; expect three different phases will appear (intact protoplast, destroyed protoplast, and other plant leaf materials). Centrifuge at 600 rpm for 10 min, setting for deceleration on "low" or "off."
14. The middle phase contains the intact protoplasts.
15. Centrifuge at 800 rpm for 5 min, deceleration setting in "low" or "off." Discard supernatant and resuspend pellet starting with 1 mL MMG solution. Use 20 μL of protoplast suspension for counting, using a hemocytometer.
16. Use 10–15 μg plasmid DNA for single transformation; for co-transformation use at least 10 ug per plasmid.
17. Use freshly prepared PEG solution. After placing 100 μL protoplast solution into 14 mL falcon tube, add pDNA right away followed by PEG solution. Add PEG slowly; let it roll down the falcon wall toward protoplasts. Mix immediately by swirling the tube, and do not vortex.
18. Centrifuge at 800 rpm for 3 min, deceleration setting on "low" or "off."
19. Extract genomic DNA from protoplast described by Collard et al. (2007) or perform direct PCR. Centrifuge protoplast suspension and discard supernatant before using pellet in direct-PCR approach. Use primers specifically designed for amplifying target region.
20. After verifying a single band in agarose gel, clean up the PCR product or gel band via commercially available kits (Zymoclean, Invitrogen, or Promega). Perform T7E1 assay:
    (a) Hybridization:

Amount [μL]	Component
X	200 ng PCR product
1	NEB2 buffer
X	Water

Mix into total volume of 20 μL and place in the thermocycler.

Thermocycler protocol

Temperature [C]	Time [min]
95	5
95	5
85	5
25	5
4	∞

(b) Digest:

Add 1 μL of T7E1 enzyme (1 U) and incubate at 37 °C for 1 h. Following the digestion add 1 μL 100 mM EDTA to stop the reaction.

Perform restriction enzyme digest, if your target destroyed a specific enzyme cutting site. Digest your PCR product with the relevant specific enzyme and check gel image for a positive result.

21. Indica immature rice embryo transformation only is described in short version; for detailed information refer to a publication by Slamet-Loedin et al. (2014) [11].
22. Perform PCR with specific primers designed for your selectable biomarker (mostly HPT) and nuclease (Cas9). Better results were obtained by using genomic DNA for screening.
23. Perform PCR for amplifying target region; same primers can be used as for screening functionality in protoplast assay. Same protocol can be applied for T7E1 assay (*see* **Note 20**).

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Chapter 4

CRISPR-Cas-Mediated Single Base Editing at More than One Locus in Rice Genome

Kutubuddin A. Molla and Yinong Yang

Abstract

Base editing is a recently developed technology that enables us to install point mutation in the genome precisely. The fusion of nucleotide deaminase with Cas9 nickase gives rise to base editors. Like the conventional CRISPR-Cas9 technology, a single-guide RNA can direct a base editor to a particular locus to change one nucleotide to another. In this chapter, we describe a stepwise protocol to perform targeted base editing experiments in rice plants.

Key words CRISPR-Cas9, Base editing, Point mutation, Rice genome editing, Adenine base editors, Crop improvement

Abbreviations

ABE	Adenine base editor
BE	Base editor
CBE	Cytosine base editor
D10A	Aspartic acid 10 alanine
HDR	Homology-directed repair
nCas9	Nickase Cas9
NHEJ	Nonhomologous end joining
PAM	Protospacer adjacent motif
TadA	tRNA adenosine deaminase

1 Introduction

The recent emergence of genome-editing technologies has revolutionized basic biological studies and practical biotechnological applications. Among the major genome-editing tools such as zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic

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repeats (CRISPR)-CRISPR-associated nuclease (Cas), CRISPR-Cas-mediated genome-editing technique has become the method of choice for most of the laboratories due to its simplicity, affordability, and high success rate. Although various laboratories around the world have rapidly adopted conventional CRISPR-Cas9-mediated approaches for targeted mutation and gene knockout via the nonhomologous end-joining (NHEJ) repair, precise genome editing with CRISPR-Cas9 remains challenging to achieve [1]. Typically, precise changes in the genome require the use of homology-directed repair (HDR), which involves the supply of an exogenous donor template with the desired changes. However, the efficiency of HDR-mediated genome editing is extremely low in higher plants due to the difficulties in delivering adequate repair templates and the low rate of homologous recombination. Moreover, HDR occurs only during S and G2 phase of the cell cycle.

Base editing is a recently developed CRISPR-Cas-mediated technology that installs precise point mutation in the genome without the need of a donor template [2]. Generally, base editing offers a much higher efficiency of single nucleotide editing than that of HDR. In this system, Cas9 nickase (D10A), which generates a nick in the target strand, is fused with a nucleotide deaminase protein to create a base editor (BE). Since BE creates a nick instead of a double-strand break (caused by wild-type Cas9) in the genome, it usually produces a very low amount of random indel mutation. There are two major types of BE developed, cytosine base editor (CBE) [3, 4] and adenine base editor (ABE) [5]. Cytidine deaminase and artificially evolved DNA adenine deaminase have been fused to nCas9 for developing CBE and ABE, respectively. CBE can install C → T/G → A mutation, whereas ABE can install A → G/T → C alteration in the genome (Fig. 1a). In our laboratory, we recently demonstrated successful alteration of two functionally important single nucleotides in two rice genes using adenine base editor [6]. Figure 1b depicts how CBE and ABE install transition point mutations in the genome.

In this chapter, we describe the stepwise protocol and minute details for conducting multiplex adenine base editing experiments in rice plants.

2 Materials

2.1 Plasmid Vectors

pKABE7.10, pKABE7.9, pKABE6.3, binary vectors for adenine base editing (Fig. 2) (*see* **Note 1**: choice of vector).

pGEM-T Easy (Promega).

pGTR: A vector to assemble multiple sgRNAs (Addgene plasmid #63143).

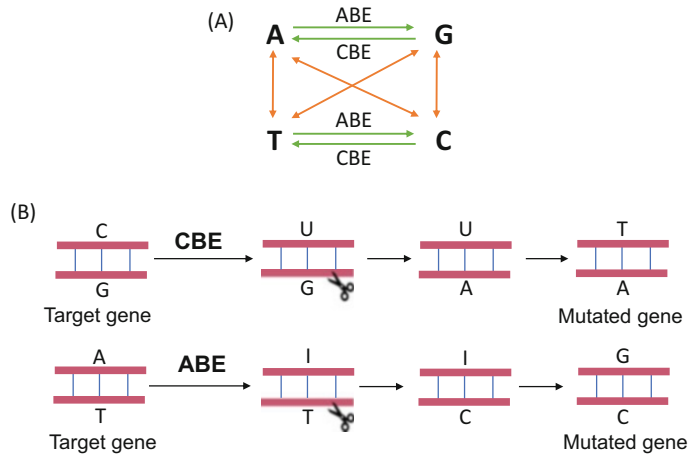

Fig. 1 (**a**) Green arrows depict the mutation types (transition) feasible with base editing. Mutation types (transversion) shown in orange arrows cannot be precisely obtained with base editors. (**b**) Schematic diagram showing how base editors (CBE and ABE) install point mutation in the genome. Scissor depicts nick in the non-edited DNA strand. nCas9 (D10A) causes nick in the target strand (complementary to the guide RNA). The nick directs cellular repair system to remove the mismatched nucleotide from the nicked strand. *CBE,* cytosine base editor; *ABE,* adenine base editor; *A,* adenine; *G,* guanine; *C,* cytosine; *T,* thymine;*I,* inosine; *U,* uracil

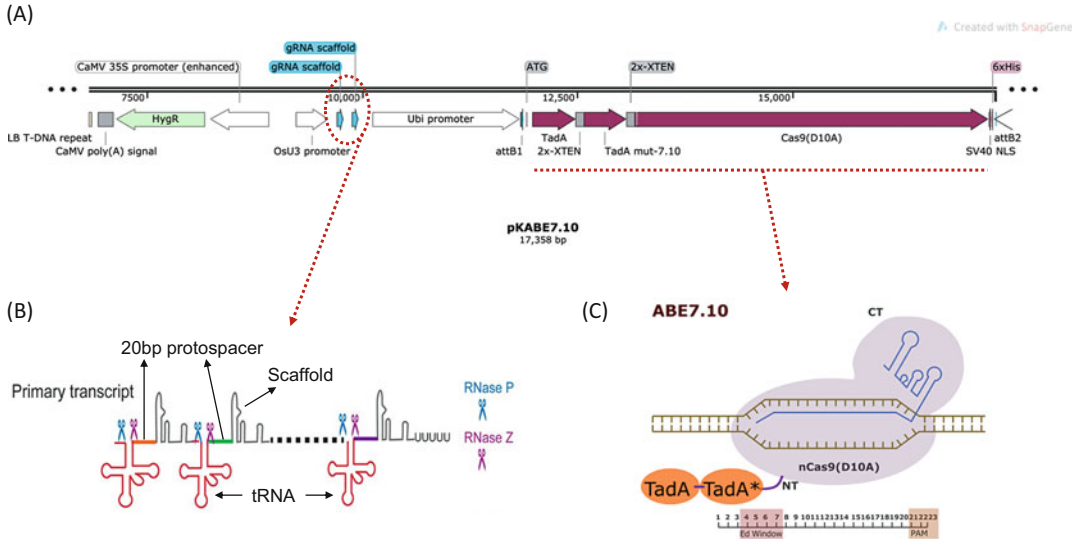

Fig. 2 (**a**) Schematic diagram of the vector pKABE7.10 (plant adenine base editor). (**b**) Primary transcript of the polycistronic tRNA-gRNA gene. PTG contains tandem array of tRNA-gRNAs. Presence of tRNA increases transcription rate from pol-III promoter (OsU3). Endogenous RNaseP and RNaseZ cleave the tRNAs and release each individual gRNA. Figure 2 was partly adapted and modified from Xie et al. [7]. (**c**) Schematic depiction of adenine base editor. Lower panel shows the highlighted editing window (Ed Window) and PAM (21–23) sequence. Protospacer, 1–20 base sequences. *ABE,* adenine base editor; *nCas9(D10A),* Cas9 nickase; *TadA*, *E.coli* tRNA adenine deaminase; *TadA*∗, artificially evolved TadA for acting on DNA; *NT,* N-terminus; *CT* carboxy-terminus

2.2 Reagents

2.2.1 Vector Construction and Detection of Editing

1. Oligonucleotide primers: *see* designing of primer section in Subheading 3.2.
2. DNase/RNase free molecular grade water.
3. dNTPs.
4. Agarose.
5. 6× DNA gel loading dye.
6. DNA ladder.
7. PCR purification kit.
8. Gel extraction kit.
9. Nanodrop/UV-spectrophotometer.
10. Enzymes: Phusion high-fidelity DNA polymerase (Thermo), Taq DNA polymerase (Promega), T7 DNA ligase (NEB), T4 DNA ligase (NEB), bovine serum albumin (NEB), BsaI (NEB), FokI(NEB).
11. 5× Green GoTaq reaction buffer (Promega).
12. Competent cells: *E. coli* Top10, *Agrobacterium tumefaciens* EHA105.
13. Electroporator.
14. Dry or water bath for 42 °C.
15. Luria-Bertani broth and solid media.
16. Antibiotic: kanamycin, ampicillin.

2.2.2 Rice Transformation

1. Mature rice seeds.
2. Commercial bleach.
3. 70% ethanol.
4. Tween-20.
5. Sterile water.
6. Sterile blotting paper.
7. Media:

Media	Components	Stock	Required quantity for 1 L	pH and note
Callus induction media (CIM)	N6 basal salt 2,4-D Modified vitamin mix Sucrose Phytagel	– 2 mg/mL 1000× – –	3.98 gm 1 mL 1 mL 30 gm 3 gm	pH—5.8. Add agar after adjusting pH
Co-cultivation media	CIM Acetosyringone	 1 M	1 L 100 μL	pH—5.8. After autoclaving and cooling to 55 °C, add acetosyringone

(continued)

Media	Components	Stock	Required quantity for 1 L	pH and note
Selection media	CIM	–	1 L	pH—5.8. After autoclaving and cooling to 55 °C, add timentin and hygromycin
	Timentin	400 mg/mL	1 mL	
	Hygromycin	50 mg/mL	1 mL	
Regeneration media	N6 basal salt	–	3.98 gm	pH—5.8. After autoclaving and cooling to 55 °C, add timentin
	Modified vitamin mix	1000×	1 mL	
	BAP	3 mg/mL	1 mL	
	NAA	0.2 mg/mL	1 mL	
	Casamino acid	–	2 gm	
	Sucrose	–	30 gm	
	Sorbitol	–	30 gm	
	Phytagel	–	4 gm	
	Timentin	400 mg/mL	0.5 mL	
Rooting media	MS basal medium with vitamins	–	4.44 gm	pH—5.8. After autoclaving and cooling to 55 °C, add hygromycin
	Sucrose	–	30 gm	
	Agar	–	8 gm	
	Hygromycin	50 mg/mL	1 mL	

3 Methods

3.1 Selection of Targets

A target base can be edited using ABE only when the base lies in a small activity window. Typically, ABE activity window ranges from fourth base to the tenth base of the protospacer sequence (PAM is counted as 21–23) (Fig. 2c). *See* **Note 1** for activity windows of different ABE variants. If NGG PAM is not present at a suitable distance from the target base, one has to opt for BEs which recognize alternative PAM (*see* Molla and Yang [2] about existing BEs with various PAM compatibility and BEs with alternative activity windows).

Unlike conventional CRISPR-Cas9 genome editing, the choice of protospacer is much more limited for base editing experiments. If there is more than one PAM found in a desired genomic locus, protospacer should be chosen in such a way that the target base falls in the middle of the activity window.

3.2 Designing of Primers

We use the polycistronic tRNA-gRNA (PTG) system to assemble more than one gRNA [7], in which all gRNAs are driven by a single OsU3 promoter (Fig. 2a). In the PTG system, a glycine tRNA sequence is fused at the upstream of each gRNA (protospacer+scaffold) (Fig. 2b). A schematic representation of the primer designing method is given below. We present here an example of base editing experiment that targets two genomic loci. BsaI overhang is used for each primer.

Protospacer1+PAM:

N_1-N_2-N_3-N_4-N_5-N_6-N_7-N_8-N_9-N_{10}-N_{11}-N_{12}-N_{13}-N_{14}-N_{15}-N_{16}-N_{17}-N_{18}-N_{19}-N_{20}-N-G-G

Protospacer2+PAM:

N_1-N_2-N_3-N_4-N_5-N_6-N_7-N_8-N_9-N_{10}-N_{11}-N_{12}-N_{13}-N_{14}-N_{15}-N_{16}-N_{17}-N_{18}-N_{19}-N_{20}-N-G-G

Primer g1F- 5'-TA-GGTCTC-N- N_9-N_{10}-N_{11}-N_{12}-N_{13}-N_{14}-N_{15}-N_{16}-N_{17}-N_{18}-N_{19}-N_{20}- gttttagagctagaa-3'

Primer g1R- 5'- CG-GGTCTC-A- N_{12}-N_{11}-N_{10}-N_9-N_8-N_7-N_6-N_5-N_4-N_3-N_2-N_1- tgcaccagccggg-3'

Primer g2F-5'-TA-GGTCTC-C- N_9-N_{10}-N_{11}-N_{12}-N_{13}-N_{14}-N_{15}-N_{16}-N_{17}-N_{18}-N_{19}-N_{20}- gttttagagctagaa-3'

Primer g2R- 5'- CG-GGTCTC-A- N_{12}-N_{11}-N_{10}-N_9-N_8-N_7-N_6-N_5-N_4-N_3-N_2-N_1-tgcaccagccggg-3'

The highlighted bases should be complementary of the respective bases.

Additionally, the following four adapter primers are needed to assemble a PTG. These primers do not vary with your targets and protospacers:

L5AD5-F-

CG GGTCTC A GGCA GGATG GGCAGTCTG GGCA ACAAAGCACCAGTGG

L3AD5-R-

TA GGTCTC C AAAC GGATG AGCGACAGC AAAC AAAAAAAAAA GCACCGACTCG

S5AD5-F

CGG GTC TCA GGC AGG ATG GGC AGT CTG GGC A

S3AD5-R

TAG GTC TCC AAA CGG ATG AGC GAC AGC AAA C

3.3 Assembly of Multiple sgRNAs through Golden Gate Assembly

1. Amplification of individual fragments:

Fragment	Primer pairs (F + R)	Amplicon size	Template
1	L5AD5-F + g1R	128 bp	pGTR plasmid
2	g1F + g2R	195 bp	pGTR plasmid
3	g2F + L3AD5-R	138 bp	pGTR plasmid

Set up a PCR reaction as follows:

Stock conc.	Reagent	Volume	PCR program
0.1 ng/μL	pGTR plasmid	1 μL	98 °C—2 min
5×	Phusion HF buffer	10 μL	
10 mM	dNTP	1 μL	98 °C—10 s

(continued)

Stock conc.	Reagent	Volume	PCR program
10 μM	Forward primer	2.5 μL	50 °C—20 s 35 cycles
10 μM	Reverse primer	2.5 μL	72 °C—20 s
2 U/μL	Phusion Taq (thermo)	0.4 μL	72 °C—2.5 min
	H_2O	32.6 μL	4 °C—Hold
Total		50 μL	

2. Check the PCR product in 2% agarose gel by loading 5 μL of each fragment. Run a 50 bp ladder to determine the size accuracy.
3. Purify the PCR fragment with a column (PCR purification kit) if only specific amplification is observed. If any nonspecific band is detected, perform band excision and gel extraction.
4. Measure the concentration using either nanodrop or any other kind of UV-spectrophotometer.
5. Dilute all three PCR fragments to 15 ng/μL with nuclease-free water.
6. Golden Gate (GG) assembly of the three fragments. Set up the GG reaction as follows:

Stock conc.	Reagents	Volume	Cycle
15 ng/μL	Fragment 1	1 μL	
15 ng/μL	Fragment 2	1 μL	
15 ng/μL	Fragment 3	1 μL	37 °C—5 min
2×	T7 DNA ligase buffer	10 μL	20 °C—10 min 45 cycles
10×	Bovine serum albumin	2 μL	
10 U/μL	BsaI	0.5 μL	20 °C—1 h
3000 U/μL	T7 DNA ligase	0.5 μL	10 °C—α
	H_2O	4 μL	
Total		**20 μL**	

7. Dilute the GG reaction product (20 μL) from **step 6** by adding 180 μL of nuclease-free H_2O.
8. Amplify the GG assembled product using S5AD5-F and S3AD5-R primer pair as follows:

Stock conc.	Reagents	Volume	Cycle
	Diluted GG product	1 μL	98 °C—2 min
5×	Green GoTaq buffer (Promega)	10 μL	
10 mM	dNTP	1 μL	98 °C—10 s
10 μM	S5AD5-F	1 μL	50 °C—20 s 25 cycles
10 μM	S3AD5-R	1 μL	72 °C—20 s
5 U/μL	*Taq* DNA pol	1 μL	72 °C—2.5 min
	H_2O	35 μL	4/10 °C—Hold
Total		50 μL	

9. Check the PCR product in 1% agarose gel. Purify the PCR fragment with a column (PCR purification kit) if only specific amplification is observed. If any nonspecific band is detected, perform gel excision and extraction.
10. Clone the purified product from **step 9** into pGEM-T Easy (Promega) or other TA cloning vectors according to the manufacturer's instructions. Perform Sanger sequencing with suitable primers to confirm the sequence of the PTG.

3.4 Cloning of sgRNAs into the Base Editor (BE) Vector

1. Digest pGEM-T-PTG with FokI restriction endonuclease enzyme as follows (*see* **Note 2**):

Stock conc.	Reagents	Volume	Cycle
100 ng/μL	Plasmid DNA (pGEM-T-PTG)	10 μL	Incubate at 37 °C for minimum 1 h
10×	Cut smart buffer (NEB)	2.5 μL	
5 U/μL	FokI	2 μL	
	H_2O	10.5 μL	
Total		25 μL	

2. Digest the base editor vectors (pKABE7.10/pKABE7.9/pKABE6.3) with BsaI as follows:

Stock conc.	Reagents	Volume	Cycle
100 ng/μL	Plasmid DNA (pKABE vector)	10 μL	Incubate at 37 °C for minimum 1 h
10×	Cut smart buffer (NEB)	2.5 μL	
10 U/μL	BsaI	1 μL	
	H_2O	11.5 μL	
Total		25 μL	

3. Electrophorese both the digested products from **steps 1** and **2** in 1% agarose gel. Excise the desired insert (PTG) and vector (pKABE) bands and extract DNA fragments using gel purification kit.
4. Measure the concentration of the purified vector and insert.
5. Ligate the FokI-digested PTG (insert) with the BsaI-digested pKABE (vector) to make the final construct as follows:

Stock conc.	Reagents	Volume	Cycle
10 ng/μL	BsaI-digested pKABE vector (~17 kb)	5 μL	Incubate at 16 °C for overnight
5 ng/μL	FokI-digested PTG (359 bp)	1 μL	
10×	T4 DNA ligase buffer (NEB)	1 μL	
400 U/μL	T4 DNA ligase (NEB)	1 μL	
	H_2O	2 μL	
Total		10 μL	

6. Transform the ligation product to *E. coli* Top10 competent cells, extract plasmid DNAs from individual bacterial clones, and perform Sanger sequencing to verify the final construct for base editing.

3.5 Transformation of Base Editing Constructs to Agrobacterium Cells

Transform the confirmed plasmid to *Agrobacterium tumefaciens* strain LBA4404/EHA105 by either electroporation or freeze-thaw transformation system. Follow the standard protocol for competent cell preparation and transformation.

3.6 Rice Transformation

1. Surface sterilize dehusked rice seeds with 70% ethanol for 90 s by gentle shaking followed by washing with 6% bleach plus one drop of Tween-20 detergent for 20 min. Rinse 3–5 times with sterile water to completely remove any residual bleach.
2. Completely dry the seeds on a sterile paper towel and transfer them to plates containing callus induction media (CIM). Incubate at 28 °C for 10–12 days.
3. Dissect the growing shoots and discard. Separate calli from seeds with scalpel. Subculture the calli for another 10 days in fresh CIM plate.
4. Streak the *Agrobacterium* strain EHA105 containing the base editing construct in a LB solid plate with kanamycin (50 mg/L) and rifampicin (50 mg/L) antibiotic. Incubate for 2 days at 28 °C.
5. Take loop full of the bacterial cells from the plate to make a bacterial suspension in liquid CIM in a test tube (OD_{600} between 0.1 and 0.3). Add acetosyringone to a final concentration of 100 μM and mix well.
6. Incubate calli with the agrobacterial suspension for 20–30 min with occasional shaking.
7. Blot dry calli with sterile filter paper, and transfer them to plates containing co-cultivation medium (CIM + 100 μM acetosyringone). Incubate the plates in the dark for 2 days at 22–25 °C.
8. Transfer calli to a 50 mL tube and wash for 5 min with sterile water. Repeat 3–4 times.
9. Wash with liquid CIM + 200 mg/L timentin for 20 min.

3.7 Selection and Regeneration

1. Blot dry calli, transfer them to hygromycin-containing selection media plates, and incubate at 30 °C with continuous light for 2 weeks. Subculture the calli in fresh selection media for an additional 2 weeks.
2. Transfer hygromycin-resistant and proliferating calli to regeneration media, and incubate for about 4 weeks under continuous light at 30 °C.

3. Transfer the regenerated green shoots to rooting media and incubate under continuous light at 30 °C. Once roots are developed, transfer the plantlets to the soil in pots and keep them in a greenhouse.

3.8 Detection of Base Editing

1. Collect leaf samples from each independent transgenic plant. Isolate genomic DNA and purify them following a previously described protocol [8].
2. Perform PCR amplification with TadA- or nCas9-specific primers to select positive transformants (*see* **Note 3**).
3. Amplify the target loci with specific primers with a typical amplicon size of 300–500 bp.
4. If the target base falls in a restriction enzyme (RE) recognition sequence or the desired base modification creates a RE site, digest the PCR product with the specific enzyme for preliminary screening and detection of successful base editing. For a practical example of how base editing success can be determined by RE digestion, see Molla et al. [6].
5. Perform Sanger sequencing of the amplicons to confirm successful base editing. *See* Fig. 3 for a representative chromatogram showing succeessful editing.

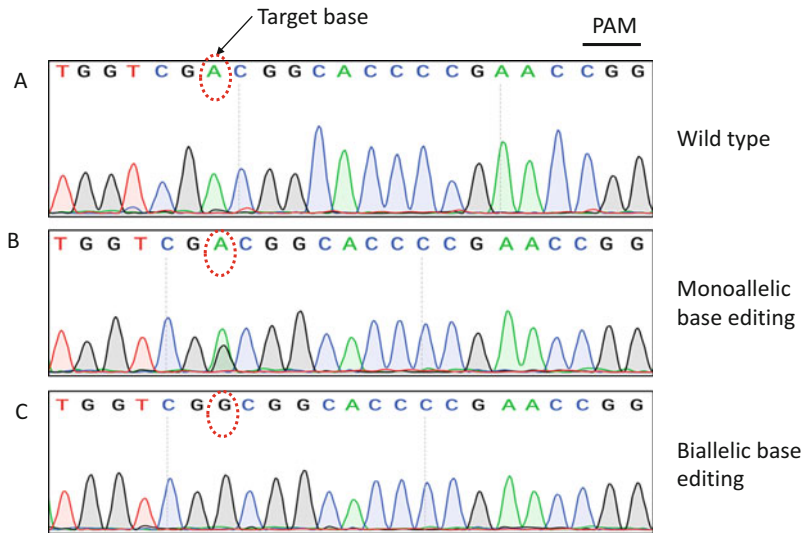

Fig. 3 Sanger sequencing chromatogram showing successful base editing. (**a**) Chromatogram showing the target 20 bp plus PAM DNA sequence from wild-type plant. The seventh "A" nucleotide is the target base and NGG (21–23) is the PAM sequence. (**b**) Chromatogram from a monoallelic mutant plant. Double peak indicates monoallelic mutation. (**c**) Chromatogram from a biallelic mutant plant

3.9 Off-Target Analysis

1. Use CRISPR-plant V2 [9] to select potential off-target sites.
2. Download the sequences from GenBank.
3. Design specific primers and amplify the off-target loci from the DNAs of regenerated plants. Sequence the PCR fragments to determine if there is any off-target base editing.

3.10 Phenotyping

If successful base editing is supposed to cause any morphological or phenotypic alterations or enhances tolerance/susceptibility to any biotic/abiotic stresses, perform subsequent phenotyping or bioassay of the base-edited plants.

4 Notes

1. Base editors prefer a range of bases in the protospacer sequence for its optimal editing activity. pKABE7.10 favors the bases from fourth to eighth position of the protospacer when PAM (NGG) is counted as position 21–23. pKABE7.9 and pKABE6.3 work better when the target base is located beyond eighth position of the protospacer. The activity window of pKABE7.9 and pKABE6.3 ranges from four to ten bases of the protospacer. However, pKABE7.10 is generally recommended when the target base falls at fourth to eighth. In our practical experience, we have seen base editing at the 12th position of the protospacer, though with very low efficiency [6].
2. Alternatively, PCR products (PTG) from **step 9** (subheading 3.3) can directly be digested with FokI enzyme. Follow similar digestion protocol as table in subheading 3.4, **step 1** except using PCR product instead of the plasmid DNA.
3. Any suitable primer for screening nCas9 is applicable. Alternatively, the following primer sets (ABE-F and ABE-R) could be used to screen for the presence of TadA-TadA∗ with an expected amplicon size of 1267 bp: ABE-F, GCTAATACGACTCACTATAGGG, and ABE-R, GGAATTAGTGCCG ATGGCTA.

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Chapter 5

CRISPR-Cas9-Mediated Genome Editing of the Model Grass Species *Brachypodium distachyon*

Deborah Petrik

Abstract

The use of CRISPR-Cas9 to generate insertions or deletions (INDELS) resulting in stable functional knockout mutants of a targeted gene of interest is explored in the model grass species *Brachypodium distachyon*. Selection of a single-guide RNA based on location in the gene and low risk of potential off-target effects is initially presented, with the goal of generating INDELS in a single-gene target. Next, the use of the polycistronic t-RNA/gRNA system to simultaneously target multiple genes, or, alternatively, to generate a large insertion within a single gene, is discussed. Methods to transform chemically competent or electrocompetent *Agrobacterium tumefaciens* AGL-1 with CRISPR vectors, followed by stable transformation of Bd21–3 *Brachypodium distachyon* embryogenic callus, followed by plant regeneration, are outlined. Lastly, two methods for genotyping are described. The first is an inexpensive alternative based on PCR product fragment sizing analysis (within a single nucleotide difference to the wild-type sequence) to quickly ascertain the number of bases inserted or deleted on each allele of the targeted gene, which can then be followed by sequencing homozygous progeny of the initial regenerated transformants.

Key words CRISPR-Cas9, *Brachypodium distachyon*, Polycistronic, *Agrobacterium tumefaciens*, Protospacer adjacent motif (PAM), Multiplex, Golden Gate cloning, Guide RNA (gRNA)

1 Introduction

The design of guide RNAs to target Cas9 to the desired site in the genome, with low risk of off-targeting, was performed using the CRISPR-PLANT webpage (https://www.genome.arizona.edu/crispr/CRISPRsearch.html). By entering the gene ID of the targeted gene, candidate guide RNA spacer sequences were listed which were classified as 0.0 or 0.1. Class 0.0 guide RNAs have no significant sequence identity with other NGG-PAM sites and have no off-target potential to NAG-PAM sites, while class 0.1 guide RNAs have no significant sequence identity with other NGG-PAM sites, but do have off-target potential to NAG-PAM sites [1]. From the list of potential guide RNA sequences, those which would guide Cas9 to sequence within the first exon of the gene, near the 5′ end

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of the cDNA transcript, were chosen. Note that it is important to map in silico the location of the guide RNAs within the genomic sequence to verify its location in the first exon, as the CRISPR-PLANT website may not accurately state the location of guide RNAs in the first or last intron in instances where a gene is located on the (−) strand. The targeting of INDELs to the first exon is preferred if one desires to create a greatly truncated protein based on creation of a premature stop codon due to a shift in the reading frame caused by the presence of inserted or deleted nucleotides. One may additionally narrow the list of potential guide RNAs by looking for those that would target a double-stranded break (DSB) to occur at a location where an insertion or deletion (INDEL) would interrupt a restriction enzyme recognition site. This may be useful for later genotyping purposes, based on the ability of a restriction enzyme to be able to cut or not cut a PCR product amplified through the region of the DSB. There are other methods, however, for subsequent genotyping of CRISPR-induced INDEL mutations, so placement of the guide RNA considering restriction enzyme recognition sites is not an absolute necessity and may not be feasible. Methods of genotyping will be discussed in a later section.

In the case where only a single-guide RNA was to be used as a targeting sequence by Cas9, with average targeting efficiency, a simplified system of cloning was used in which the partial BsaI recognition sequence 5′ GGCA was added to the 5′ end of the forward primer and the partial BsaI recognition site 5′ AAAC was added to the 5′ end of the reverse primer. The dissolved primers are then heated together and slowly cooled to allow their complementary annealing. The double-stranded guide RNA sequence is then ligated into the BsaI-digested pRGEB32 vector, transformed into *E. coli* Top10 cells, and selected using LB + 60 ug/mL kanamycin plates. Plasmid preps from selected colonies were sequenced using the M13R (−48) primer.

A method for increasing the efficiency of Cas9 targeting to desired genomic DNA sites, which also allows for multiplexed targeting of multiple genomic sites simultaneously, was described by Xie et al. [2]. Per guide RNA sequence, two oligos were designed. The forward oligo was designed as follows: 5′-ta-GGTCTC-N-N9-N10-N11-N12-N13-N14-N15-N16-N17-N18-N19-N20-gttttagagctagaa-3′. The reverse oligo was designed as follows: 5′- cg-GGTCTC-N-N12-N11-N10-N9-N8-N7-N6-N5-N4-N3-N2-N1 (the red color indicates the reverse complementary sequence to the guide RNA spacer sequence). One can see that the forward and reverse oligos have N9–N12 as a 4-base overlap with each other, in a location where each oligo's 5′ GGTCTC sequence together with the subsequent N and N9–N12 create BsaI sites for Golden Gate cloning. The two oligos upon Golden Gate Assembly create the full guide RNA targeting sequence.

2 Materials

2.1 Materials for Cloning of CRISPR Vector

2.1.1 Materials for Brachypodium distachyon Embryo Dissection

1. Partially filled, still green *Brachypodium distachyon* Bd21-3 immature seeds.
2. Fine-tipped forceps.
3. Seed sterilization solution: 10% bleach, 0.1% Triton X-100.
4. Sterile water.
5. Sterile microcentrifuge tubes and tube rack to hold them.
6. Sterile petri dishes.
7. Callus induction media (CIM): To 800 mL ddH_2O, add 4.73 g Linsmaier and Skoog (LS) pH-buffered basal salts, 30 g sucrose, and 1 mL of 0.6 mg/mL $CuSO_4$. pH to 5.8 with 0.1 N KOH. Bring solution up to 1 L, and transfer to 2 L bottle. For plates, add 2.5 g Phytagel. Autoclave bottle on media cycle with cap loose. Let cool to 60 C. Transfer to sterile hood. If preparing CIM plates to grow uninfected *Brachypodium* callus, add 500 μL of 5 mg/mL 2,4-D to the 1 L of media, swirl to mix, and pour plates.

2.1.2 Materials for Transformation and Regeneration of Brachypodium distachyon

1. Glycerol stock of *Agrobacterium tumefaciens* strain AGL-1 transformed with CRISPR-Cas9 plasmid vector.
2. MG/L media (hereafter referenced to as AGL-1 media so as to not confuse the original name with MilliGrams/Liter): Per 1 L, add 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 5 g mannitol, 0.1 g $MgSO_4$, 0.25 g K_2HPO_4, and 1.2 g glutamic acid. Adjust the pH to 7.2 with 1 N NaOH. Add 15 g agar per 1 L media if making plates. Autoclave media on liquid cycle with cap loose, and let cool to 65 C in water bath. Bring media to sterile hood, add appropriate antibiotics, and pour into petri dishes.
3. CIM + 2,4,D + timentin + hygromycin solid media plates: If preparing CIM plates to grow and select *Agrobacterium tumefaciens*-infected *Brachypodium* callus, use the same recipe as described in Subheading 2.1.2, **Item 7**. For the gelling agent, use 2.5 g/L Phytagel if using hygromycin or BASTA transgenic plant selection. If using a vector with nptII selection gene and planning to use paromomycin selection, use 5 g/L of phytoagar instead of Phytagel. This is because paromomycin will precipitate in media containing Phytagel. Once the autoclaved media has cooled to 60 C, in addition to adding the 500 μL of 5 mg/mL 2,4-D to 1 L media, also add 1 mL of 150 mg/mL timentin stock solution and 1 mL of 40 mg/mL hygromycin stock solution (if using hygromycin selection). Swirl to mix, and pour plates.

4. Regeneration media (RM): To 800 mL ddH_2O, while mixing, add 4.73 g pH-buffered LS basal salts and 30 g maltose. pH to 5.8 using 0.1 N KOH. Bring water up to 1 L, transfer to 2 L bottle, and add 2.5 g Phytagel (as long as using hygromycin or BASTA selection; use 5 g phytoagar if using a vector that contains nptII plant selectable marker and planning to use paromomycin selection). Autoclave on media cycle for 45 min with cap of bottle loose, and allow to cool to 60 C. Transfer media to sterile hood. Before pouring, add 1 mL 150 mg/mL timentin stock solution, 1 mL 0.2 mg/mL kinetin solution, and 1 mL 40 mg/mL hygromycin solution (if using hygromycin plant selection which is the marker contained in pRGEB32). Note that the [3] protocol suggests adding 2 mL of 150 mg/mL timentin per L of media, but 1 mL of 150 mg/mL timentin per L of media works sufficiently to gradually kill *Agrobacterium*; overgrowth of *Agrobacterium* has not been observed.
5. MS media: To 800 mL ddH_2O, add 4.42 g Murashige and Skoog (MS) basal medium with vitamins and 30 g sucrose while mixing. pH to 5.7 using 0.1 N KOH. Bring water up to 1 L. Pour media into 2 L bottle, add 1.75 g Phytagel, leave cap loose, and autoclave on media cycle for 45 min. Allow to cool to 60 C in a water bath, and transfer bottle to sterile hood. Although the original protocol by Bragg et al. [3] recommends adding 1 mL 150 mg/mL timentin stock solution and the appropriate antibiotics to select transgenic callus, it has been found that the 3-weeks that the transgenic callus is selected on CIM + 2,4,D + timentin + hygromycin, plus the 2–3 weeks that the transgenic callus is incubated (in total) on regeneration media also containing 150 μg/mL timentin (final concentration), is sufficient to effectively kill the *Agrobacterium tumefaciens*. Further, although hygromycin can be included in the MS media for longer transgenic plant selection, this will significantly slow the growth of roots in the MS media containing magenta boxes. This could be problematic, particularly in cases where mutations induced by CRISPR already are expected to affect cell elongation. Therefore, one may choose to transfer shoots from regeneration media that contained timentin and hygromycin to magenta boxes containing MS media lacking these antibiotics. Lastly, in cases where the mutated gene may affect rooting, one can add 1 mL of 0.2 mg/mL NAA rooting hormone per L of MS media before pouring media into magenta boxes.
6. Stock solutions:
   (a) 150 mg/mL timentin stock solution (1000×). Add 1.5 g timentin to 10 mL sterile water, and mix. Filter sterilize

using 0.2 μm nylon filter, aliquot into 1 mL aliquots in microcentrifuge tubes, and store at −20 °C.

(b) 40 mg/mL hygromycin B stock solution (1000×): Dissolve 0.4 g hygromycin B into a total of 10 mL of water, and mix. Filter sterilize using 0.2 μm nylon filter, aliquot into 1 mL aliquots in microcentrifuge tubes, and store at −20 °C.

(c) 200 mM acetosyringone solution: Dissolve 0.392 g acetosyringone in 10 mL DMSO, and mix. Filter sterilize using 0.2 μm nylon filter, aliquot into 1 mL aliquots in microcentrifuge tubes, and store at −20 °C.

(d) 10% Pluronic F-68 (100×) (Caisson Labs, PFL01-100 mL). Already comes filter sterilized.

(e) 9.3 mM (0.2 mg/mL) kinetin stock solution (1000×). A 20 mg/mL kinetin stock solution can be made in DMSO and filter sterilizing. One can then dilute an aliquot 1:100 down to 0.2 mg/mL in DMSO, and refilter sterilize. Use a 0.2 μm nylon filter, as DMSO can dissolve some other types of filters including cellulose filters. Aliquot into 1 mL aliquots and freeze at −20 C. Avoid multiple freeze-thaws.

(f) 0.6 mg/mL $CuSO_4$: Can store at room temperature.

3 Methods

3.1 Cloning of Single gRNA into pRGEB32

1. Design forward and reverse guide RNAs for the gene of interest using CRISPR-PLANT website.
2. Design the primers to anneal to genomic DNA sequence within the first intron of the gene.
3. Choose the online parameters to select against off-target effects – namely, guide RNAs of classes 0.0 or 0.1.
4. Add the adapter sequence 5′ GGCA- to the 5′ end of the forward primer and the adapter sequence 5′ AAAC to the 5′ end of the reverse primer.
5. Dissolve the primers to 100 μM.
6. Into a PCR tube, add:

   1 μL 100 μM forward primer

   1 μL 100 μM reverse primer

   1 μL 10× T4 DNA ligase buffer

   $7\,\mu L$ sterile water

   10 μL total

Perform the following incubation:

37 °C 60 min.

95 °C 10 min.

Slowly cool to 25 °C at a rate of 0.1 °C/s.

7. When this annealing reaction is complete, dilute a fraction of the reaction 1:200.
8. Prior to performing the ligation reaction of the annealed oligos into the pRGEB32 vector, one must digest pRGEB32 with BsaI and purify it:

	μL
~400 ng/μL pRGEB32	5.0 (2 μg)
10× NEB#4 buffer	2.0
100× bovine serum albumin	0.2
10 U/μL BsaI (NEB)	1.0
Sterile water	*11.8*
Total	20.0

Incubate at 37 C for 2 h, heat inactivate enzyme at 65 C 20 min, and 4 C hold.

Purify digested vector using DNA purification column, and quantify using NanoDrop 2000 C spectrophotometer. Dilute an aliquot if necessary to achieve a concentration of 50–100 ng/μL.

9. Perform the following ligation reaction of diluted annealed guide RNA oligonucleotides into the BsaI-digested pRGEB32 vector:

	μL
50–100 ng/μL BsaI cut PRGEB32 vector	1.0
Oligo duplex (diluted 1:200)	1.0
10× T4 DNA ligase buffer	0.5
T4 DNA ligase	1.0
Sterile water	*1.5*
Total	5.0

Ligate overnight at 4 C using a heated lid to prevent evaporation and condensation on the lid of the PCR tube.

10. Transform 1 μL of the ligation into 50–100 μL of *E. coli* Top10 chemically competent cells according to manufacturer's protocol. Briefly, thaw the cells 10 min on ice, add 1 μL of the ligation to the cells, and incubate on ice 30 min. Heat shock the cells at 42 C for 30 s, and then immediately return to ice for 2 min. Add 500 μL of SOC recovery medium to the cells and incubate at 37 C with shaking for 1 h. In a sterile hood, plate 100 μL of the recovered cells onto LB + 60 μg/mL kanamycin plate, allow plate to dry, and place plate lid side down in a 37 C incubator overnight.
11. Inoculate kanamycin-resistant colonies into 5 mL of LB + 60 μg/mL kanamycin, and incubate cultures at 37 C overnight with shaking.
12. Perform bacterial plasmid DNA miniprep procedure according to manufacturer's recommendations. Sequence plasmid for the presence of the guide RNA sequence using the M13R (−48) primer, which anneals in the pRGEB32 plasmid backbone.

    Or, instead of Subheading 3.1, perform Subheading 3.2 to target two genomic regions simultaneously.

3.2 Cloning of Two Guide RNAs Spaced Between Two tRNAs to Boost Cas9 Efficiency (tRNA-gRNA3-tRNA-gRNA4) into pRGEB32

As described in Xie et al. (2015), Cas9 can be targeted to two genomic locations in the same gene by designing two guide RNAs within the same gene, thereby increasing the probability of a large deletion occurring between the two double-stranded break (DSB) sites. Alternatively, multiple guide RNAs can be used to multiplex gene targeting simultaneously. Using the Level 1 Golden Gate Assembly method described in the Xie et al. (2015) supporting information, two to six genes can be targeted by Cas9 for CRISPR-mediated INDELs simultaneously. Interspersing tRNA sequences between each guide RNA sequence can increase the efficiency of expression of the guide RNAs. These alternating tRNA-gRNA modules are created using Golden Gate Assembly. Provided below is a protocol for the assembly of PTG3 (tRNA-gRNA3-tRNA-gRNA4). To keep the naming convention the same as the original publication, the guide RNA targeting the first gene to be mutated is named gRNA3, and the guide RNA targeting the second gene to be mutated is named gRNA4.

Step 1. For each gRNA, forward and reverse oligos are designed, preferably targeting genomic DNA sequence close to the 5′ end of exon 1 of each gene.

For each guide RNA, the forward and reverse oligos are composed of a part of the 20 nt total guide RNA sequence (N1–N20).

Forward primer:

5′ ta-GGTCTC-N-**N9-N10-N11-N12**-N13-N14-N15-N16-N17-N18-N19-N20-gttttagagctagaa-3′

Reverse primer:

5′ cg-GGTCTC-N-**N12-N11-N10-N9**-N8-N7-N6-N5-N4-N3-N2-N1-tgcaccagccggg-3′

(Letters in red represent the reverse complement of the N12-N1 nucleotides of the entire N1 through N20 guide RNA sequence in the 5′ to 3′ orientation.)

Step 2. Set up 50 μL PCR to amplify each part needed for the polycistronic tRNA-guide RNA construction of PTG3, which is composed of 5′-the last 10 bp of the U3P promoter:: pre-tRNA sequence::gRNA3::gRNA scaffold::pre-tRNA sequence::gRNA4:: gRNA scaffold::pol III terminator sequence-3′. To assemble this PTG, the following Golden Gate Assembly Level 1 parts need to be synthesized first using PCR:

(A) Level 1 part L5AD-gR3:

	μL
0.1 ng/μL pGTR plasmid	1.0
5× Phusion HF buffer	10.0
10 mM dNTPs	1.0
10 μM L5AD5-F primer	2.5
10 μM gRNA3-R primer	2.5
Phusion DNA pol. (2 U/μL)	0.5
Sterile H_2O	*32.5*
Total	50.0

The sequence of L5AD5-F primer used in (A) is:

5′- CGGGTCTCAGGCAGGATGGGCAGTCTGGGCAACAAAGCACCAGTGG-3′

(B) Level 1 part gRNA3-gRNA4.

	μL
0.1 ng/μL pGTR plasmid	1.0
5× Phusion HF buffer	10.0
10 mM dNTPs	1.0

(continued)

	μL
10 μM gRNA3-F primer	2.5
10 μM gRNA4-R primer	2.5
Phusion DNA pol. (2 U/μL)	0.5
Sterile H_2O	*32.5*
Total	50.0

(C) Level 1 part gRNA4-L3AD.

	μL
0.1 ng/μL pGTR plasmid	1.0
5× Phusion HF buffer	10.0
10 mM dNTPs	1.0
10 μM gRNA4-F primer	2.5
10 μM L3AD5-R primer	2.5
Phusion DNA pol. (2 U/μL)	0.5
Sterile H_2O	*32.5*
Total	50.0

The sequence of L3AD5-R primer used in (C) is:

5′-TAGGTCTCCAAACGGATGAGCGACAGCAAACAAAAAAAAAAGCACCGACTCG-3′

Note that the L5AD5-F and L3AD5-R primers contain FokI sites (underlined) to generate compatible overhangs (marked in red) for cloning into pRGEB32.

Step 3. Run each PCR using the same program:

1. 98 °C 2 min.
2. 98 °C 10 s.
3. 50 °C 20 s.
4. 72 °C 20 s.
5. Repeat **steps 2–4** 35 times.
6. 72 °C 2.5 min.
7. 4 °C hold.

Step 4. Run each PCR product on a gel to verify successful amplification of each Level 1 assembly part based on its size, then purify each amplification product from the agarose gel slice, and quantify the concentration using a NanoDrop spectrophotometer. The gel electrophoresis will assist in separating any undesired DNA

(such as primers or the pGTR plasmid) from the desired amplification products prior to later cloning steps.

Step 5. Perform the following Golden Gate Assembly reaction:

Level 1 part L5AD-gR3	25–50 ng (same amount for each part)
Level 1 part gRNA3-gRNA4	25–50 ng
Level 1 part gRNA4-L3AD	25–50 ng
2× T7 DNA ligase buffer	10.0 μL
Bovine serum albumin (1 mg/mL)	2.0 μL
BsaI (10 U/μL)	0.5 μL
T7 DNA ligase (3000 U/μL, NEB)	0.5 μL
Sterile water	Enough to bring total reaction volume to 20 μL
Total volume	20 μL

Step 6

Incubate the Golden Gate Assembly reaction:

1. 37 °C 5 min.
2. 20 °C 10 min.
3. Cycle between **steps 1** and **2** 30–50 cycles.
4. Hold at 20 °C for 1 h.

Step 7

Dilute this 20 μL Golden Gate Assembly reaction 1:20 by adding 180 μL of sterile water.

Step 8

Amplify the Golden Gate Assembly product (to increase the yield of fully assembled product) using the following 50 μL PCR:

	μL
(1:10) dilution of the Golden Gate assembly product	1.0
5× go green PCR buffer (Promega)	10.0
10 mM dNTPs	1.0
10 μM S5AD5-F primer	1.0
10 μM S3AD5-R primer	1.0
GoTaq DNA polymerase (2 U/μL, Promega)	1.0
Sterile water	*35.0*
Total	50.0

The sequence of the S5AD5-F primer is:
5′-CGGGTCTCAGGCAGGATGGGCAGTCTGGGCA-3′
The sequence of the S3AD5-R primer is:
5′-TAGGTCTCCAAACGGATGAGCGACAGCAAAC-3′

Step 9

Use the following amplification program to amplify the Golden Gate Assembly product:

1. 95 °C 2 min.
2. 95 °C 10 s.
3. 60 °C 20 s.
4. 72 °C 1 min/kb. The size of PTG3 should be approximately 371 bp, so a 30-s extension is sufficient.
5. Cycle through **steps 2–4** 35 times.
6. 72 °C 5 min.
7. 4 °C hold.

Step 10

Run 5 μL/50 μL PCR on 1% agarose gel to verify the expected size (~420 bp in the case of PTG3, including the sequence added using the S5AD5-F and S3AD5-R primers).

Then, column purify the remaining 45 μL of the PCR using a PCR purification column (the Machery Nagel PCR cleanup columns provide excellent yield). Elute using 30 μL elution buffer.

Step 11

Digest 1.5–2.0 μg of the purified amplification product with FokI:

	μL
~250.0 ng/μL PCR bulked up Golden Gate assembly product	6.0
Sterile water	36.0
10× cut smart buffer (NEB)	5.0
5 U/μL FokI	*3.0*
Total	50.0

Incubate at 37 °C for 3 h, and then heat inactivate the enzyme at 65 °C for 20 min, followed by 4 °C hold.

Step 12

Run the 50 μL FokI digest on a 1% agarose gel, extract the digestion product (~360 bp in the case of PTG3), and purify DNA from the agarose slice using a gel extraction kit. Elute in 30 μL elution buffer, and quantify DNA concentration using NanoDrop spectrophotometer.

Step 13

Prior to performing the ligation reaction of the annealed oligos into the pRGEB32 vector, one must digest pRGEB32 with BsaI and purify it:

	μL
~400 ng/μL pRGEB32	5.0 (2 μg)
10× NEB#4 buffer	2.0
100× bovine serum albumin	0.2
10 U/μL BsaI (NEB)	1.0
Sterile water	*11.8*
Total	20.0

Incubate at 37 C for 2 h, heat inactivate enzyme at 65 C 20 min, and 4 C hold.

Purify digested vector using DNA purification column, and quantify using NanoDrop 2000 C spectrophotometer. Dilute an aliquot if necessary to achieve a concentration of 50–100 ng/μL.

Step 14

Ligate the FokI-digested Golden Gate Assembly product PTG3 into the BsaI-digested pRGEB32:

	μL
~ 60 ng/μL BsaI-digested pRGEB32	1.25
~ 40 ng/μL FokI-digested PTG3	1.0
Sterile water	6.25
10× T4 DNA ligase buffer (NEB)	1.0
T4 DNA ligase (NEB)	*0.5*
Total	10.0

Incubate at 16 C overnight.

Step 15

Transform 2 μL into 50 μL *E. coli* Top10 cells according to manufacturer's protocol (Thermo Fisher), plate two different volumes of the recovered cells onto LB + 60 μg/mL kanamycin plates, and incubate at 37 C overnight.

Step 16

Inoculate 5 mL LB + 60 μg/mL kanamycin cultures with colonies picked from the transformation plate. Incubate with shaking at 37 °C overnight. Isolate plasmid DNA from the cultures as per manufacturer's instructions. Quantify DNA using spectrophotometer.

4 Sequencing of CRISPR Constructs

Both the pRGEB32 plasmid with a single-guide RNA inserted and the pRGEB32 plasmid with a Golden Gate Assembled polycistronic tRNA-gRNA insert can be sequenced using the M13R (−48) primer. Alternatively, the polycistronic tRNA-gRNA (PTG) can be sequenced with the gRNAHind3R primer.

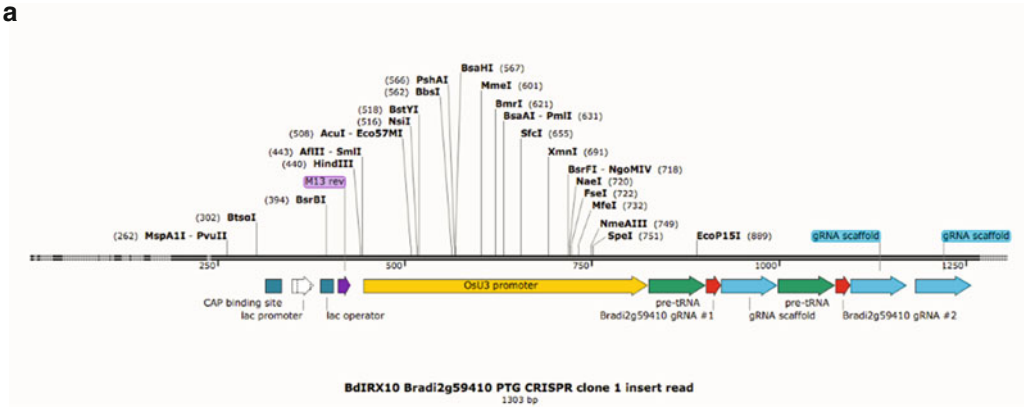
Here is an example of a single gRNA cloned into the BsaI site of pRGEB32 and sequenced with M13R (−48):

NNNNNNNNNNTGNTTACGCCAAGCTTAAGGAATCTTTAAACATACGAACAGATCACTTAAAG
TTCTTCTGAAGCAACTTAAAGTTATCAGGCATGCATGGATCTTGGAGGAATCAGATGTGCAGT
CAGGGACCATAGCACAAGACAGGCGTCTTCTACTGGTGCTACCAGCAAATGCTGGAAGCCGG
GAACACTGGGTACGTTGGAAACCACGTGATGTGAAGAAGTAAGATAAACTGTAGGAGAAAAG
CATTTCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGGCCGGCCCATTACG
CAATTGGACGACAACAAAGACTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTG
ATTTAAAAGAGTTGTGCAGATGATCCGTGGCATGAGTCGCTCATCAAGTCTCGTTTTAGAGCT
AGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGG
TGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTG
CATGCCTGCAGGTCCACAAATTCGGGTCAAGGCGGAAGCCAGCGCGCCACCCCACGTCAGCA
AATACGGAGGCGCGGGGTTGACGGCGTCACCCGGTCCTAACGGCGACCAACAAACCAGCCAG
AAGAAATTACAGTAAAAAAAAAGTAAATTGCACTTTGATCCACCTTTTATTACCTAAGTCTCA
ATTTGGATCACCCTTAAACCTATCTTTTCAATTTGGGCCGGGTTGTGGTTTGGACTACCATGAA
CAACTTTTCGTCATGTCTAACTTCCCTTTCAGCAAACATATGAACCATATATAGAGGAGATCG
GCCGTATACTAGAGCTGATGTGTTTAAAGGTCGTTGATTGCACGAGAAAAAAAAATCCAAATC
GCAACAATAGCAAATTTATCTGGTTCAAAGTGAAAAGATATGTTTAAAGGTAGTCCAAAGTA
AAACTTATAGANNNAAATGTNGNCCAAAGCGTAATTCACTCAAAAAAAATNANGAGACGTGT
ACNAACGGANANAANNNATNNNNCGAANNTTNCCANNNTCGNTCGCCNNCNNGNNNNCNNA
ACNNGNNNGNTTCANCNNGNNGNNNNNNNNNNANNGNNNNTNNNNNNNNNNNNTCNANNN
NNNNNCNTNANTNCANNCNNNANNNNNNNNNNNNNNNNNNCNNCNNNAAAANNNNCTNN
NNANNNNNNNNN

Light blue: Promoter U3.
Red: 20 nt guide RNA sequence targeting desired gene.
Gray: gRNA scaffold.

Here is an example of the map (Fig. 1a) of tRNA-gRNA3-tRNA-gRNA4, followed by its sequence as determined by using gRNAHind3R as the sequencing primer (Fig. 1b). The sequence data received was reverse complemented prior to comparison to

a



b

NNNTNNNNCAANGNNNNNNNNNGNNNNNNCNNNCNTTNANNCGNNNNNNNNNNNNNNNNN
NNNNNGNNNNNNNNNNANNNNNNNNNNNNNTCNNNNNNNNNNNNNNTGNNNNAAANGTNNN
AANNNNNNNNNTNTNNNNNNNNNGNNNNNNTTNNANNNTTNNNNNNNAGNNNNNANNNNT
NNNNNNNCNNNCATGNTNNCAAGNNNCTCNAGCCANNACGCAANCNNNCTNNNCCCNNNNC
GTTGGCNNNTNATTAATGCAGCTGNCACGACAGGTTTCCNNACTGGAAAGCGGGCAGTGAGC
GCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCC
GGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACAT
GATTACGCCAAGCTTAAGGAATCTTTAAACATACGAACAGATCACTTAAAGTTCTTCTGAAGC
AACTTAAAGTTATCAGGCATGCATGGATCTTGGAGGAATCAGATGTGCAGTCAGGGACCATA
GCACAAGACAGGCGTCTTCTACTGGTGCTACCAGCAAATGCTGGAAGCCGGGAACACTGGGT
ACGTTGGAAACCACGTGATGTGAAGAAGTAAGATAAACTGTAGGAGAAAAGCATTTCGTAGT
GGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGGCCGGCCCATTACGCAATTGGACGA
CAACAAAGACTAGTATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAAGAG
TTGTGCAGATGATCCGTGGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCAC
GGTACAGACCCGGGTTCGATTCCCGGCTGGTGCATGCTGTTCTCGTTGCTGCTGGTTTTAGAGC
TAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG
GTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGT
TCGATTCCCGGCTGGTGCAACATGTGGTTCAGGCATCTAGTTTTAGAGCTAGAAATAGCAAGT
TAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTTGT
TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCtAGtCCgTTATCAACTTGAAAAAGTGGCACC
GAGTCGGTGCTTTTNGNNAGANNNNNCNNNNNNNNNNNNNNNNNNTNNNNNNNNNN

Fig. 1 (**a**) Partial map of a PTG consisting of tRNA-Bradi2g59410 gRNA #1-gRNA scaffold-tRNA-Bradi2g59410 gRNA #2-gRNA scaffold in pRGEB32. (**b**) Plasmid sequenced with gRNAHind3R, then sequence data reverse complemented. That reverse complemented sequence is shown above

predicted sequence, because that primer anneals on the bottom strand, 3′ of the PTG insert, and reads backward into the insert.

Magenta: Rice OsU3 promoter.

Blue: Pre-tRNA.

Red: Bradi2g59410 gRNA #1 and Bradi2g59410 gRNA #2, respectively.

Yellow: gRNA scaffold.

Gray: Pol III terminator.

5 Preparation of Chemically Competent *Agrobacterium tumefaciens* AGL-1

Agrobacterium tumefaciens strain AGL-1 works well for *Brachypodium distachyon* transformation. AGL-1 has the C58, *RecA* chromosomal background, and the pTiBo542ΔT Ti plasmid. AGL-1 has been engineered to contain the hypervirulent Ti plasmid, pTiBo542, which contains additional virulence genes originating from the *Agrobacterium* strain A281.

Chemically competent *Agrobacterium tumefaciens* strain AGL-1 can be prepared by:

1. Culture a single colony of AGL-1 in 5 mL of LB + 100 μg/mL carbenicillin. Incubate at 28 C with shaking overnight.
2. Use 1 mL of the 5 mL culture to inoculate 50 mL of LB +100 μg/mL carbenicillin. Incubate with shaking until OD600 = 0.5–0.8.
3. Transfer culture to 50 mL Falcon tube, and incubate 30 min on ice.
4. Centrifuge at 4 C, ~3500 rpm, 15 min.
5. Discard supernatant, and add 10 mL sterilized and pre-chilled 20 mM $CaCl_2$ to resuspend pellets. Resuspend gently by pipetting.
6. Centrifuge at 4 C, ~3500 rpm, 15 min.
7. Discard supernatant, and add 2 mL sterile pre-chilled 20 mM $CaCl_2$ and 10% sterile glycerol. The presence of glycerol prevents cell lysis during freezing and thawing.
8. Aliquot 100 μL of resuspended cells into each of 20 pre-chilled microcentrifuge tubes, snap freeze in liquid nitrogen, and store at −80 C.

6 Transformation of *Agrobacterium tumefaciens* AGL-1 by Heat Shock

1. Place an aliquot of frozen AGL-1 cells on ice to slowly thaw for 10 min.
2. Add 1–2 μL of plasmid to the 100 μL of AGL-1 competent cells, and allow to incubate on ice for 30 min.

3. Freeze in liquid nitrogen for 5 s. You will see the cells turn an opaque white color in the tube.
4. Immediately put the tube in the 37 C water bath for 5 min.
5. Add 1 mL liquid LB without any antibiotics, and shake at 28 C, 150 rpm for 3–5 h.
6. Plate 250 μL of cells onto LB + the appropriate antibiotic for selection of the recombinant plasmid (kanamycin 60 in the case of the use of pRGEB32 plasmid). Incubate at 28 C for 3 days in dark.
7. Pick colonies to inoculate into LB or YEP with the appropriate antibiotics in order to prepare glycerol stocks. The glycerol stocks can be made using 700 μL of culture +300 μL of sterile 50% glycerol and flash frozen in liquid nitrogen before storing at −80 C. One may also desire to verify the plasmid in the transformed *Agrobacterium* by PCR or restriction enzyme digestion. If one desires to verify by PCR, a plasmid DNA isolation directly from the *Agrobacterium* culture can be used. If one desires to use restriction enzyme digestion analysis to verify no recombination of the plasmid, the concentration of the plasmid DNA obtained from the *Agrobacterium* culture will be too low for this purpose; instead one may take a fraction of the plasmid DNA isolated from *Agrobacterium* and use it to transform *E. coli*. One would then isolate plasmid DNA from a transformed *E. coli* single colony and perform restriction enzyme analysis.

7 Generation of *Brachypodium distachyon* Embryogenic Callus

The methods outlined below for the generation of *Brachypodium distachyon* embryogenic callus and its subsequent transformation and regeneration are those described by Bragg et al. [3], with minor modification:

1. Identify spikelets that have florets that are filling, but none or, at most, only the florets at the very bottom of the spikelet are slightly tan because they are starting to senesce. You actually are looking for florets in the spikelets that are starting to fill but the lemmas are still completely green (Fig. 2a).
2. Peel off the lemma from each floret as you are separating the florets from each spikelet (Fig. 2b). Place the peeled seeds into microcentrifuge tubes, approximately 20 per tube.
3. In a sterile tissue culture hood, sterilize each tube of seeds with 1 mL of 10% bleach and 0.1% Triton X-100 for 10 min. Then rinse 3× with sterile water. After the last rinse, dispense 1 mL of sterile water into each tube. This will make it easier to pour out

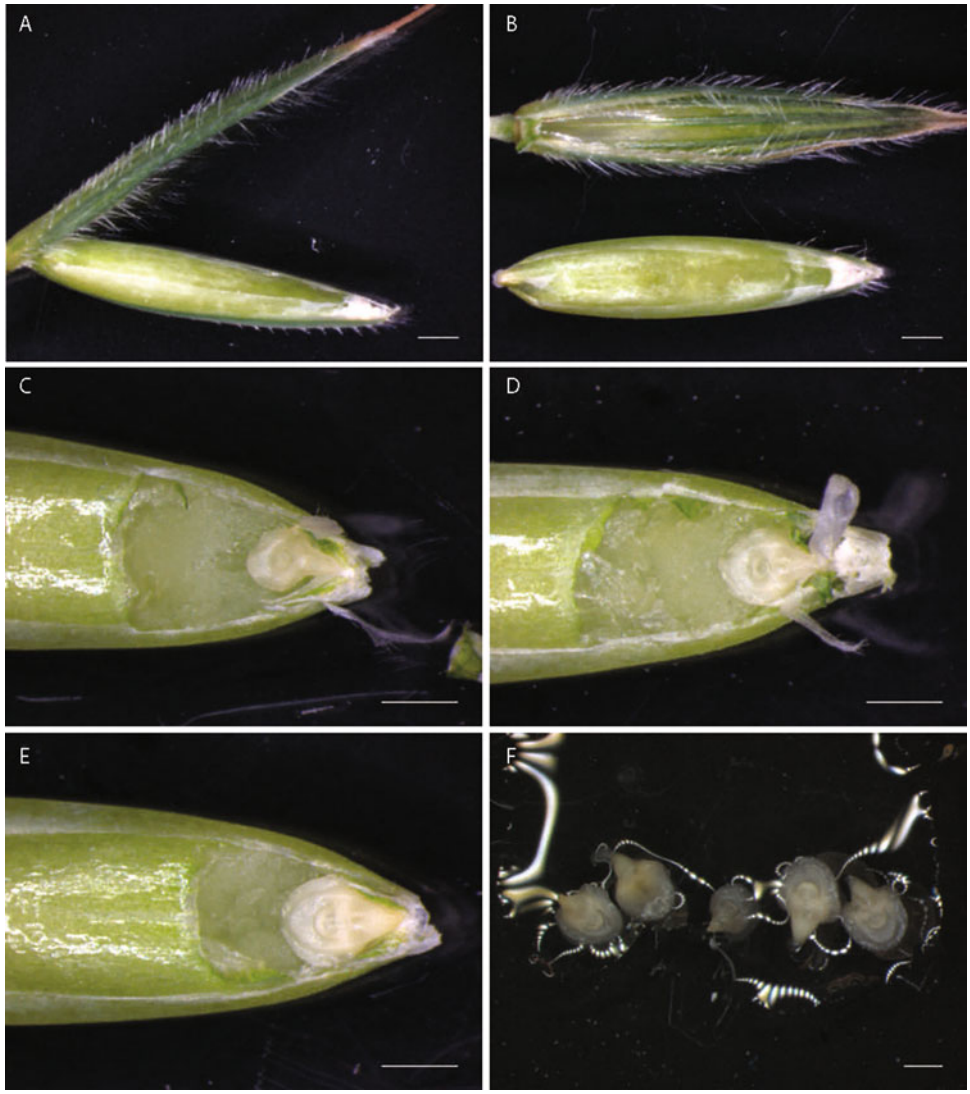


Fig. 2 Dissected Bd21-3 embryos. (**a**) Bd21-3 immature seed with lemma peeled but still attached. 8× magnification; Scale bar = 1 mm. (**b**) Bd21-3 immature seed with lemma peeled and placed side by side with immature seed. 8× magnification; Scale bar = 1 mm. (**c**) Dissected embryo of desired stage, still inside seed. 22.5× magnification; Scale bar = 0.5 mm. Note that embryo scutellumn was slightly bent with forceps during dissection. (**d**) Dissected embryo of slightly older but still usable stage, still inside seed. 22.5× magnification; Scale bar = 0.5 mm. (**e**) Dissected embryo that is too mature, still inside seed. 22.5× magnification; Scale bar = 0.5 mm. (**f**) Range of dissected embryos. 15× magnification; Scale bar = 0.5 mm. Embryo third from left is at ideal developmental stage

the seeds into a sterile petri dish for subsequent embryo dissection.

4. Pour out sterilized seeds into a sterile petri plate. With sterilized forceps, move the seeds toward the inside edge of the plate so they are not spending an extended amount of time

directly under the light of the microscope. This will help the seeds from drying out as you are individually dissecting the embryos. Of course, each seed will need to be placed under the light of the microscope as you are dissecting the embryo from that individual seed.

5. With sterile forceps, place each seed so that its palea is face down, i.e., the curved side of the seed is facing upward toward you. You will be able to see the embryo at the basal end of the seed, slightly apical to where the floret had connected to the spikelet.
6. With the sterile fine-tipped forceps, pinch and pull back the epidermis of the seed starting somewhat apical to the location of the embryo. Peel away the epidermis, pulling it away from the base of the seed, revealing the embryo underneath (Fig. 2c).
7. Rather than grasping the embryo with the fine-tipped forceps, it may be less damaging to use one tip of the open forceps to get underneath the embryo and gently scoop it out of the seed. The embryo will gently adhere to the forcep tip because it is slightly wet. The embryo can then be gently placed on the surface of the petri plate containing CIM + 2,4,D media, also in the sterile tissue culture hood. Figure 2c shows an embryo of the desired size and stage of development that is still translucent. Figure 2d shows a slightly older embryo that is somewhat larger and turning opaque white. This stage of embryo will also generate callus. Figure 2e shows an embryo which is too late in development. Note that it is turning yellow at the tip. Callus generated from embryos at this stage of development will have a low frequency of regenerating shoots.
8. The frequency of callus generation from a dissected embryo is dependent on the stage of development of the embryo. Callus will form from embryos at the stages circled. The embryo located third from the left is at the optimal stage (Fig. 2f).
9. Incubate the dissected embryos on CIM + 2,4,D media for 2 weeks in the dark at 28 C.
10. Often, the dissected embryos will germinate during the 2 weeks on CIM + 2,4,D media plates. When this occurs, in the sterile tissue culture hood, use sterile forceps to pinch and remove the germinated coleoptile and radicle. Then, place the dissected embryo onto a fresh plate of CIM + 2,4,D media. Incubate for an additional week to 10 days at 28 C in the dark.
11. At this point, clumps of yellow callus should begin to appear growing from the embryos (Fig. 3a). Clumps of callus can be transferred to fresh CIM + 2,4,D plates every week to 10 days. It usually takes a total of 5–6 weeks (2 weeks for dissected embryos on first set of CIM + 2,4,D plates plus three 1-week

transfers onto fresh media) to get enough callus for a transformation.

12. One should plant 5–10 pots containing 4 Bd21–3 plants per pot weekly, and dissect embryos weekly. Once a week is encountered in which a high percentage of seeds have embryos at the perfect stage of development; dissect twice within that week to 10 days. Following these two batches of dissected embryos through the subsequent transfers onto fresh CIM + 2,4,D media for the initial 2-week incubation plus two to three subsequent 1-week incubations on CIM + 2,4,D will provide enough callus to perform two infections simultaneously. Therefore, it is best to plant wild-type *Brachypodium* weekly, and dissect embryos weekly in order to maintain a steady pipeline of incoming callus.

8 *Agrobacterium* Infection of *Brachypodium* Callus

1. Two days prior to the planned infection in a sterile hood, spread 200 μL of the *Agrobacterium tumefaciens* glycerol stock onto an LB (or YEP) plate with the appropriate antibiotic for the bacterial selection of the transformation vector plasmid. One does not want to obtain individual colonies, but rather a confluent lawn of bacteria. Incubate the plate for 2 days at 28 C in the dark.
2. Using a sterile inoculation loop, scrape the transformed AGL-1 lawn of bacterial cells into 5 mL of liquid CIM (no need to add the 2,4,D for this purpose). Vortex gently to resuspend the cells. Use a separate aliquot of liquid CIM to blank a spectrophotometer at A_{600}. Make a dilution of the resuspended AGL-1 cells and read the optical density at A_{600}. Calculate the optical density of the undiluted AGL-1 liquid suspension.
3. Dilute a fraction of the AGL-1 liquid suspension to obtain 20 mL of suspended cells that are at $OD_{600} = 0.6$. To this 20 mL, add 20 μL of 200 mM acetosyringone and 200 μL of 10% Pluronic F68, and then mix. One can use sterile 50 mL Falcon tubes for this process.
4. Add the *Brachypodium distachyon* callus pieces into the Falcon tube containing the transformed AGL-1 liquid suspension at OD600 of 0.6 to which the acetosyringone and Pluronic F68 have been added. If you are performing more than one transformation, the callus for the first transformation can be left in the first tube with liquid AGL-1 suspension while transferring callus into the second tube of AGL-1 suspension transformed with the second construct (such as an empty vector control).

5. When all infected *Brachypodium* callus has incubated in their respective transformed AGL-1 liquid suspensions for at least 5 min to allow adequate saturation, use a 25 mL serological pipet to remove the excess AGL-1 liquid suspension. Use new pipets for each Falcon tube of transformed cells.
6. Pour the transformed callus into a sterile 150 x 15 mm petri dish. Remove remaining liquid that was not captured using the serological pipet earlier.
7. Into new sterile petri dishes, place 1 piece of sterile Whatman filter paper. Use filter discs that are slightly smaller than the 10 cm diameter of 100 mm x 15 mm petri dishes, or the filter won't sit on the bottom of the dish.
8. Using sterile forceps, transfer the infected *Brachypodium distachyon* callus onto the sterile filter paper discs sitting on the bottom of petri dishes. Leave small spaces between the pieces of callus so that it can dry. This may take multiple petri dishes per transformation depending on the amount of callus used per 20 mL of AGL-1 liquid suspension. Repeat for each transformation performed.
9. Place lids on plates, and label plates with the construct transformed and the infection date. Seal plates with parafilm. Wrap plates with aluminum foil.
10. Incubate plates for 3 days at 22 C.

9 Regeneration of Transformed *Brachypodium* Plants

1. Perform a total of three passes of infected *Brachypodium* callus onto fresh CIM + 2,4,D + timentin + hygromycin plates. Transfer the callus onto fresh media every 7 to 10 days. After each pass, wrap the plates with parafilm and then cover with aluminum foil. Incubate at 28 C for each 7- to 10-day incubation. The uninfected regions on a clump of callus will turn brown and then black, while the transformed callus will stay white to pale yellow (Fig. 3b).
2. After the end of the third incubation on CIM + 2,4,D + timentin + hygromycin, transfer the infected callus to shoot regeneration media including timentin and hygromycin. Infected callus cells will begin to turn green after the first week to 10-day incubation at 28 C in the light. Transfer to fresh shoot regeneration media including timentin plus kinetin plus hygromycin every week to 10 days (Fig. 3c). Shoots normally begin to form during the second incubation, although it may take a third incubation in order for shoots to be large enough to safely transfer to MS media in magenta boxes without damaging them.

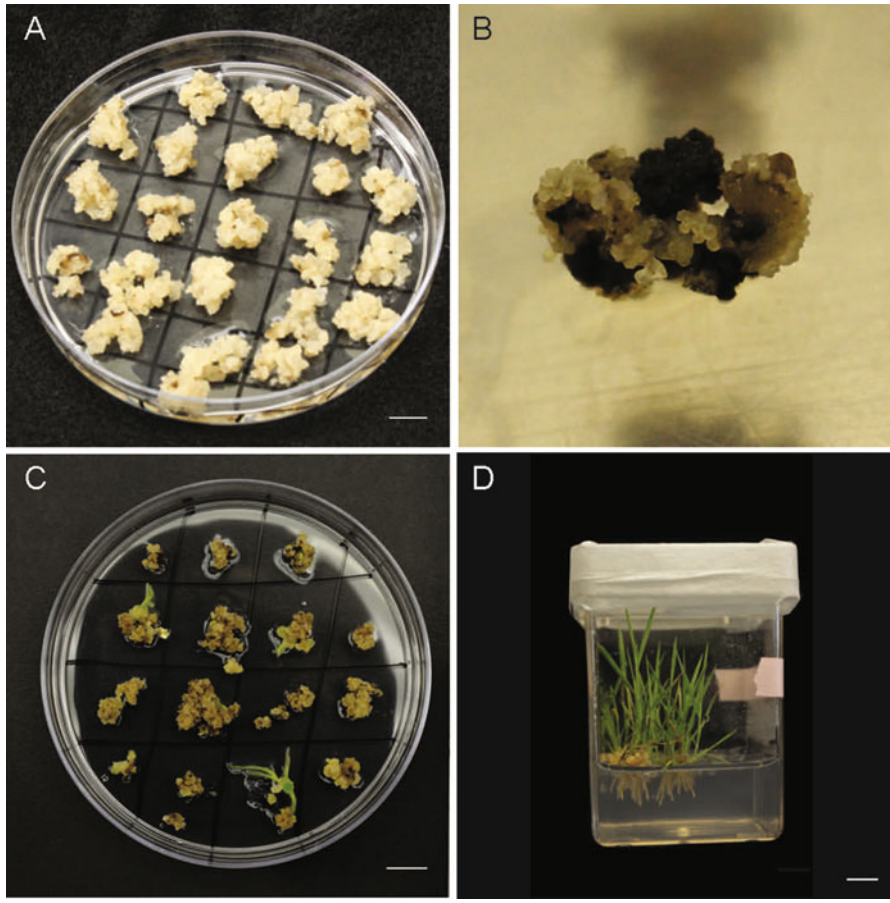


Fig. 3 *Brachypodium distachyon* stages of transformation. (**a**) *Brachypodium distachyon* Bd21-3 uninfected Callus. Scale bar = 1.0 cm. (**b**) Higher magnification view of infected callus undergoing hygromycin selection. (**c**) Transformed shoots on Shoot Regeneration media including timentin, kinetin, and hygromycin. Scale bar = 1.0 cm. (**d**) Plantlets in magenta boxes that have developed roots. Scale bar = 1.0 cm

3. Once regenerated shoots are approximately 1 cm long, transfer the shoot and attached callus to MS media in magenta boxes. Typically, inclusion of hygromycin, kinetin, or timentin is no longer necessary. This will allow faster root growth and escapes after 5 prior weeks of selection and is unusual to occur. In cases where the transgene may affect cell elongation, one may include NAA rooting hormone (1 mL of 0.2 mg/mL NAA per 1 L of MS) in the MS media in order to encourage root formation.
4. After roots have grown >2 cm long, one can transfer regenerated seedlings to soil.

10 Genotyping of Transformed *Brachypodium* CRISPR Plants

Design PCR primers on either side of the Cas9 double-stranded break (DSB) site. Extract genomic DNA from CRISPR plants and perform PCR, and then sequence the purified PCR product using one of the PCR primers as the sequencing primer. Keep in mind that different mutations may occur on each allele of the gene or that only one of the two alleles may be mutated. The PCR products amplified off of the two alleles of the gene in these cases would likely be only a few base pairs different in size, appearing as a single band on a gel. Co-purifying these PCR products as a single band and sequencing it will cause the sequencing read to be clean until it reaches the mutation site, after which one allele's sequence will be shifted relative to the other, causing a messy read of two different peaks on top of one another. One may want to allow the transformed and regenerated *Brachypodium* to self-pollinate, allowing homozygotes to be generated which will have the same mutated sequence on both alleles of the gene.

11 Notes

1. Note that it has been incorrectly stated in some past literature that the carbenicillin resistance gene is present on the Ti plasmid, but both Rif and Carb R genes are present on the C58, *RecA* bacterial chromosome (https://www.intactgenomics.com/product/agl1-electrocompetent-agrobacterium/) [4]. The streptomycin resistance gene is present on the pTiBo542ΔT Ti plasmid (https://www.lifescience-market.com/strains-c-95/agl1-agrobacterium-strain-p-63291.html) [5]. (Therefore, one should not use plant transformation vectors using streptomycin resistance when planning to use the AGL-1 *Agrobacterium tumefaciens* strain.) Since both Rif and Carb R genes are present on the bacterial chromosome, the use of only carbenicillin is sufficient, as long as sterile technique is used to verify no accidental contamination with Carb R recombinant *E. coli*.
2. Carbenicillin can be additionally added to the media (in addition to the antibiotic for transformation vector selection) when growing up the transformed AGL-1 culture, in order to select for the AGL-1 chromosome. The final carbenicillin concentration in the media should be 100 ug/mL. However, this greatly slows the growth of AGL-1. Typically, I simply make sure I am using sterile technique and solely use 60 ug/mL kanamycin to select for the nptII gene on the pRGEB32 plasmid when selecting for transformed AGL-1. It is recommended to use carbenicillin

selection when initially growing up AGL-1 cultures to make AGL-1 competent cells.

Supplementary Protocols: Plasmid DNA isolation from *Agrobacterium tumefaciens*:

Borrowed from https://web.csulb.edu/~bruss/documents/AgrobacteriumPlasmidprep.doc

Agrobacterium Plasmid Prep

1. Grow *Agrobacterium* cells overnight (20–24 h) at 28 °C and 250 RPM in 2 mL YEP medium containing an appropriate conc. of antibiotics (e.g., 50 μg/mL kanamycin for pBI101.1, plus 50 μg/mL gentamycin and 50 μg/mL rifampicin for *Agrobacterium*).
2. Transfer the culture to a 1.5 mL tube and pellet by centrifugation for 1 min (4 °C, 14 K). Take out all the supernatant with a drawn-out Pasteur pipette.
3. Resuspend cells in 100 μL of ice-cold Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris–HCl, pH 8.0, 10 mg/mL lysozyme [added fresh; stored at −20 °C])
4. Incubate for 15 min at RT.
5. Add 200 μL of Solution II (0.2 N NaOH, 1% SDS) and invert gently to mix.
6. Incubate for 5 min at RT.
7. Add 30 μL phenol (Tris-phenol, pH 8.0) equilibrated with 2 volumes of Solution II:
   (a) Mix well by inversion.
8. Add 150 μL 3 M sodium acetate (pH 4.8) and mix by inversion.
9. Incubate at −20 °C for 20 min.
10. Centrifuge for 15 min (4 °C, 14 K). Transfer the supernatant with a P200 into a new 1.5 mL tube.
11. Add 1 mL ice-cold 95% ethanol to the supernatant. Mix by inversion and store at −80 °C for 20 min (or at −20 °C overnight).
12. Centrifuge for 15 min (4 °C, 14 K) and remove supernatant.
13. Add 1 mL 80% ethanol centrifuge for 10 min and remove supernatant.
14. Resuspend pellet in 20 μL TE. ∗ Store DNA sample at −20 °C until transformation of:
   (a) *E. coli*.
15. ∗The copy number of oriV plasmid in *Agrobacterium* is very low so we cannot analyze plasmid DNA at this stage.

16. Thaw 100 μL of competent *E. coli* (e.g., DH5α) cells on ice. *Alternatively, the standard* E. coli *electroporation with 2 μL of Agro DNA can be done, followed by the standard plasmid prep.*
17. Add 2 μL of *Agro* DNA, mix, and incubate on ice for 15 min.
18. Heat shock at 42 °C for 90 sec, and place on ice for 2 min.
19. Add 800 μL S.O.C. (RT and no antibiotics), and shake cells for 50 min at 37 °C.
20. Plate 200 μL on LB plate containing appropriate antibiotics, and incubate ON at 37 °C.
21. Single colonies can be picked and inoculated in media plus appropriate antibiotics for plasmid analysis (16 h at 37 °C and 200 RPM).

YEP Liquid Medium (per Liter)
10 g Bacto Tryptone.

5 g yeast extract.

5 g NaCl.

(add 12 g bactoagar for plates only)

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Chapter 6

CRISPR-Cas9-Mediated Gene Editing of the Plant Pathogenic Oomycete *Phytophthora palmivora*

Miaoying Tian, Natasha Navet, and Dongliang Wu

Abstract

Phytophthora palmivora is a destructive oomycete pathogen that infects a large number of plant species. An effective functional genomic tool is required to understand the molecular mechanisms underlying its pathogenicity and broad host range. Gene knockout using homologous recombination has been unviable in oomycetes due to their ploidy (diploidy to polyploidy) and low rates of homologous recombination. CRISPR-mediated genome editing offers a boon for oomycete functional genomics allowing accelerated dissection of gene functions. *Agrobacterium*-mediated transformation (AMT) represents an advantageous and efficient gene delivery system over other transformation methods. In this chapter, we describe a detailed protocol for generating *P. palmivora* mutants via AMT to deliver single-guide RNA and Cas9, the components required for gene editing. This protocol can be used directly for CRISPR-Cas9-mediated gene editing of *P. palmivora* and modified for other culturable oomycete species.

Key words *Phytophthora palmivora*, Gene editing, CRISPR-Cas9, Oomycete transformation, *Agrobacterium*-mediated transformation

1 Introduction

Phytophthora palmivora is a destructive broad-host-range oomycete plant pathogen that infects many economically important host species such as cacao and papaya [1]. Understanding the molecular mechanisms underlying its pathogenicity requires an effective functional genomic approach. As *P. palmivora* is tetraploid [2] and heterothallic with a low rate of oospore germination [3], the functional genomic studies of *P. palmivora* had been very challenging until the advent of CRISPR-Cas9-mediated gene editing technology. This technology is able to simultaneously mutate two/multiple alleles in the first generation of transformants [4, 5] and thus presents an ideal approach to generate *P. palmivora* mutants for gene function analyses.

Fang et al. [6, 7] adapted the CRISPR-Cas9 technology for oomycete gene editing by using oomycete-derived nuclear

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localization signal to direct the nuclear localization of Cas9 and oomycete-derived promoters for the expression of Cas9 and single-guide RNA (sgRNA). This system is compatible with plasmid DNA uptake-based transformation methods, such as PEG/$CaCl_2$-mediated protoplast transformation, electroporation of zoospores, and microprojectile bombardment, but not *Agrobacterium*-mediated transformation (AMT). Comparing to other transformation methods, AMT is easier to perform and usually generates single-copy integration instead of multi-copy integration with the latter likely disrupting multiple loci of the genome and complicating gene function analyses [8]. To perform gene editing of *P. palmivora* using AMT, we have established a simple and efficient AMT protocol for *P. palmivora* [8] and constructed an "all-in-one" binary plasmid pCB301TOR-CRISPR [9] to express Cas9 and sgRNA using the elements from the oomycete gene editing plasmids developed by Fang et al. [6]. This binary plasmid allows direct cloning of a 20-nt sgRNA target sequence together with the hammerhead (HH)-ribozyme sequence to NheI and BsaI sites, which are upstream of sgRNA scaffold and HDV ribozyme [9]. By varying the 20-nt sgRNA target sequence, the resulted plasmid can be used for editing of a specific target gene using AMT. Through CRISPR-Cas9-mediated gene editing via AMT, we have successfully generated homozygous mutants of the *P. palmivora* extracellular cystatin-like protease inhibitor PpalEPIC8 [9]. We have also successfully generated mutants for two *P. palmivora* candidate effector genes (M. Tian et al. unpublished).

In this chapter, we describe a detailed method for generating *P. palmivora* mutants using CRISPR-Cas9-mediated gene editing via *Agrobacterium*-mediated transformation, including procedures on sgRNA design and cloning to pCB301TOR-CRISPR (*see* Subheading 3.1), *Agrobacterium*-mediated transformation of *P. palmivora* (*see* Subheading 3.2), isolation of single zoospore-derived transformants (*see* Subheading 3.3), detection of the T-DNA integration in genomes of *P. palmivora* transformants (*see* Subheading 3.4), and detection of mutations in the targeted gene (*see* Subheading 3.5). This protocol can be used directly for CRISPR-Cas9-mediated gene editing of *P. palmivora* and modified for other culturable oomycete species.

2 Materials

2.1 sgRNA Design and Cloning

1. sgRNA design software: Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT, http://grna.ctegd.uga.edu).

2. *P. palmivora* whole genome sequence: downloaded from NCBI GenBank under Accession Number NCKW00000000.1, reported by Ali et al. [2].
3. RNA secondary structure prediction: RNAstructure (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.htmL).
4. Oligos: custom synthesized by IDT or other companies.
5. Restriction enzymes NheI and BsaI (New England Biolabs, NEB).
6. T4 DNA ligase and buffer (NEB).
7. Plasmid pCB301TOR-CRISPR, a binary vector used to express sgRNA, Cas9, and NPTII in oomycetes via *Agrobacterium*-mediated transformation [9].
8. *Escherichia coli* DH5α competent cell, made as described previously [10].
9. QIAquick Gel Extraction Kit (Qiagen).
10. QIAprep Spin MiniPrep Kit (Qiagen).
11. *Agrobacterium tumefaciens* EHA105 competent cell, made as described previously [11].
12. Electroporator: various types suitable for electroporation of bacteria.
13. Electroporation cuvette (1 or 2 mm electrode gap).

2.2 Antibiotics Stock Solution

1. Kanamycin (50 mg/mL), G418 (30 mg/mL), cefotaxime (200 mg/mL): dissolved in water, filter sterilized, aliquoted, and stored at −20 °C.
2. Rifampicin (15 mg/mL): dissolved in DMSO, aliquoted, stored in −20 °C, and protected from light.

2.3 Bacterial Growth Media

1. Luria-Bertani (Miller) (LB) liquid medium: add LB powder (25 g/L) to water, autoclave, and store at room temperature (RT).
2. Luria-Bertani (Miller) (LB) agar: add LB powder (25 g/L) and agar (15 g/L) to water, autoclave, and add appropriate antibiotics to make plates.

2.4 Media and Supplies for P. palmivora Growth and Transformation

1. Unclarified 10% V8 agar: For 500 mL, add 50 mL V8 Original 100% vegetable juice, 450 mL Milli-Q water, and 0.5 g $CaCO_3$, stir to mix well, add 7.5 g agar, autoclave and pour plates (add antibiotics stock solution when needed), store at 4 °C, or use fresh.
2. K-buffer (1 M, pH 5.3): Make 1 M of KH_2PO_4 (136.09 g/L) and K_2HPO_4 (174.18 g/L), add one solution to the other until pH 5.3 is reached, autoclave, and store at RT.

3. MN buffer: Dissolve 30 g $MgSO_4 \cdot 7H_2O$ and 15 g NaCl in 1 L water, autoclave, and store at RT.
4. 1% $CaCl_2$: Dissolve 0.5 g $CaCl_2$ in 50 mL water (*see* **Note 1**), autoclave, and store at RT.
5. 0.25% $FeSO_4$: Dissolve 0.125 g $FeSO_4$ in 50 mL water (*see* **Note 2**), filter sterilize, and store at 4 °C with bottle wrapped in foil to protect it from light.
6. Spore elements: Dissolve 100 mg $ZnSO_4 \cdot 7H_2O$, 100 mg $CuSO_4 \cdot 5H_2O$, 100 mg H_3BO_3, 100 mg $MnSO_4 \cdot H_2O$, and 100 mg $Na_2MoO_4 \cdot 2H_2O$ in 1 L water, filter sterilize, and store at RT.
7. 20% NH_4NO_3: Dissolve 10 g of NH_4NO_3 in 50 mL water, filter sterilize, and store at RT.
8. 50% glycerol: Mix equal volume of glycerol and water, autoclave, and store at RT.
9. 20% glucose: Dissolve 10 g glucose in 50 mL water, filter sterilize, and store at RT.
10. 1 M MES (pH 5.3): For 1 L, add 195.2 g MES in 800 mL water, titrate to pH 5.3 with NaOH solution (*see* **Note 3**), add water to make 1 L, filter sterilize, and store at 4 °C under dark conditions.
11. Acetosyringone (AS) (200 mM): Dissolve 40 mg acetosyringone in 1 mL DMSO, make fresh, and protect it from light.
12. *Agrobacterium* induction media (AIM) (40 mL): Add 36 mL water, 400 μL K-buffer (1 M, pH 5.3), 800 μL MN buffer, 40 μL 1% $CaCl_2$, 16 μL 0.25% $FeSO_4$, 200 μL spore elements, 100 μL 20% NH_4NO_3, 400 μL 50% glycerol, 400 μL 20% glucose, 1.6 mL 1 M MES (pH 5.3), and 40 μL of AS (200 mM), mix well, and keep at RT with tubes wrapped in foil to protect it from light (*see* **Note 4**).
13. *Agrobacterium*-zoospore co-cultivation agar (AZCA): For 600 mL, add 9 g agar in 543 mL Milli-Q water, and then autoclave; when temperature drops to 50–55 °C, add 6 mL 1 M K-buffer (pH 5.3), 12 mL MN buffer, 600 μL 1% $CaCl_2$, 240 μL 0.25% $FeSO_4$, 3 mL spore elements, 1.5 mL 20% NH_4NO_3, 6 mL 50% glycerol, 3 mL 20% glucose, 24 mL 1 M MES (pH 5.3), and 600 μL of AS (200 mM), mix well, pour plates, and cover them with black cloth or foil to protect from light (*see* **Note 5**).
14. Plich agar: To prepare 500 mL, in 500 mL Milli-Q water, add 0.25 g KH_2PO_4, 0.125 g $MgSO_4 \cdot 7H_2O$, 0.5 g L-asparagine, 2.5 g glucose, 0.25 g yeast extract, 5 μL of thiamine solution (100 mg/mL in water, stored at 4 °C), and 5 mg β-sitosterol (dissolve in 1.5 mL ethanol before adding). When all

components are dissolved, add 7.5 g agar, autoclave, add appropriate antibiotics as needed when cooldown, and pour plates (*see* **Note 6**).

15. Hemocytometer (0.1 mm depth).
16. Hybond N^+ membranes (GE Healthcare Amersham Hybond™-N^+): Cut the membrane together with the protective sheets to 5 × 5 cm square pieces, wrap them in aluminum foil, and autoclave.

2.5 Detection of the T-DNA Integration and Targeted Gene Mutations *in* P. palmivora Transformants

1. DNeasy PowerLyzer Microbial Kit (Qiagen).
2. FastPrep-24 Instrument (MP Biomedicals).
3. Taq DNA polymerase.
4. Phusion high-fidelity polymerase (New England Biolabs).
5. QIAquick Gel Extraction Kit (Qiagen).
6. ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific).
7. PCR thermocycler.
8. DNA sequence analysis software.

3 Methods

3.1 sgRNA Design and Cloning

3.1.1 Selection of 20-Nucleotide (nt) sgRNA Target Sequences

1. Identify 20-nt sequences directly upstream of Cas9 protospacer adjacent motif (PAM) 5′-NGG-3′ from both DNA strands of a target gene using Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (http://grna.ctegd.uga.edu) [12]. Choose SpCas9 for "RNA guided nuclease selection." Select "Custom genome," and upload *P. palmivora* whole genome sequence as the custom genome to identify potential off-targets. Paste the protein encoding sequence and click "Get guide RNA." Choose the 20-nt sequences upstream of PAM as candidate sgRNA target sites if they (1) are located near or upstream of the central region of the encoding sequence, (2) do not span an intron in the corresponding genomic DNA, (3) do not have off-targets in the genome, and (4) have total scores exceeding 0.5.
2. Analyze RNA secondary structures of the above selected candidate sgRNA target sequences using RNAstructure web server (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html) [13]. Select one to two candidates with least complex secondary structures (with three or less hydrogen bonds based on MaxExpect and ProbKnot results) for cloning.

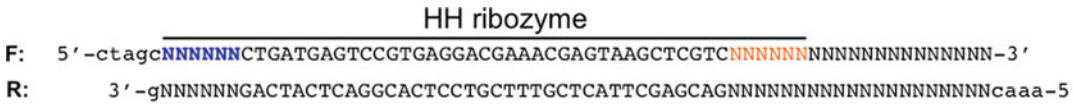

Fig. 1 Sequences of oligos F and R for annealing and cloning to pCB301TOR-CRISPR

3.1.2 Cloning of sgRNA Target Sequences

1. Design two oligos for cloning of the selected 20-nt sgRNA target sequence together with the flanking HH-ribozyme sequence to NheI and BsaI sites of pCB301TOR-CRISPR. The sequences of two oligos are shown in Fig. 1. In the oligo F, fill the 20-nt sgRNA target sequence in the 20 Ns close to 3′ end, and fill the 6 Ns close to 5′ end (shown in blue) with the reverse complementary sequence of the first 6 nucleotides (shown in red) of the 20-nt sgRNA target sequence. In the oligo R, fill all Ns with the complementary nucleotides corresponding to Ns in the oligo F. The nucleotides in lower case are the overhangs compatible with the ends of linearized vectors generated with NheI and BsaI. The oligos are custom synthesized and subjected to polyacrylamide gel electrophoresis (PAGE) purification.
2. Anneal the two oligos to generate a double-stranded DNA fragment with overhangs. Set up a 20 μL annealing reaction in a 1.5 mL centrifuge tube containing:

   5 μL 10 μM oligo F

   5 μL 10 μM oligo R

   2 μL 10 × T4 DNA ligase buffer

   8 μL H_2O

   Fill a 1 L beaker with water and heat to 95 °C, then float the tube in the water bath, and allow the temperature to cool down gradually to RT (*see* **Note 7**)
3. Digest the plasmid pCB301TOR-CRISPR with NheI and BsaI restriction enzymes in a 50 μL digestion reaction containing:

   30 μL pCB301TOR-CRISPR (about 3–5 μg)

   5 μL 10 × CutSmart buffer (NEB)

   1 μL NheI (10,000 units/mL, NEB)

   1 μL BsaI (10,000 units/mL, NEB)

   13 μL H_2O

   Incubate at 37 °C for 4–5 h.

   Run the reaction on 0.8% agarose gel with non-digested plasmids as a control to show successful digestion. The linearized plasmids are extracted from gel using QIAquick Gel Extraction Kit (Qiagen)
4. Ligate the annealed product (**step 2**) and linearized plasmids (**step 3**) in a 20 μL ligation reaction containing:

80 ng linearized pCB301TOR-CRISPR (**step 3**)

4 μL annealed product (**step 2**)

2 μL 10 × T4 DNA ligase buffer (NEB)

1 μL T4 DNA ligase (400,000 units/mL, NEB)

Add H_2O to 20 μL

Also set up a control ligation without the addition of annealed products

Leave the reaction tubes at RT for 2 h up to overnight

5. Transform 100 μL *E. coli* DH5α competent cells with 7.5 μL ligation products using the standard heat shock transformation method. Under the same treatments, the appearance of few colonies for the control ligation and more colonies for the ligation with the annealed products (sample ligation) suggests successful cloning.
6. Isolate plasmids from three to four colonies derived from sample ligation using QIAprep Spin MiniPrep Kit (Qiagen), and sequence them using the oligo RPL41pro-seqF1 (5′-GAACATCGTTCGGGGTAAGTG-3′), which is located in the RPL41 promoter and 117 bp upstream of NheI site in pCB301TOR-CRISPR.
7. Transform the plasmid with the right insert (pCB301TOR-CRISPR+sgRNA) into *Agrobacterium tumefaciens* strain EHA105 using electroporation [11].

3.2 Agrobacterium-Mediated Transformation of P. palmivora

3.2.1 Preparation of Agrobacterium tumefaciens

1. Streak *Agrobacterium tumefaciens* strain EHA105 carrying pCB301TOR-CRISPR+sgRNA (stored at −80 °C) on LB agar plate supplemented with rifampicin (15 μg/mL) and kanamycin (50 μg/mL), and grow at 28 °C for 2 days (*see* **Note 8**).
2. Collect bacteria using the bottom of a sterile 1.5 mL microcentrifuge tube, and spread on a new LB agar plate with antibiotics; grow at 28 °C overnight.
3. Scrape the cells from the overnight culture with a 1 mL pipette tip, and resuspend in 1 mL *Agrobacterium* induction medium (AIM) by pipetting up and down. Dilute the cells to $OD_{600} = 0.4$ with AIM and make a minimum of 5 mL.
4. Incubate the *Agrobacterium* suspension with the tube wrapped in foil (*see* **Note 9**) for 2 h at room temperature on a platform shaker with gentle agitation (70 rpm).

3.2.2 Preparation of Phytophthora palmivora Zoospores

1. Grow *P. palmivora* on 10% unclarified V8 agar (*see* **Note 10**) under 12-h light/12-h dark for 7–8 days at 25 °C.
2. After 1.5 h of incubation of *Agrobacterium tumefaciens* (**step 4** of Subheading 3.2.1), flood the *P. palmivora* plate with 10 mL

ice-cold sterile Milli-Q water, and incubate at 4 °C for 15 min, followed by 15 min at RT (*see* **Note 11**).

3. Transfer the released zoospore suspension (about 7–8 mL) into a 50 mL sterile centrifuge tube with a pipette without disturbing the mycelia and sporangia.
4. Take 30 μL of zoospore suspension and dilute tenfold with water to measure spore concentration. Vortex for 1 min to encyst the zoospores followed by counting with a hemocytometer. Multiply a factor of 10 to get the zoospore concentration of the zoospore sample (**step 3**). For the *P. palmivora* strain isolated from papaya, we routinely obtain a concentration of ~3×10^6 /mL.

3.2.3 Co-Incubation of Agrobacterium and Zoospores

1. In a sterile 50 mL tube, mix 5 mL of *Agrobacterium* (**step 4** of Subheading 3.2.1.) and 5 mL of zoospore suspension (**step 3** of Subheading 3.2.2.), and incubate at RT for 2 h with tubes covered with foil (*see* **Note 9**).
2. Before the end of the incubation, place 5 × 5 cm pieces of sterilized Hybond N^+ membranes onto *Agrobacterium*-zoospore co-cultivation agar (AZCA) plates, which are made on the day of transformation and stored in dark.
3. After 2 h of incubation, evenly spread 330 μL of the *Agrobacterium*-zoospore mixture onto each Hybond N^+ membrane atop of AZCA plates.
4. Allow the mixture on membranes to air-dry for about 10 min in the hood. It is not necessary to dry the membrane completely (*see* **Note 12**). Incubate the plates at 25 °C under dark conditions for 2 days.

3.2.4 Selection of G418-Resistant Transformants

1. After 2 days, transfer the Hybond N^+ membranes using sterilized forceps from AZCA plates upside down to Plich agar plates supplemented with 20 μg/mL G418 (*see* **Note 13**) and 200 μg/mL cefotaxime.
2. Squeeze out any air bubble between the membrane and the agar plates using the bottom of a sterile 1.5 mL microcentrifuge tube (*see* **Note 14**).
3. Incubate the plates under 12-h light/12-h dark at room temperature for 3 days.
4. Remove the membranes using sterile forceps and continue to incubate the plates under the same condition. The transformants usually appear 1–3 days after membrane removal.
5. Transfer the transformants onto 10% V8 agar plates with 20 μg/mL G418 (*see* **Note 13**) and 200 μg/mL cefotaxime, and grow them for 7 days under the same condition.

6. G418-resistant transformants (*see* **Note 15**) are ready for isolation of single zoospore-derived transformants (3.3). They can also be stored (**step 7**) for processing at a later time.
7. Stock the transformants by placing agar plugs of sporulating culture in sterile Milli-Q water in cryogenic vials.

3.3 Isolation of Single Zoospore-Derived Transformants

1. Use the culture from (**step 5** of Subheading 3.2.4) or grow new culture from stock (**step 7** of Subheading 3.2.4) on 10% V8 agar plate with 20 μg/mL (*see* **Note 13**) under 12-h photoperiod for 7–8 days at room temperature.
2. Scrape some sporangia and swirl into 500 μL ice-cold sterile water in a 1.5 mL microcentrifuge tube with a pipette tip. Incubate at 4 °C for 15 min and then at RT for 15 min to release zoospores.
3. Transfer 100 μL zoospore suspension in a new microcentrifuge tube, and vortex for 1 min to encyst the zoospores, followed by spore counting using a hemocytometer.
4. Dilute zoospores from the original suspension (not the one after vortexing) to a final concentration of 1000–2000 zoospores/mL.
5. Mark small circles (about 0.4 cm in diameter) on the back of nonselective Plich agar plates (*see* **Note 6**).
6. Drop 1 μL of diluted zoospore suspension on the surface of Plich agar plate within each circle. Incubate the plates under 12-h photoperiod at RT.
7. Four hours later, observe each circle under the microscope, and identify those circles containing a single germinating zoospore.
8. Cut the agar circles having a single germinated zoospore with the end of a sharp pipette tip, and transfer it to 10% V8 agar plate containing 20 μg/mL G418. Grow for 7–8 days under 12-h photoperiod at RT. We usually isolate three to five single zoospore-derived transformants for each original transformant.
9. Stock the transformants by placing plugs of sporulating culture in sterile Milli-Q water in cryogenic vials.

3.4 Detection of the T-DNA Integration in the Genomes of P. palmivora Transformants

3.4.1 Isolate Genomic DNA from Transformants Using DNeasy PowerLyzer Microbial Kit (Qiagen)

1. Add 300 μL PowerBead solution to a glass PowerBead tube.
2. Collect appropriate amount of *P. palmivora* mycelia/sporangia of single zoospore-derived transformants using a sterile applicator stick with the end pre-wetted by dipping it in the PowerBead solution, and resuspend them in PowerBead solution.
3. Add 50 μL of solution SL.
4. Homogenize the sample using FastPrep-24 instrument (MP Biomedicals) for 20 s at level 4.
5. Perform the following procedures as described in the instruction manual.

3.4.2 PCR

1. Verify the T-DNA integration by PCR using primers RPL4/Pro-SeqF1 (5′-GAACATCGTTCGGGGTAAGTG-3′) and sgRNA scaffold-R (5′-TGTCCCATTCGCCATGCCGAAG-3′), which are located within the sgRNA expression cassette in the promoter and sgRNA scaffold, respectively.
2. Perform PCR using 1 μL of gDNA as a template and Taq DNA polymerase with annealing temperature 55 °C. Wild-type (WT) *P. palmivora* is used as a negative control.
3. Analyze PCR amplification products by gel electrophoresis. The amplification of a DNA fragment of about 350 bp in the transformants but not in the WT suggests the T-DNA integration in the genome.

3.5 Detection of Mutations in the Targeted Gene

1. Amplify the gDNA fragment of the targeted gene from the WT and single zoospore-derived transformants using primers flanking the sgRNA target sequence and at least 200 bp away and Phusion high-fidelity DNA polymerase.
2. Purify the PCR products by gel purification using QIAquick Gel Extraction Kit (Qiagen) or enzymatic cleanup using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific).
3. Sequence the PCR products using Sanger sequencing, and identify the mutations that occur at 3 nt upstream of PAM, which is the cleavage site of Cas9, using Sequencher or other DNA analysis software.

4 Notes

1. If using a hydrated $CaCl_2$, make the corresponding adjustment, for example, add 0.5 g $CaCl_2 \cdot 2H_2O$ in 37.7 mL water.
2. If using $FeSO_4 \cdot 7H_2O$, dissolve 0.23 g in 50 mL water.
3. Initially use 10 N NaOH solution; when the pH is close and the chemical powder is almost completely dissolved, use 1 N NaOH solution.
4. Make fresh; use within several hours.
5. Make on the same day of transformation.
6. If used for isolation of single zoospore-derived transformants, make a thin layer of agar for the ease of observation under microscope.
7. Condensation is often seen on the inner side of the tube lid; it does not affect the result. Do a quick spin to collect all contents to the bottom of the tube and gently mix. Other annealing methods such as using a PCR machine should work as long as the temperature cools down gradually.

8. The culture can be stored at 4 °C up to 1 week before use.
9. Loose the lid of the tube to allow air in; use tape to prevent the lid from falling off.
10. Use freshly made 10% unclarified V8 agar plate to increase sporulation and zoospore production.
11. If a *P. palmivora* isolate does not produce high concentration of zoospores, the incubation time at 4 °C and RT can be extended to 30 min. In addition, multiple plates can be used. We have used three plates for a *P. palmivora* cacao isolate. In this case, add 12 mL ice-cold sterile Milli-Q water to the first plate, gently scrape sporangia/mycelia using a glass spreader, transfer the suspension to the second plate, then scrape sporangia from the second plate, and transfer suspension to the third plate. Incubate the third plate at 4 °C for 30 min, followed by 30 min at RT. Use a pluriStrainer (mesh size 20 μm) (pluriSelect) to filter the suspension, the zoospores will pass through the membrane, while the mycelia and empty sporangia will be blocked. Collect the flowthrough as the clean zoospore suspension.
12. As soon as the solution on the membrane stops flowing around, the plate can be closed.
13. Adjust the concentration of G418 based on the *P. palmivora* isolate used for transformation. Different isolates may have different levels of sensitivity. For the isolate we use, 10 μg/mL G418 is able to completely inhibit its growth. 20 μg/mL G418 is used for a stringent selection of transformants.
14. Hold the tube in horizontal position with the lid slightly higher than the bottom, and gently press and slide over the surface of the membrane.
15. Real transformants will sporulate, while false positives only grow under the agar surface. Always include wild-type strain as control.

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Chapter 7

An Effective CRISPR-Cas9 Technology for Efficiently Isolating Transgene-Free Mutants in *Arabidopsis*, *Brassica napus*, Strawberry, and Soybean

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Abstract

The CRISPR-Cas9 technique is a highly valuable tool in creating new materials for both basic and applied researches. Here, we inserted a fluorescent tag (sGFP) into the CRISPR-Cas9 vector *pKSE401* to facilitate a visual screening of mutants. This modified vector was named *pKSE401G* and tested in several dicot plant species, including *Arabidopsis*, *Brassica napus*, *Fragaria vesca* (strawberry), and *Glycine max* (soybean). Consequently, the candidate mutants were isolated through fluorescence screening, and the transgene-free mutants were sufficiently identified in *Arabidopsis* and *B. napus* in the next generation based on the absence of GFP fluorescence. Collectively, *pKSE401G* provides us an effective tool to readily identify positive primary transformants and transgene-free mutants in later generations in a wide range of dicot plant species.

Key words CRISPR-Cas9, GFP, Genome editing

1 Introduction

The CRISPR-Cas9-mediated genome-editing technology provides unprecedented tools to precisely edit DNA sequences in animals and plants [1, 2]. This technology requires expression of the Cas9 protein, production of a guide RNA (gRNA) that complements the target DNA sequences, and the existence of an NGG protospacer adjacent motif (PAM) site in the target sequence [2, 3]. Briefly, genome editing mediated by CRISPR-Cas9 utilizes a 20-bp gRNA that directs the Cas9 nuclease to the target site by base pairing. Cas9 cuts the target site to generate a double-strand break (DSB). Mutations are introduced during the DNA repairing process. Because of its simplicity, CRISPR-Cas9 has been widely adopted. Several CRISPR vectors have been developed for genome editing in plants [3–8]. It was proven that CRISPR-Cas9-mediated genome-editing technology could successfully generate various heritable mutations in plant species [9].

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After transformation, the first mission is to isolate primary transformants with expected mutations. To this end, restriction enzyme digestion or sequencing of the PCR amplicons is usually performed [8–10]. However, both methods are time- and money-consuming and laborious. Next, mutants without the T-DNA insertion may need to be identified, which are favored in both basic and applied researches because of the following reasons. First, prolonged existence of CRISPR-Cas9 in the mutants would greatly increase the risk of producing off-target mutations. Second, transgene-free materials are more easily accepted by the public. Cas9-free mutants could be obtained by self-crossing or backcrossing. In *B. napus*, for instance, by using gene-specific primers (such as *Cas9*), 10.9% (58/530) of the mutant plants in the T1 generation lost the Cas9 transgene by self-crossing [8], a ratio lower than what is expected, which need larger populations to get Cas9-free mutant. Therefore, an easy method to screen for the mutants in need in both primary transformants and their offspring is highly required.

As previously reported, making use of fluorescent proteins as visible markers would not only speed up the screening of positive transformants but also help to isolate transgene-free offspring [11, 12]. In this study, we developed a new vector used for visual screen of transgenic plants by adding a *35S::sGFP* cassette in the CRISPR-Cas9 vector *pKSE401*. This modified vector was named *pKSE401G* and successfully applied in *B.* napus, *Arabidopsis*, strawberry, and soybean, respectively. The Cas9-free mutants could be readily isolated according to the absence of GFP fluorescence, and the mutations were stably inherited into the next generation. Our strategy of using GFP and dual gRNAs provides an efficient tool to screen for edited plants and isolate Cas9-free mutants with the desired mutations.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cmat 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Plant and Bacteria Material

Arabidopsis thaliana: The ecotype *Col-0* is used for *Arabidopsis* transformation.

Brassica napus: *Westar* cultivar is used for *B. napus* transformation.

Strawberry: The strawberry variety Ruegen (Ru F7-4, red-fruited) [13] is used for agrobacterium transfection.

Soybean: The variety Williams 82 (W82) is used as wild types used for agrobacterium transfection.

The plants were cultivated in a growth room under a light intensity of 100 μmol m^{-2} s^{-1} with a 16/8-h light/dark photoperiod at 22 °C (*B. napus*, *Arabidopsis*, and strawberry) or 25 °C (soybean).

***E. coli* strain**: *E. coli* DH5α strain carrying the *pCBC-DT1T2* intermediate plasmid and *pKSE401G* binary plasmid which are used for preparation of construct(s) with sgRNA expression cassette (s) for plant transformation with appropriate U6 promoters for dicot plant(s).

Agrobacterium strain: The agrobacterium strain *GV3101*, carrying a binary vector harboring the gene of interest and a selectable marker such as kanamycin.

2.2 Bacteria Growth Media

LB agar plates containing 50 μg mL^{-1} kanamycin.

LB agar plate containing 100 μg mL^{-1} chloramphenicol.

LB liquid medium containing 50 μg mL^{-1} kanamycin.

LB liquid medium containing 100 μg/mL mL^{-1} chloramphenicol.

Universal primer set for amplifying the sgRNA constructs.

Shaking incubator or tube roller.

Gene Pulser Xcell Electroporation System (BioRad, Cat# 617BR1-08950) with 1-mm electroporation cuvettes.

2.3 PCR and Cloning Relative Reagent

BSA (NEB, Cat#B9001).

BsaI (NEB, Cat#R0535S).

T4 DNA ligase with 10 × buffer (NEB, Cat# M0202s, 400 U/μL).

KOD Plus high-fidelity DNA polymerase and 10× buffer containing 250 μM dNTPs (Toyobo, Cat# KOD-201).

PCR product purification kit (Sangon Biotech, Cat#SK8142).

PCR thermal cycler.

2.4 Arabidopsis Transformation Media

MS 2.2 g L^{-1}, sucrose 5 g L^{-1}, 6-BA 1 mg mL^{-1}, Silwet-77 0.04% (v/v)

2.5 B. napus Tissue Culture Media

Seed germination medium: MS 2.2 g L^{-1}, sucrose 10 g L^{-1}, and Phytagel 4 g L^{-1}. Adjust the pH to 5.8 before addition of Phytagel.

Cocultivation medium (M1): MS 4.43 g L^{-1}; sucrose 30 g L^{-1}; acetosyringone 100 mM L^{-1}; pH 5.8–5.9.

Callus induction medium (M2): MS 4.43 g L^{-1}; sucrose 30 g L^{-1}; acetosyringone 100 mM L^{-1}; mannitol 18 g L^{-1}; 2,4-D 1 mg L^{-1}; kinetin 0.3 mg mL^{-1}; pH 5.8–5.9.

Shoot initiation medium (M3): MS 4.43 g L^{-1}; glucose 10 g L^{-1}; xylose 0.25 g L^{-1}; zeatin 2 mg L^{-1}; IAA 0.1 mg L^{-1}; Timentin 270 mg L^{-1}; pH 5.8–5.9.

Root initiation medium (M4): MS 2.22 g L^{-1}; sucrose 10 g L^{-1}; IBA 0.5 mg L^{-1}; Timentin 135 mg L^{-1}; pH 5.8–5.9.

2.6 Strawberry Infiltration Media

MS 4.43 g L^{-1}; sucrose 20 g L^{-1}.

2.7 Soybean Hairy Root Transformation Media (CCM)

MS 0.443 g L^{-1}; 3.9 g L^{-1} morpholino ethanesulfonic acid; 150 mg L^{-1} cysteine; and 150 mg L^{-1} dithiothreitol.

3 Methods

The workflow of generation reliable genome-editing transgenic plants is shown in Fig. 1.

3.1 Selection of sgRNA Targeting Sequences and Design of Target Adaptors

The target sgRNA sequences are designed using the web server CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) (Fig. 2a) [14]; within either strand of the target gene sequences, select regular target site(s) with 5′-N_{20}NGG-3′ as the candidate target (s). Then the primers are designed as Fig. 2b.

To obtain high editing efficiency, the GC content of candidate target sequences should be around 50% to 70%. Target sequences

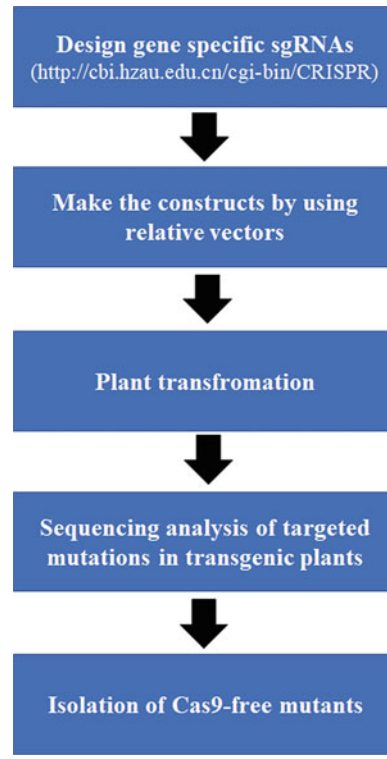

Fig. 1 The workflow used in this study. The workflow of generation CRISPR-Cas9 genome-editing transgenic plants, from target sgRNAs designing, plant transformation, and isolating positive mutant plants

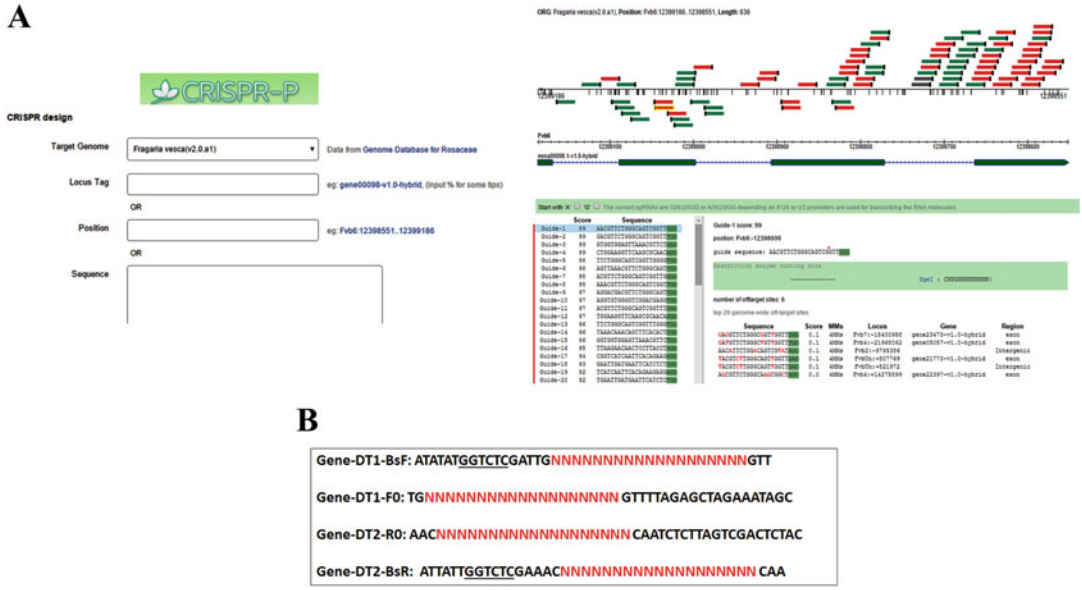

Fig. 2 Design of the primer sets containing gene-specific sgRNAs. (**a**) At the "submit" page, select a genome you are studying, and input gene locus, chromosome position, or DNA sequence in FASTA format. Other parameters are set as default. A few seconds after submission, the result is shown in web browser, which contains sgRNA sequence information, including GC content, on-target score, off-target score, and restriction endonuclease sites. (**b**) Choose the sgRNA sequences, and replace 19-nt N in the forward primers with 19-nt target sequences in front of PAM (NGG) and 19-nt N in the reverse primers with reverse complement sequences of 19-nt target sequences in front of PAM (NGG)

having four or more consecutive T nucleotides should be avoided [6]. In addition, the formation of hairpin or stem structure of longer than continuous 6 bp between a target sequence and the sgRNA sequence may decrease editing efficiency. Therefore, conduct a secondary structure analysis of the candidate target-sgRNA sequences using a program (such as http://skl.scau.edu.cn/) [15] to exclude target candidates that form hairpins or stems of longer than 6 bp with the sgRNA sequence. Furthermore, the candidate sgRNAs target sequences are blasted to the reference genome using BLASTN at NCBI (http://blast.ncbi.nlm.nih.gov) to avoid the nonspecific off-targeting.

3.2 Isolation of the Binary Plasmids and sgRNA Intermediate Plasmids

Streak the *E. coli* DR5α strain(s) carrying the selected binary plasmid *pKSE401G* (Fig. 3a) [16] onto LB agar plate(s) containing 50 μg mL^{-1} kanamycin. Streak the *E. coli* DR5α strain(s) carrying the selected sgRNA intermediate plasmids *pCBC-DT1T2* (Fig. 3b) [17] onto LB agar plates containing 100 μg mL^{-1} chloramphenicol. Incubate the plates at 37 °C overnight.

Pick single colonies of each strain to inoculate 2-mL LB liquid medium containing the appropriate antibiotic. Grow the cells overnight at 37 °C with shaking at 200 rpm or on a tube roller.

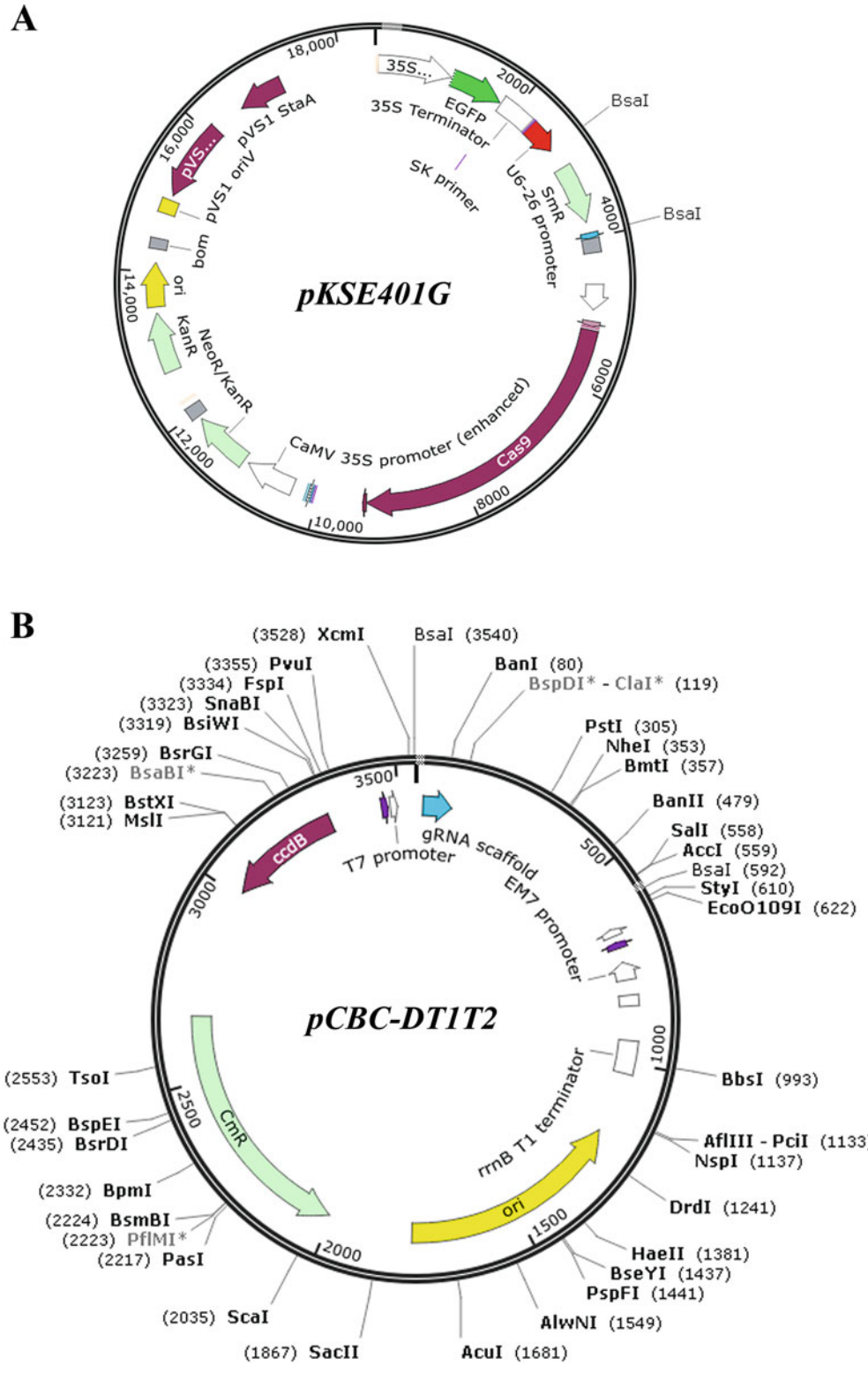

Fig. 3 Construction of *pKSE401G.* (**a**) The CRISPR-Cas9 vector *pKSE401G* is modified from *pKSE401* [17]. The *35S-sGFP-terminator* cassette is amplified from *pK7GWIWG2D* [21] using primers *35S-GFP-Ter-F* and *35S-GFP-Ter-R* and inserted into the *PmeI* site of *pKSE401* by the Gibson Assembly method [22]. (**b**) The map of *pCBC-DT1T2* [17]

The plasmids are isolated from 1.5 to 2 mL of culture using a plasmid miniprep isolation method (Molecular Cloning, third edition), precipitate the recovered plasmid DNA solutions using a standard isopropanol precipitation protocol, wash the pellets twice with 70% ethanol, and air-dry, and dissolve in 30 μL ddH_2O.

3.3 Assembly of sgRNA Expression Cassettes into a CRISPR-Cas9 Binary Construct

Using *pCBC-DT1T2* as the template, two *AtU6 promoter-sgRNA-AtU6 terminator* cassettes are amplified by PCR using the primers listed in Table 1 (Fig. 2b). Set up PCR (50 μL) containing PCR reagents as follows:

Components	Volumes (μL)
KOD plus	1
KOD plus buffer	5
$MgSO_4$	3.5
dNTP(2 mM)	5
DMSO	2.5
pCBC-DT1T2 (100 times dilution)	5
Gene-DT1-BsF	5
Gene-DT1-F0	0.25
Gene-DT2-R0	0.25
Gene-DT2-BsR	5
ddH_2O	17.5
Total	50

Perform PCR using the following thermal cycling program:

- 1 cycle: 5 min 95 °C
- 30 cycles: 30 s 95 °C (denaturation)
  30 s 60 °C (annealing)
  60 s 68 °C (extension)

Optionally check 4 μL of PCR product on a 1.2% agarose gel. Confirm the specificity and estimate the quantity of each PCR product.

Then the PCR fragments are inserted into *pKSE401G* by Golden Gate Assembly (Fig. 4) [17] containing reaction reagents as follows:

Table 1
The list of primers used in this study

Primer name	Primer sequence (5′ to 3′)	Purpose of the primers
RPK1-DT1-BsF	ATATATGGTCTCGATTGCTAACTGTATCGGTCACGGGTT	Making RPK1 CRISPR construct
RPK1-DT1-F0	TGCTAACTGTATCGGTCACGGGTTTTAGAGCTAGAAATAGC	Making RPK1 CRISPR construct
RPK1-DT2-R0	AACAAGGAGATTTGGGGGTTGGCAATCTCTTAGTCGACTCTAC	Making RPK1 CRISPR construct
RPK1-DT2-BsR	ATTATTGGTCTCGAAACAAGGAGATTTGGGGGTTGGCAA	Making RPK1 CRISPR construct
BnaARF2-DT1-BsF	ATATATGGTCTCGATTGAGGCTTTTTGGCATTCCTCGTT	Making BnaARF2 CRISPR construct
BnaARF2-DT1-F0	TGAGGCTTTTTGGCATTCCTCGTTTTAGAGCTAGAAATAGC	Making BnaARF2 CRISPR construct
BnaARF2-DT2-R0	AACCGAAGGCATTTGCTTCAGACAATCTCTTAGTCGACTCTAC	Making BnaARF2 CRISPR construct
BnaARF2-DT2-BsR	ATTATTGGTCTCGAAACCGAAGGCATTTGCTTCAGACAA	Making BnaARF2 CRISPR construct
FvMyb10-DT1-BsF	ATATATGGTCTCGATTGGTTATTTCGGTGTGAGAAAGTT	Making FvMyb10 CRISPR construct
FvMyb10-DT1-F0	TGGTTATTTCGGTGTGAGAAAGTTTTAGAGCTAGAAATAGC	Making FvMyb10 CRISPR construct
FvMyb10-DT2-R0	AACCTGCAAACTCTCCTCTCTTCAATCTCTTAGTCGACTCTAC	Making FvMyb10 CRISPR construct
FvMyb10-DT2-BsR	ATTATTGGTCTCGAAACCTGCAAACTCTCCTCTCTTCAA	Making FvMyb10 CRISPR construct
GmNFR1a-DT1-BsF	ATATATGGTCTCGATTGGCTTCCTACTATGTCAGTCGTT	Making GmNFR1a CRISPR construct
GmNFR1a -DT1-F0	TGGCTTCCTACTATGTCAGTCGTTTTAGAGCTAGAAATAGC	Making GmNFR1a CRISPR construct
GmNFR1a -DT2-R0	AACATACAGTCACAGGGGAATGCAATCTCTTAGTCGACTCTAC	Making GmNFR1a CRISPR construct
GmNFR1a -DT2-BsR	ATTATTGGTCTCGAAACATACAGTCACAGGGGAATGCAA	Making GmNFR1a CRISPR construct
GmNFR1a–checking-F	ACCCTGTATAGAAAGTCCTAG	Checking GmNFR1a gene editing
GmNFR1a–checking-R	CATTAACCCTGGCATTTGCAG	Checking GmNFR1a gene editing
AtRPK1-DS1_sgR1-F	CTAACTGTATCGGTCACGG	Checking RPK1-sgRNA1 gene editing
AtRPK1-DS1_sgR1-R	TCTCGGTTTCGCTCGCATGGTAAC	Checking RPK1-sgRNA1 gene editing

AtRPK1-DS2_sgR2-F	CGAGAGTGGTGGTGGTCTCCACG	Checking RPK1-sgRNA2 gene editing
AtRPK1-DS2_sgR2-R	AACCCCCAAATCTCCTTTGG	Checking RPK1-sgRNA2 gene editing
FvMyb10-checking-F	TTCTCTTTCGTAAAGTAATTAATG	Checking FvMyb10 gene editing
FvMyb10-checking-R	TAGCTTTGGAATTCCAGAAG	Checking FvMyb10 gene editing
BnaC9.ARF2-sgRNA1-F	CTTCTAACCCATTTTCCTGAGTTTTGG	Checking BnaC9.ARF2-sgRNA1 gene editing
BnaC3.ARF2-sgRNA1-F	CTGTTTTTGTTAGATCATACTATTATTCAAGAAGCC	Checking BnaC3.ARF2-sgRNA1 gene editing
BnaA9.ARF2-sgRNA1-F	CACTTGCTTCTTATTTGTATTGAAACATATTTGAC	Checking BnaA9.ARF2-sgRNA1 gene editing
BnaA6.ARF2-sgRNA1-F	GCAGAGTATAACAGGTTGATCATATTATTCAAG	Checking BnaA6.ARF2-sgRNA1 gene editing
BnaARF2-sgRNA1-R	GCCTTACACCCACACGTAATTC	Conserved primer for BnaARF2-sgRNA1 site
BnaARF2-sgRNA2-F	AACTGGCCAATACGTCCAC	Conserved primer for BnaARF2-sgRNA2 site
BnaC9.ARF2-sgRNA2-R	AAGTTATTAATTCAGAAGAACACACAAGTATAA	Checking BnaC9.ARF2-sgRNA2 gene editing
BnaC3.ARF2-sgRNA2-R	CTCTGCGTTACAAAAGTCTCGTTAGT	Checking BnaC3.ARF2-sgRNA2 gene editing
BnaA9.ARF2-sgRNA2-R	CTCTCTAGTAACCTGTGAACACATATGTA	Checking BnaA9.ARF2-sgRNA2 gene editing
BnaA6.ARF2-sgRNA2-R	AACACCCAAGTCTCATTTGTCTGTAAT	Checking BnaA6.ARF2-sgRNA2 gene editing
35S-GFP-Ter-F	CTGTCAAACACTGATAGTTTTGCATGCCTGCAGGTCAAC	Amplify 35S-GFP-terminator cassette
35S-GFP-Ter-R	TCGTTTCCCGCCTTCAGTTTTCGACCATATGGGAGAGCTC	Amplify 35S-GFP-terminator cassette
Cas9-s	AGACCGTGAAGGTTGTGGAC	Cas9 gene checking primer
Cas9-r	TAGTGATCTGCCGTGTCTCG	Cas9 gene checking primer
NPTII-s	ATGGGGATTGAACAAGATGGAT	NPTII gene checking primer
NPTII-r	CAGAAGAACTCGTCAAGAAGGCG	NPTII gene checking primer
U626-s	TGTCCCAGGATTAGAATGATTAGGC	pKSE401 fragment checking primer
U629-r	GTCAGGCTGCAGTAGTTTCCATTAA	pKSE401 fragment checking primer
GFP-s	ATGGTGAGCAAGGGCGAGGAGC	GFP gene checking primer

(continued)

Table 1
(continued)

Primer name	Primer sequence (5′ to 3′)	Purpose of the primers
GFP-r	TTACTTGTACAGCTCGTCCATGC	GFP gene checking primer
BnaActin-s	ATGTGATGTGGATATCAGGAAGGAT	qPCR primer of *B. napus* internal control gene
BnaActin-r	ACGGTCCAGATTCGTCATACTCA	qPCR primer of *B. napus* internal control gene
Atactin2-s	GTTCCAGCCCTCGTTTGTG	qPCR primer of *Arabidopsis* internal control gene
Atactin2-r	CAAGTGCTGTGATTTCTTTGCTC	qPCR primer of *Arabidopsis* internal control gene

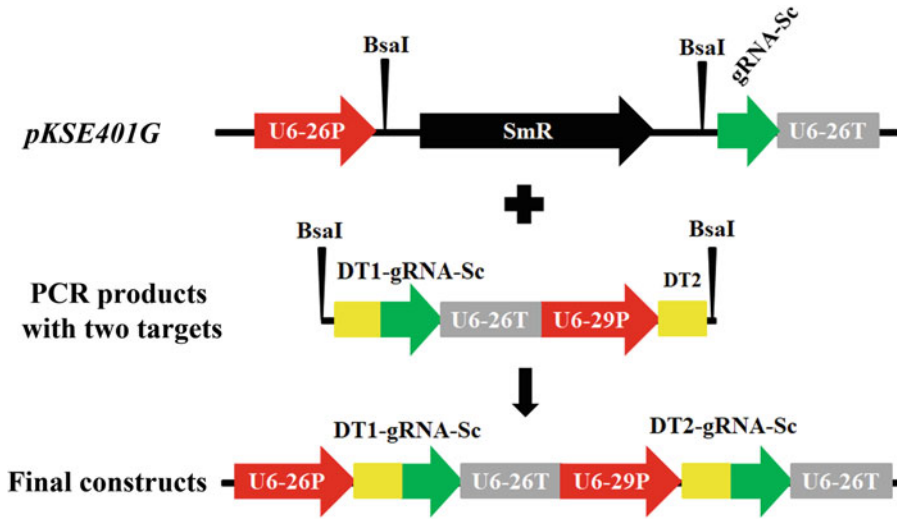

Fig. 4 The gRNA modules used for the assembly of two gRNA expression cassettes. Examples of the assembly of two-gRNA expression cassettes for dicots using the gRNA modules. Using *pCBC-DT1T2* as the template, two *AtU6 promoter-sgRNA-AtU6 terminator* cassettes were amplified by PCR, and the PCR fragments were then inserted into *pKSE401G* by Golden Gate Assembly. U6-29p and U6-26p are two *Arabidopsis* U6 gene promoters; U6-29t and U6-26t, corresponding *Arabidopsis* U6 gene terminators with downstream sequences, respectively. gRNA-Sc, gRNA scaffold; DT1/2, dicot target-1/2

Components	Volumes (μL)
PCR fragments (626 bp)	2
pKSE401	2
10× NEB T4 buffer	1.5
10× BSA	1.5
BsaI	1
T4 ligase	1
ddH_2O	6
Total	15

Perform the reaction in a thermal cycler using the following program:

- 50 cycles: 3 min 37 °C
  4 min 16 °C
- 1 cycle: 5 min 50 °C
  5 min 80 °C
  5 min 12 °C

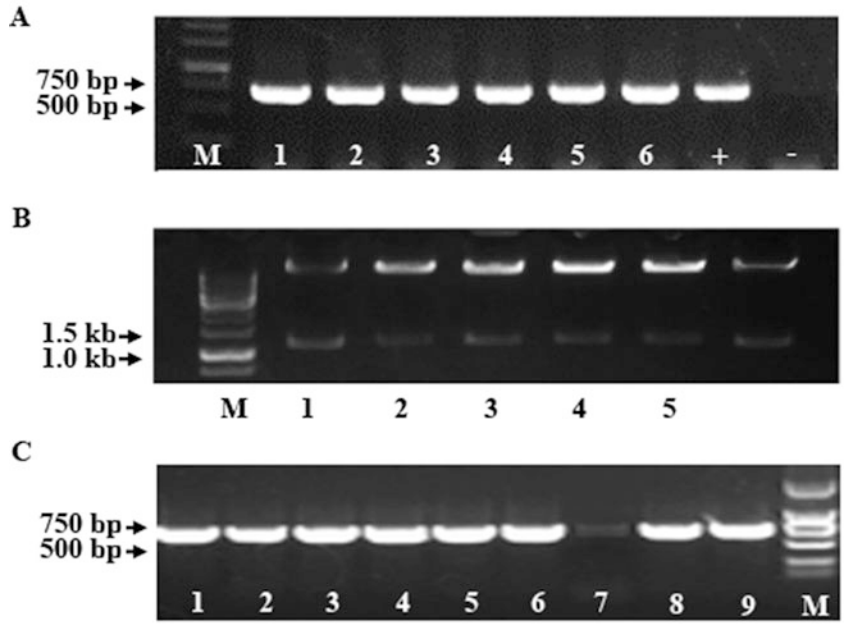

Fig. 5 Gel image showing the positive clones by cloning PCR and restriction enzyme digestion. (**a**) Gel image showing the positive clones after *E. coli* transformation. (**b**) Gel image showing the positive clones by HindIII (restriction enzyme) digestion. (**c**) Gel image showing the positive clones after agrobacterial transformation. The number indicated different clones. "+" indicated positive control, and "-" indicated negative control. The arrows indicated size of DNA ladders

3.4 Transformation of E. coli *and* A. tumefaciens *Strains*

For *E. coli* transformation, 5 μL of the ligation product is mixed with 20 μL *E. coli* DH5α competent cells and is kept on ice for 30 min. Then, the competent cells are shocked by water bath (42 °C) for 90 s, and add 1-mL LB media (without antibiotics), and after place on ice for 2 min. Shake the cells at 200 rpm at 37 °C for 1.5 h. Plate the cells on a LB agar plate containing 50 μg mL^{-1} kanamycin.

Pick several colonies for a plasmid miniprep (2 mL LB culture). To screen positive clones, the PCR are set up (20 μL, using Taq enzyme) using the target adapter primers to form the primer sets: U626-F/U629-R. After PCR, check the products on a 1% agarose gel (Fig. 5a). Use the following reaction mix:

Components	Volumes (μL)
Taq polymerase	0.3
1× Taq buffer	2
$MgCl_2$	1.5
dNTP(2 mM)	0.5
Plasmid	0.1

(continued)

Components	Volumes (μL)
U626-F	1
U629-R	1
ddH_2O	14.6
Total	20

Perform the PCR using the following thermal cycling conditions:

- 1 cycle: 5 min 95 °C
- 30 cycles: 30 s 95 °C (denaturation)
  30 s 60 °C (annealing)
  60 s 72 °C (extension)

Optionally check 4 μL of PCR product on a 1.2% agarose gel. Confirm the specificity and estimate the quantity of each PCR product.

Further, the positive plasmids are analyzed by restriction digestion with HindIII (Fig. 5b).

Optional but recommended: Sequence the construct with primer U626-F.

For *A. tumefaciens* transformation, 1 μL of the plasmid is mixed with 20 μL *A. tumefaciens* competent cells GV3101 using standard electroporation conditions (voltage of 1500–1600 V for a 1-mm cuvette or 2500 V for a 1-mm cuvette), and immediately add 1 mL LB at 28 °C for 2 h before plating. Plate the cells on LB agar medium with 50 μg mL^{-1} kanamycin and 25 μg mL^{-1} gentamycin, and incubate plate at 28 °C for 2 days. The positive clones are confirmed by PCR as *E. coli* colonies (Fig. 5c).

3.5 Plant Transformation

Use the *A. tumefaciens* cells containing the intact construct for plant transformation with a suitable method.

For *Arabidopsis* transformation, the CRISPR-Cas9 constructs are transformed into *Arabidopsis* wild-type *Col-0* through floral dipping [18]. The procedure of *Agrobacterium*-mediated *B. napus* transformation is carried out as previously described [8]. Briefly, the explants were incubated in the *Agrobacterium*-infection buffer (MS 4.43 g L^{-1}; sucrose 30 g L^{-1}; acetosyringone 100 mM L^{-1}; pH 5.8–5.9) for 20 min, and then transfer to M1 medium plates and keep in dark for 48 h. Afterward, the explants are transferred to M2 medium plates with proper selection antibiotics to induce callus growth. The calli are transferred to M3 and followed by M4 medium to let shoot and root regenerate, respectively.

The strawberry fruit transient transformation assay is performed as described [19]. Briefly, a single agrobacterium

(GV3101) colony is picked and grown in 2 mL of liquid LB medium until OD_{600} reached around 0.8–1.0. Then the culture is spun down and resuspended in the infiltration buffer to reach an OD_{600} of 0.8. Fruits at the white stage are used for injection. The color phenotype is examined 1 week after injection.

A. rhizogenes (K599)-mediated hairy root transformation is performed on soybean W82 as previously described [20]. In brief, a single colony is picked and grown in 10 mL of liquid LB medium until OD_{600} reached around 0.4–0.6. Then the culture is spun down and resuspended in the CCM buffer to reach an OD_{600} of 0.2–0.3. Then the roots are incubated in the solution for 2–3 days. The phenotype was observed 4 weeks after transformation.

3.6 Sequencing Analysis of Targeted Mutations in Transgenic Plants

All the mutants are first screened by GFP fluorescence, which is examined using a dissecting microscope equipped with a GFP filter (Olympus, SZX16; Excitation/Emission 488 nm/509 nm) (Fig. 6). There are two alleles for each gene, both of which might be mutated by CRISPR-Cas9. Thus, CRISPR-Cas9 could produce five genotypes: homozygote (the two alleles have the same mutation), biallele (the two alleles have different mutations), heterozygote (only one allele is mutated), chimera (more than two different mutations exist), and WT type (no mutation). Traditional Sanger-sequencing chromatograms of plants with biallelic, heterozygous, and homozygous mutations can be decoded by the Web-based tool DSDecode [15]. On the other hand, genotyping of chimeric mutations requires cloning of the amplicons followed

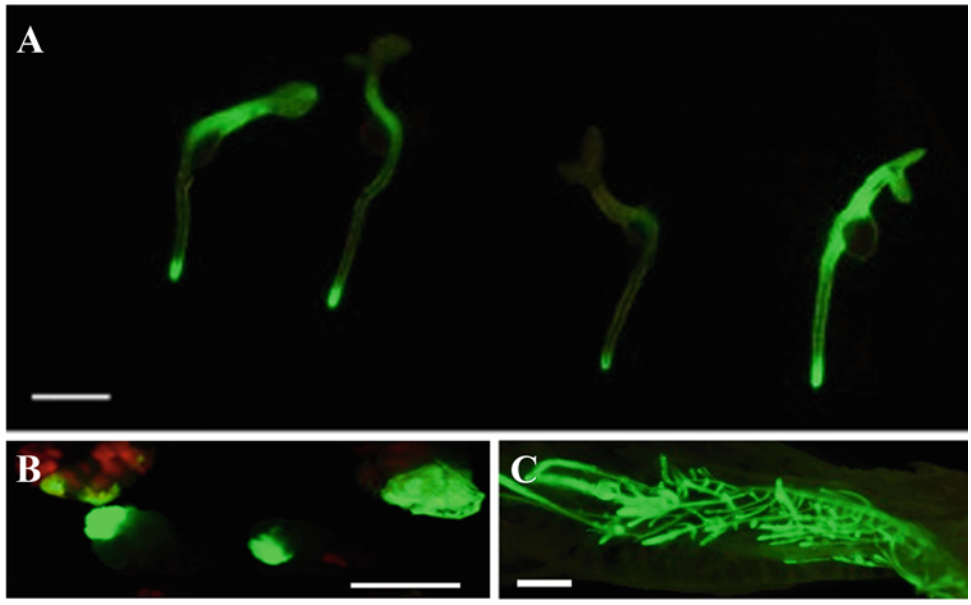

Fig. 6 Visual screen of positive transgenic plants. (**a**) Visual screen of positive transgenic *Arabidopsis* in the T1 generation. GFP signal was visualized in seedlings. Visual screen of positive transgenic *B. napus* in calli (**b**), and roots (**c**). From **a**–**c**, the bar was indicated 5 mm

by sequencing of multiple clones or requires deep sequencing (such as Illumina sequencing) of the amplicons.

Specific PCR primer pairs are designed which are located at about 150–250 bp upstream and downstream of the target sites, respectively. For multiplex targeting involving two or more closely arrayed sites, design specific PCR primer pairs for amplification of the genomic fragments (about 2–3 kb or shorter for each fragment) containing these target sites.

The target-containing genomic fragments are amplified from transgenic plants using standard PCR with Taq DNA polymerase. PCR products are purified by agarose gel electrophoresis and a purification kit.

All types of mutations except for chimeric ones, i.e., biallelic, heterozygous, and homozygous mutations, can be resolved automatically by DSDecode. This tool only needs to copy/paste a wild-type reference sequence containing the target site and covering the sequencing range and upload a sequencing chromatogram file (abi format) into the program. A decoding task, from data upload to result display, requires few seconds.

3.7 Isolation of Cas9-Free Mutants

Genetically manipulated materials without T-DNA insertions are largely favored for crop improvement because they are classified as non-GMO plants in the USA, Germany, and Sweden. The transgenic lines from previous generation are screened by dissecting microscope with GFP filter, and the Cas9-free candidate mutants are isolated based on the fluoresce signal "on" or "off." The GFP-negative plants are further checked by PCR using *Cas9*-, *GFP*-, and *NPTII*-specific primers, respectively.

4 Conclusions

Compared to traditional mutagenesis strategies, CRISPR-Cas9-targeted genome editing is precise and efficient simultaneously knocking out a group of genes. Therefore, CRISPR-Cas9 is extremely useful to create ideal materials for functional studies in plant species and crop domestication [1]. The fluorescent proteins as visible markers would not only speed up the screening of positive transformants.

Then, mutants without the T-DNA insertion may need to be identified in next generation, which are favored in both basic and applied researches because of the following reasons. First, prolonged existence of CRISPR-Cas9 in the mutants would greatly increase the risk of producing off-target mutations. Second, transgene-free materials are more easily accepted by the public. Cas9-free mutants could be obtained by self-crossing or backcrossing. Base on the fluorescence "on" or "off," the Cas9-free offspring is easily isolated.

In summary, we demonstrate that the CRISPR-Cas9 is a highly efficient tool for genome editing in several plant species. We find that sgRNAs derived from conserved sequences could simultaneously induce homozygous or biallelic mutations at multiple loci. Moreover, the targeted gene modification mediated by CRISPR-Cas9 is safer for both human health and the environment because it is possible to remove the foreign DNA following the mutagenesis of the target DNA. Thus, CRISPR-Cas9 is useful for both basic and applied research in plants.

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Chapter 8

An Optimized RNA-Guided Cas9 System for Efficient Simplex and Multiplex Genome Editing in Barley (*Hordeum vulgare* L.)

Sebastian Gasparis and Mateusz Przyborowski

Abstract

The ability to create genetic mutants is crucial for genomics studies in plants and for crop improvement. However, until recently, knockout mutants were created mainly by random mutagenesis, which produced many undesirable mutations and genomic rearrangements. With the emergence of programmable site-specific nucleases, mutations can be introduced precisely at specific genomic locations. CRISPR-Cas9 technology represents the most versatile and efficient genome editing tool to date, and the number of species in which this technology has been applied is still rising. Recently, many efforts have been made to adapt the CRISPR-Cas9 system for efficient genome editing of economically important crop species. At present, CRISPR-Cas9-based targeted mutagenesis of single and multiple target genes can be utilized in barley with near 90% efficiency. Cas9 and sgRNA can be introduced to barley plants in a single T-DNA construct via stable *Agrobacterium*-mediated transformation of immature embryos. Mutations induced at the target loci are detected in T_0 plants, and homozygous transgene-free mutants segregate in the T_1 and T_2 generations. Here, we present methods and strategies for the induction of single and multiple knockout mutations in barley. The protocol covers all important steps, from target sequence selection and construct optimization and assembly to mutation detection and selection of nontransgenic mutant lines.

Key words Barley, Cas9, sgRNA, PTG, Mutant, Mutation detection

1 Introduction

CRISPR-Cas9 has emerged as an alternative to the ZFN (zinc finger nuclease) and TALEN (transcription activator-like effector nuclease) genome editing technologies and was implemented in plants in 2013 shortly after its discovery [1–3]. The simplicity, efficiency, and versatility of this tool have revolutionized the genome engineering of many eukaryotic organisms. It was adapted from the bacterial type II adaptive immune system in which clustered regularly interspaced short palindromic repeat (CRISPR) loci consist of multiple short fragments (spacers) that were acquired from invading foreign DNA, such as bacteriophages. Transcripts of

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the CRISPR array are processed to noncoding molecules of guide RNA that form complexes with the Cas9 endonuclease protein [4, 5]. Due to the sequence specificity of the gRNA, the Cas9/gRNA complex can capture and cut the foreign DNA at specific target sites. The sequences of the Cas9 gene and sgRNA (single-guide RNA) are the only elements of the CRISPR-Cas9 system that need to be expressed in plant cells, and they can be introduced in a single T-DNA construct. sgRNA is a synthetic RNA chimera created by fusing native crRNA with tracrRNA, and it preserves the full functionality of the bacterial archetype. Specificity to target DNA is conferred by the guide sequence located at the 5′ end of the sgRNA. The canonical length of the guide sequence is 20 bp. Therefore, by modifying this 20 bp fragment, it is possible to create sgRNAs specific to any 20 bp target sequence followed by the consensus NGG sequence of the PAM (protospacer adjacent motif). DNA double-strand breaks (DSBs) generated by Cas9/sgRNA site-specific DNA cleavage induce DNA repair. In somatic cells, DNA is repaired mostly via the NHEJ pathway (nonhomologous end joining), which causes random insertions and deletions, which in turn may result in frameshift mutations.

Just after the emergence of the CRISPR-Cas9 system, it was successfully applied to model plants, such as *Arabidopsis* and tobacco, where it worked very efficiently, although the coding sequence of Cas9 was not optimized for plants [1, 6, 7]. However, in many crop species, especially monocots, the respective elements of the CRISPR-Cas9 system require some optimization to work with a similar efficiency to that in model plants. There are three key prerequisites to achieve efficient Cas9/sgRNA-based genome editing in barley. These are (i) knowledge of the target genes' sequences and structures, (ii) correct design of monocot-optimized expression cassettes, and (iii) use of efficient methods for stable genetic transformation. The first prerequisite should not be problematic because the whole genome sequence of barley is deposited in public databases, and most of the known genes have been mapped and annotated. Moreover, several online tools have been developed recently to facilitate the selection of optimal target sequences and to predict potential nonspecific "off-targets," and some of these tools are recommended in our protocol. Additionally, many Cas9/sgRNA platforms for plant genome editing have been developed, and some of them are available from plasmid repositories; however, not all of these platforms will be suitable for application to barley. As mentioned before, Cas9 and sgRNA are the two necessary elements of the CRISPR-Cas9 system that need to be introduced into host cells. Both elements need to be efficiently expressed to work effectively, and this can be achieved with properly designed expression cassettes. The Cas9 is a monomeric protein with endonucleolytic activity that was first discovered in the bacterium *Streptococcus pyogenes*. Because of its prokaryotic origin, there are

significant differences in coding sequence, in terms of both GC content and codon usage, between the native Cas9 and coding sequences of monocot plants. For this reason, it is a common practice to use a synthetic Cas9 gene with a codon-optimized sequence corresponding to the codon usage observed in the host plant. The positive relationship between codon usage and translation efficiency of heterologous proteins in plants has been established, and properly optimized sequences may lead to as much as 100-fold increases in protein accumulation [8, 9]. It has also been indicated that the use of plant codon-optimized *Cas9* under monocot-specific promoters, such as the maize *Ubi* promoter, results in a greater mutation rate, ranging from 60 to 100% [10–14], compared to an efficiency of 8.6–45.3% with the human codon-optimized Cas9 and/or 35S promoter [15–17]. The expression of *Cas9* can be additionally enhanced by using other regulatory elements, such as introns. We used the *UBQ10-i1* intron from *Arabidopsis thaliana* for intron-mediated enhancement of Cas9 [14]. This type of promoter-proximal intron may increase the expression levels of transgenes in plants when placed in the 5′ coding region [18]. *UBQ10-i1* is among the best studied enhancing introns in plants, and its enhancing effect has already been proven in barley [19]. sgRNAs in plants are transcribed by RNA polymerase III, and their expression is driven by U3 or U6 small nuclear RNA gene promoters [3]. For application to barley, a monocot-derived U3 or U6 promoter should be used for sgRNA expression rather than their counterparts from *Arabidopsis* or other dicots.

Finally, the methods of delivery of Cas9 and sgRNAs into plant cells is the third most important factor affecting the final efficiency of genome editing. For barley, the stable *Agrobacterium*-mediated transformation of immature embryos is the most suitable method because it allows for the generation of a large number of fertile transgenic plants. A spring cultivar, Golden Promise, is the most susceptible genotype, and using well-established agro-transformation protocols, it is possible to achieve up to 25% transformation efficiency [20, 21].

Here, we present methods and strategies for CRISPR-Cas9-based genome editing in barley. The procedure covers all steps from target sequence selection and construct assembly to detection of transgene-free mutant lines. The main part of the protocol is devoted to the design and assembly of monocot-optimized Cas9 and sgRNA constructs. To facilitate the generation of multiple sets of Cas9/sgRNA constructs, we propose a strategy in which the sgRNA expression cassette and Cas9 expression cassette are assembled in two separate vectors that are compatible with Gateway LR recombination. This approach allows for the elimination of multiple cloning steps with potentially incompatible restriction enzymes and ensures greater versatility of the system. We also describe a

strategy for multiplex genome editing using the tRNA processing system proposed by Xie et al. [10]. The main advantage of this system is that multiple sgRNAs for different target sequences are simultaneously produced from a single polycistronic tRNA-gRNA gene (PTG); therefore, stacking of separate sgRNA cassettes in one vector can be avoided. Simultaneous editing of multiple target sequences can be used for deletion of large chromosomal fragments and is also particularly useful for knockout of multiple homologous or homeologous genes in polyploid species. Moreover, it has been demonstrated that the PTG-based system is more efficient in targeted mutagenesis than single sgRNAs [10, 22–24].

The identification of transgene-free mutant plants is an important step of genome editing experiments in which mutant lines are produced by stable genetic transformation. This process can be laborious, time-consuming, and costly when a large number of genome-edited plants are generated. Therefore, we propose three simple methods of mutation detection that can be used alternatively for fast screening of a large number of transgenic plants.

Using the strategies described in this protocol, we developed a Cas9/sgRNA platform for both simplex and multiplex genome editing in barley [14]. The platform was developed based on a modified pBract211 vector; however, the procedure can also be adapted to other vectors. From 1000 immature embryos, we obtained on average above 70 independent transgenic plants with mutation rates up to 88%. From 10 to 20, T_0 mutant lines can be selected for segregation of homozygous mutant plants in the T_1 generation. By screening from 10 to 20 T_1 plants from each line, we were able to find at least 1 homozygous mutant per line.

2 Materials

2.1 Selection of Target Sequences

1. The whole genome sequence of barley is available at Ensembl Plants (http://plants.ensembl.org/Hordeum_vulgare/Info/Index), where the transcript tables and splice variants are provided for annotated genes.
2. Online tools such as CRISPOR (http://crispor.tefor.net/) or CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2/) may be helpful for selection of target sequences.

2.2 Prediction of off-Target Sites

1. Tools for prediction of potential off-target sites are available either as standalone software, e.g., CasOT, or web-based tools, e.g., CRISPR-PLANT v2 (https://www.genome.arizona.edu/crispr2/CRISPRsearch.html).
2. CasOT is available at http://casot.cbi.pku.edu.cn/download.php.

3. Barley genome sequence file in FASTA format, e.g., from http://plants.ensembl.org/index.html.
4. Exon annotation file in GTF format, e.g., from http://plants.ensembl.org/index.html.
5. Target sequence file in FASTA format (sequence of your protospacer).
6. Perl is available at http://www.perl.org/get.html.

2.3 Construct Assembly

1. Many customizable Cas9 vectors are available from Addgene (https://www.addgene.org). *See* Subheading 3.1 for guidance on choosing the appropriate Cas9 variant (*see* **Note 1**).
2. The native sequence of the Cas9 gene from *Streptococcus pyogenes* can be accessed at NCBI (GenBank accession AMH03982).
3. The sequence of the wheat U6 promoter can be accessed at NCBI (GenBank accession X63066.1 (1..363)).
4. PCR primers and DNA oligonucleotides can be purchased from Sigma-Aldrich (dry format is recommended).
5. *Bsa*I enzyme (New England Biolabs, R3733).
6. T4 DNA ligase (New England Biolabs, M0202).
7. Water bath or heat block.
8. PCR thermocycler with ramp temperature option.
9. NanoDrop or similar spectrophotometer for DNA concentration measures.
10. Molecular biology grade agarose.
11. PCR clean-up kits for purification of DNA from enzymatic reactions.
12. DNA gel extraction kits for purification of DNA from agarose gels.

2.4 Cloning

1. Plasmid vector compatible with AT cloning; we recommend the pGEM-T Easy vector from Promega (A1360).
2. Taq DNA polymerase.
3. *E. coli* DH5α electrocompetent cells are available from Thermo Fisher Scientific (11319019).
4. 2 mm electroporation cuvettes are available from VWR (732–1136).
5. Bio-Rad Gene Pulser II or equivalent device for electroporation.
6. SOC medium: 20 g/L tryptone, 5 g/L yeast extract, 0.5 g/l NaCl, 0.19 g/L KCl; adjust the pH to 7.0 with 1 N NaOH, sterilize by autoclaving at 121 °C, and prepare 100 mL

aliquots. Prior to use, add to 100 mL SOC medium: 1.8 mL of sterile 20% (w/v) glucose solution, 0.5 mL of sterile 2 M $MgCl_2$ solution.

7. LB medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl; for solid medium, add 15 g/L of bacteriological agar. Adjust the pH to 7.0 and sterilize by autoclaving at 121 °C for 20 min.
8. Ampicillin (1000×): 100 mg/mL stock in ddH_2O. Filter-sterilize and store 0.5 mL aliquots at −20 °C.
9. X-Gal: 2% (w/v) stock in DMF (N,N-dimethylformamide). Dissolve 20 mg in 1 mL DMF. Store at −20 °C in darkness.
10. IPTG: 20% (w/v) stock in ddH_2O. Dissolve 200 mg in 1 mL H_2O. Filter-sterilize and store 50 μL aliquots at −20 °C in darkness.
11. Kits for plasmid DNA extraction.
12. Microbiology incubator.

2.5 Mutation Detection in Target Loci

2.5.1 Genomic DNA Extraction (See **Note 2**)

1. CTAB buffer—to prepare 100 mL, mix 2 g CTAB, 17.53 g NaCl, 0.037 EDTA g, 20 mL of 0.5 M Tris–HCl pH 8.0, and 0.6 mL of β-mercaptoethanol; add ddH_2O to a final volume of 100 mL. Prepare from sterile water, and do not sterilize by autoclaving.
2. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
3. RNase A.
4. Chloroform.
5. Isoamyl alcohol.
6. Isopropanol.
7. 3 M sodium acetate: dissolve 24.609 g in 100 mL ddH_2O.
8. 70% ethanol.

2.5.2 PCR and Electrophoresis

1. High-fidelity DNA polymerase, e.g., Q5® High-Fidelity DNA Polymerase (New England Biolabs).
2. dNTP mix (10 mM each).
3. Specific primers (10 μM each).
4. Nuclease-free water.
5. Standard thermocycler.
6. 1 × TAE buffer: add 4.844 g/L Tris base, 1.21 mL/L acetic acid, and 0.372 g/L EDTA.
7. Molecular biology grade agarose.
8. Ethidium bromide or alternative dye for DNA staining in agarose gels.
9. Commercially available loading buffer.

10. Electrophoresis tanks and power supply.
11. System for gel visualization and documentation.

2.5.3 Restriction Analysis of PCR Products

1. Restriction enzyme specific to the Cas9 cleavage site in target sequence (*see* Subheading 3.6.3).
2. Nuclease-free water.
3. Water bath or heat block.

2.5.4 T7 Endonuclease I Cleavage Method

1. T7 endonuclease I.
2. Nuclease-free water.

2.5.5 qPCR with Dual Labeled Molecular Probes

1. Universal PCR Master Mix for qPCR with dual labeled probes, e.g., Luna® Universal qPCR Master Mix, NEB.
2. Specific primers (10 μM each) (*see* **Note 3**).
3. Control probe—a probe labeled with, e.g., 5′-[HEX] and [TAM]-3′.
4. "Mutant" probe—a probe labeled with, e.g., 5′-[6FAM] and [BHQ1]-3′.
5. Nuclease-free water.
6. qPCR thermocycler with two separate channels compatible with the fluorescent dyes.

2.5.6 Identification of Transgene-Free Mutant Lines

1. Petri dishes, 100 mm.
2. 3 MM Whatman paper or glass beads 3 mm in diameter.
3. Plastic pots 180–200 mm in diameter, 200 mm deep with drainage holes.
4. Soil—we use universal flower soil mixed with sand in a ratio of 10:1.

3 Methods

3.1 Design of the Cas9 Gene

Many plasmid vectors for plant genetic transformation that contain different variants of the Cas9 gene are available from plasmid repositories such as Addgene. It should be emphasized, however, that not all Cas9 variants are optimized for use in monocot plants, which may lead to weak expression and insufficient editing efficiency of the Cas9/sgRNA complex. The most important features of Cas9 may help to design custom synthetic Cas9 genes or to choose the appropriate variant from available sources.

1. The coding sequence of Cas9 should be optimized for expression in monocot plants in terms of both GC content and amino acid codon usage. For example, the GC content in the wild-type Cas9 DNA sequence of *S. pyogenes* is 35.1%, while the

average GC content observed in barley transcripts is 56.9%. The amino acid codons should also be adjusted to resemble the codon frequencies observed in barley (see the codon table for *Hordeum vulgare* at https://www.kazusa.or.jp/codon). The optimization of the Cas9 coding sequence can be performed using dedicated software, such as GeneOptimizer (Thermo Fisher Scientific).

2. The presence of the nuclear localization signal at the N terminus of the Cas9 protein is obligatory to facilitate its translocation from the cytoplasm to the nucleus. For that purpose, the sequence MAPKKKRKVG of NLS-SV40 (simian virus 40 nuclear localization signal) can be used.
3. For high-level expression of Cas9 in barley plants, monocot-specific promoters, such as the maize ubiquitin promoter (*ZmUBI*), should be used.
4. Although it is not obligatory, the expression of the Cas9 gene in monocot plants can be additionally boosted by intron-mediated enhancement. It has been observed that some introns, when placed at the 5′ proximal region of the heterologous gene, can greatly enhance its expression in host cells. The enhancing effect of the *Arabidopsis* UBQ10 first intron was previously demonstrated in barley [19]. In the case of the Cas9 gene, this intron can be placed at the +196 position within an AGGT canonical splice site [14].
5. Analyze the sequence of the Cas9 gene for the presence of internal restriction sites that are the same as those used for ligation with the vector of your choice. If such restriction sites exist, they should be mutagenized, preserving the coding sequence of Cas9.

3.2 Selection of Target Sequences

The genomic target sequence recognized by the Cas9-sgRNA complex is a 20 bp array followed by the consensus NGG sequence of the PAM motif. In plants, sgRNAs are transcribed using RNA polymerase III promoter U3 or U6, which have defined transcription start nucleotides A and G, respectively. The genomic target sequence should conform to pattern $A(N)_{19}NGG$ when the U3 promoter is to be used or $G(N)_{19}NGG$ when the U6 promoter is to be used (*see* **Note 4**). In this protocol, the U6 promoter is used as an example in all further steps.

Recently, several online tools have been developed to aid the selection of optimal target sequences; however, the functionality of the Cas9/sgRNA complex depends on additional factors that must be considered before choosing the appropriate target sequence.

1. A target sequence that conforms to the $G(N)_{19}NGG$ pattern can be identified using dedicated online tools, e.g., CRISPOR (http://crispor.tefor.net/), or software such as Vector NTI.

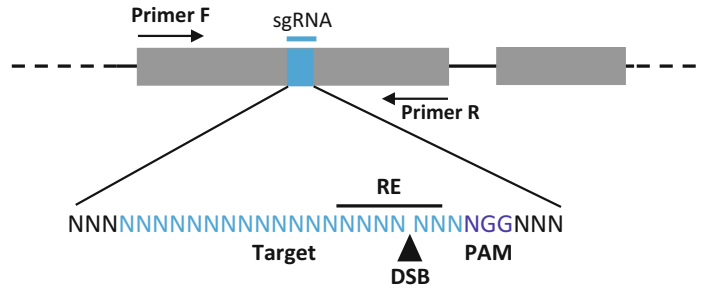

Fig. 1 Schematic presentation of the target locus. sgRNA was designed to target the first exon of a gene. The forward and reverse primers can be used for PCR-RE screening or sequencing of transgenic lines. The target sequence is marked in blue; RE, restriction site; DSB, double-strand break generated by Cas9; PAM, palindromic adjacent motif

The target sequence can be located on either the forward or reverse DNA strand.

2. Check the Ensembl Plants database for the splice variants of your target gene. The target sequence should be located in the first exon to increase the chance of frameshift mutations and premature stop codons.
3. If the first exon is removed by alternative splicing, there is still a chance that this truncated transcript may encode a functional protein. In this case, the target sequence should be located in the next exon that is shared by all other splice variants.
4. [Optional] Target sequences that possess a restriction site that overlaps the cut site of Cas9 (3 bp upstream of PAM; *see* Fig. 1) are advantageous because they allow screening for mutant plants by restriction analysis. However, this is not an obligatory feature because other fast screening methods exist (*see* Subheading 3.6).
5. Evaluate your selected target sequences for the presence of potential off-target sites (*see* Subheading 3.3).
6. If the selected target locus meets the above criteria, its sequence should eventually be confirmed by sequencing. This allows for the identification of potential sequence variations in the target sequence, because Golden Promise may differ slightly from the reference genome of cv. Morex. Design the PCR primers flanking the target sequence. Use the genomic DNA of Golden Promise for PCR. The reaction should give a single strong band on the agarose gel. Clean the reaction mixture using a PCR clean-up kit, and submit the sample for Sanger sequencing.

Table 1
Excerpt from the result table generated in the CasOT output file with the original column labels preserved

# Location	Site	Target	Mm.Type	PAM	Mm.All	Exon_info
chr7H:546588019-546588043:-	GGTTGGGCA_CCTTTGAGACGG-CGGA	nud	A00	A:NGG	0	(HORVU7Hr1G089930)
chr2H:510091074-510091098:+	GGcTtGGCA_CCTTTGAcACGG-CGGA	nud	A12	A:NGG	3	(HORVU2Hr1G071470)
chr1H:156317542-156317566:+	cGTccGGCA_CCcTTGAGACGG-AGGA	nud	A13	A:NGG	4	

Location, chromosomal coordinates for the detected sequence; Site, a sequence with full or partial homology to sgRNA; Target, sgRNA sequence name; Mm.Type, mismatch type; PAM, PAM sequence type; Mm.All, the sum of all mismatches; Exon_info, name of gene or potential gene from the Ensembl Plants database

3.3 Prediction of Potential off-Target Sites

CasOT is a program designed for genome-wide searches of Cas9/gRNA off-targets [25]. It can be used to search the whole genome of barley for "off-target" sequences, which can be located in locations other than the target locus and are similar to the protospacer sequence in sgRNA (*see* **Note 5**).

1. Download the genome sequence file for barley from Ensembl Plants: ftp://ftp.ensemblgenomes.org/pub/release-42/plants/fasta/hordeum_vulgare/dna/Hordeum_vulgare.IBSC_v2.dna_sm.toplevel.fa.gz
2. Download the exon annotation file for barley from Ensembl Plants: ftp://ftp.ensemblgenomes.org/pub/release-42/plants/gff3/hordeum_vulgare/Hordeum_vulgare.IBSC_v2.42.gff3.gz
3. To ensure that all commands were input correctly, we suggest using a tool prepared by the authors of the program, which is available at http://casot.cbi.pku.edu.cn/option.php#general. The results of the searches are provided in an output file. An example of an output file is described in Table 1.

3.4 Assembly of the sgRNA Construct

The sgRNA cassette contains the RNA polymerase III promoter (U3 or U6), a 20 bp spacer matching the target sequence (protospacer), and an 82 bp gRNA scaffold. Usually, such a construct is designed to contain a cloning site between the U3/U6 promoter and the gRNA scaffold, which allows for the introduction of a spacer sequence in the form of a hybridized oligo duplex (*see* Fig. 2). It is also recommended to clone the sgRNA cassette into either a Gateway entry vector or a Golden Gate level 0 vector because many CRISPR-Cas vectors are compatible with these systems, and thus, the cloning steps are minimized to only one reaction. The example below demonstrates the assembly and cloning of sgRNA into a Gateway-compatible vector, which is a more convenient system. In this strategy, the target consensus sequence in the form of annealed oligos is cloned between the wheat U6 promoter and gRNA scaffold using *Bsa*I cleavage sites. This is the easiest way to prepare a set of multiple different sgRNAs that can be later cloned into the Cas9 destination vector in a Gateway recombination reaction (Fig. 3).

1. The wheat U6 promoter and gRNA scaffold can be synthesized as a single sequence using available commercial services or assembled by overlapping extension PCR. The following *Bsa*I cloning site insert, 5′-CGAGACCAACACAAGGTCTCC-3′, should be precisely located between the U6 promoter and the gRNA scaffold (*see* Fig. 2 and Table 2).
2. Clone the assembled sequence into a Gateway entry vector. Keep in mind that the vector backbone must not contain *Bsa*I restriction sites (*see* **Note 6**).

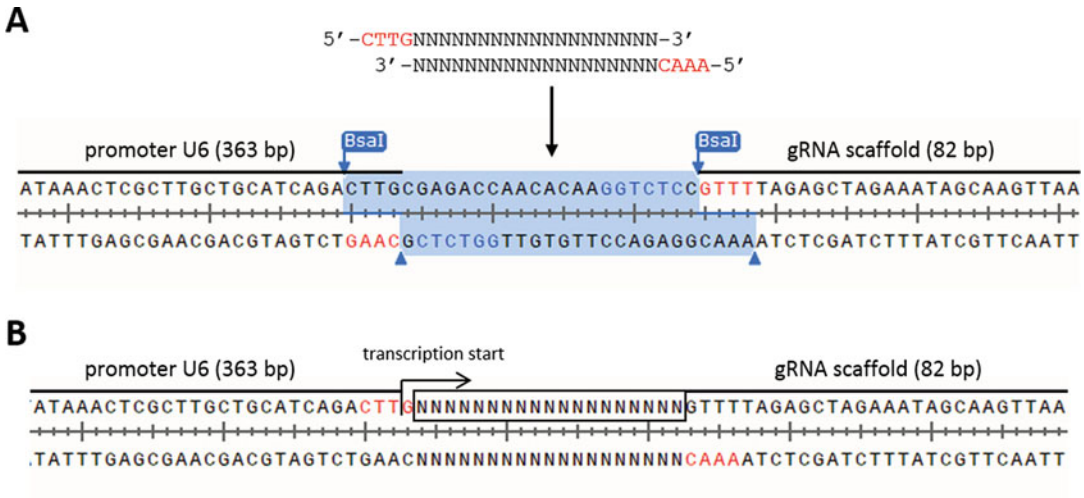

Fig. 2 (**a**) Fragment of the sgRNA cassette with the *BsaI* cloning site. A custom oligo DNA duplex can be cloned between the U6 promoter and gRNA scaffold using overhangs generated by the *BsaI* restriction enzyme. (**b**) Fragment of the sgRNA cassette with oligo DNA duplex cloned in frame with the gRNA scaffold

3. After ligation, the vector should be cloned into the *E. coli* strain and verified by restriction analysis or sequencing. Take 1 μL from the ligation reaction and add it to 50 μL of DH5α electrocompetent cell suspension. Use a 2 mm electroporation cuvette, and set the following parameters on the Bio-Rad Gene Pulser II, 200 Ω, 25 μF, and 2.5 kV, or refer to the manufacturer's instructions for other strains. After applying the electrical pulse, resuspend the cells immediately in 1 mL of SOC medium, and incubate the tube at 37 °C for 1 h with shaking at approx. 500 rpm.
4. Plate the transformed *E. coli* cells on LB agar containing the antibiotic specific to your vector, and incubate the plates overnight at 37 °C. Pick ten colonies for inoculation of liquid LB medium in separate tubes. Incubate the cultures overnight at 37 °C with shaking. Isolate the plasmid DNA using a plasmid extraction kit and following the manufacturer's instructions.
5. Cut 2–3 μg of isolated plasmid with *Bsa*I restriction enzyme according to the manufacturer's instructions.
6. The linearized plasmid should be separated from the excised *Bsa*I insert or uncut residues by electrophoresis on agarose gel (*see* **Note 7**). Excise the band corresponding to the linearized vector, and extract the plasmid using a gel purification kit. The purified vector is ready for ligation with oligo DNA fragment. Proceed to **step 9** to perform the ligation reaction (*see* **Note 8**).
7. Design a pair of oligonucleotides that match your previously selected target sequence. Important—do not include the PAM motif in the oligo sequence. In addition, keep in mind that an

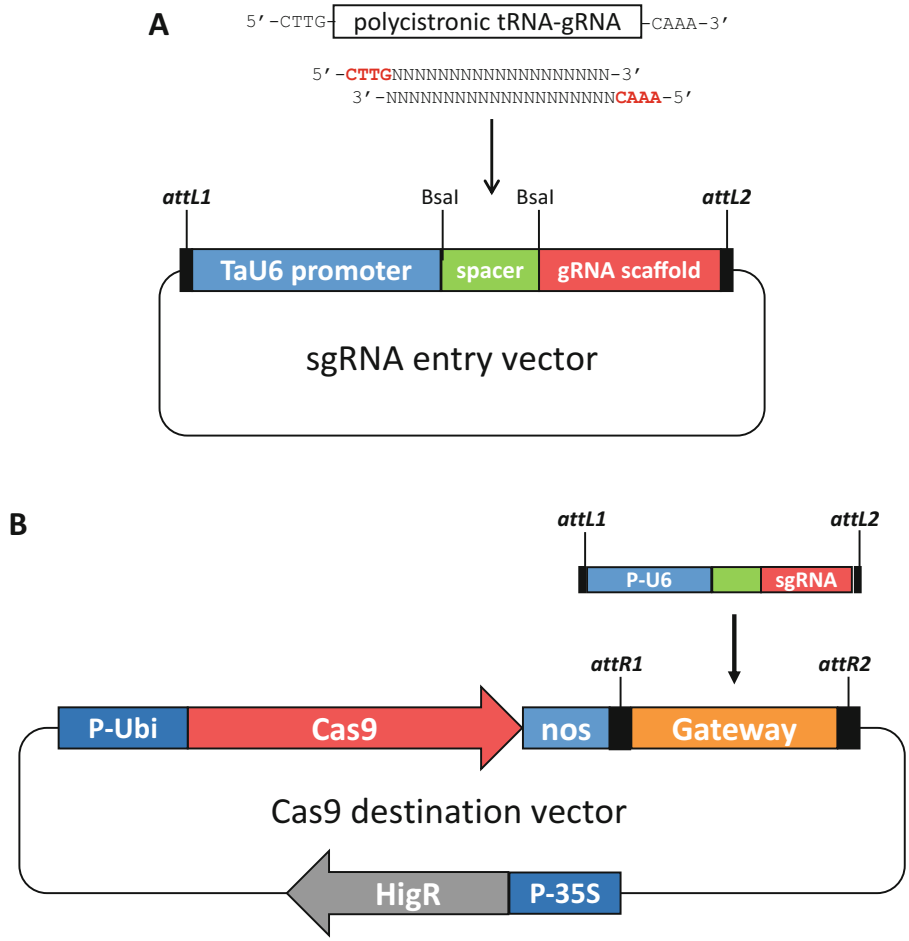

Fig. 3 Schematic structures of the sgRNA and Cas9 constructs. (**a**) The sgRNA expression cassette can be assembled in a Gateway entry vector; oligo DNA duplex or polycistronic tRNA-gRNA gene (PTG) is inserted into the sgRNA cassette using the *BsaI* cloning site. (**b**) Gateway destination vector containing the Cas9 expression cassette. The sgRNA expression cassette is cloned into this vector via LR recombination reaction

Table 2
Sequences used for assembly of the sgRNA cassette ready for *Bsa*I cloning

Name	Sequence/accession
TaU6 promoter	X63066.1 (1..363)
*Bsa*I insert	5′-CGAGACCAACACAAGGTCTCC-3′
gRNA scaffold with terminator	5′-GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT-3′

initial nucleotide G is already present in the U6 promoter sequence. Both oligonucleotides should contain 5′ overhangs, which are complementary to overhangs generated after *Bsa*I cleavage of the vector. The sense oligonucleotide, 5′-CTTG $(N)_{19}$–3′, is consensus to the target sequence, while the antisense oligonucleotide, 5′-AAAC$(N)_{19}$–3′, should be in reverse complement form.

For example, if the target sequence is 5′-GATCACCG CGGCGTCTCCTATGG-3′ (PAM motif is underlined), then the sequence of the sense oligonucleotide should be 5′-CTTGATCACCGCGGCGTCTCCTA-3′, and the sequence of the antisense oligonucleotide should be 5′-AAA CTAGGAGACGCCGCGGTGAT-3′.

8. The oligonucleotides are synthesized in the same way as ordinary PCR primers, and they do not require any modifications. Synthesis scale of 0.025–0.05 μmol is sufficient. HPLC purification is recommended for cloning.
9. Dilute the synthesized oligonucleotides to 100 μM concentration in H_2O or 10 mM Tris–HCl pH 8.0. Perform the annealing reaction as shown in Table 3 (*see* **Note 9**).
10. Dilute the annealed oligonucleotides 200 × in H_2O, and use 1 μL for ligation with the linearized vector from **step 5**. Perform the ligation reaction as shown in Table 3.
11. Take 1 μL of the ligation mixture and perform the electroporation procedure as described in **step 3** above.
12. Incubate the plates overnight at 37 °C. Pick a single colony from each plate and inoculate 10 mL of liquid LB medium in separate tubes with antibiotics. Incubate the cultures overnight at 37 °C. Prior to plasmid isolation, prepare a glycerol stock

Table 3
Reaction conditions for oligo DNA duplex formation and ligation

Annealing		**Reaction conditions**
100 μM forward oligo	1 μL	Incubation in thermocycler: **Step 1**, 95 °C/5 min; **step 2**, decrease temp. By 0.2 °C/s to 70 °C; **step 3**, decrease temp. by 0.1°C/s to 25 °C
100 μM reverse complement oligo	1 μL	
T4 ligase buffer 10× (NEB)	1 μL	
H_2O	7 μL	
Ligation		Reaction conditions
Annealed oligos diluted 200×	≈ 0.15 pmol ends	Incubation at 22 °C for 10 min or at 16 °C overnight
Vector 50 ng/μL	≈ 0.05 pmol ends	
T4 ligase buffer 10× (NEB)	2 μL	
T4 ligase (NEB)	1 μL	
H_2O	to 20 μL	

from each culture; to 2 mL cryovial, add 400 μL of sterile 50% (v/v) glycerol and 600 μL of bacteria suspension. Gently vortex the cryovial, freeze it in liquid nitrogen, and store at −80 °C. After screening the colonies, the glycerol stocks from positive colonies can be kept at −80 °C for long-term storage and reused if necessary.

13. The integrity of the assembled sgRNA cassette should be verified by Sanger sequencing using vector-specific primers flanking the insert. Alternatively, the sgRNA-specific primers, i.e., forward U6 5′-GACCAAGCCCGTTATTCTGA-3′ and reverse gRNA 5′-AAAAAAAGCACCGACTCGGTGCCAC-3′, can also be used.
14. If the sequence of the sgRNA is correct, perform the Gateway recombination reaction with the Cas9 destination vector according to the manufacturer's protocol.

3.5 Assembly of the PTG Construct for Multiplex Genome Editing

PTG is a polycistronic tRNA-gRNA construct consisting of multiple sgRNAs that are preceded by tRNA modules (Fig. 4). Similar to single sgRNAs, the PTG construct requires the U3 or U6 promoter for expression in plant cells. After transcription, the sgRNA modules are excised from the primary transcript and form a complex with the Cas9 protein. The below procedure depicts the design of the PTG construct, which can be cloned into previously made sgRNA vectors using *Bsa*I-generated overhangs (*see* Subheading 3.4, **step 5**).

1. De novo synthesis of the DNA sequence of the tRNA-gRNA array is the fastest and most convenient method (*see* **Note 10**).
2. The design of the tRNA-gRNA array is shown in Fig. 4 and Table 4. Considering that the spacer sequences are 20 bp long in this case, it is not necessary for them to start with G as a first nucleotide. Moreover, only the last gRNA scaffold in the array must end with the terminator sequence, and its sequence is already present in the sgRNA vector.
3. The synthesized DNA fragments are usually provided in plasmid vectors from which they can be released by cutting with appropriate restriction enzymes. In our case, the synthesized tRNA-gRNA array must be cut out with *Bsa*I enzyme.

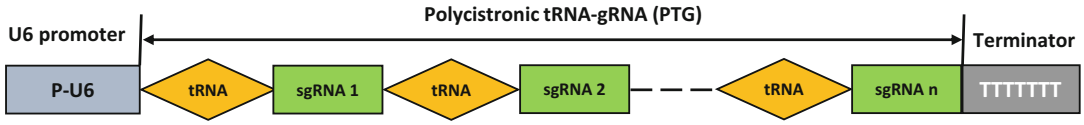

Fig. 4 Schematic structure of the polycistronic tRNA-gRNA gene (PTG) for multiplex genome editing. As a result of intracellular tRNA processing, sgRNAs are excised from the PTG transcript and can hybridize with the Cas9 molecules

Table 4
Sequences used for assembly of the PTG construct

Name	Sequence / accession
TaU6 promoter	X63066.1 (1..363)
tRNA-gRNA (for 2 targets)	5′- GGTCTCCCTTGCAACAAAGCGCATCTGGTGTAGTGGTATCATAGTACCCTCCCACG GTACTGACCAGGGTTCGATTCCCTGGATGCGCANNNNNNNNNNNNNNNNNNNNGT TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAA AGTGGCACCGAGTCGGTGCCAACAAAGCGCATCTGGTGTAGTGGTATCATAGTAC CCTCCCACGGTACTGACCAGGGTTCGATTCCCTGGATGCGCANNNNNNNNNNNNN NNNNNNNGTTTCGAGACC-3′
gRNA scaffold with terminator	5′-GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTA TCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT-3′

The tRNA sequence is marked in yellow, the 20 bp protospacer is marked in blue, the sequences of the *BsaI* restriction sites are underlined, and the 5′ and 3′ overhangs are indicated in red

4. Separate the excised tRNA-gRNA fragment from the vector backbone by electrophoresis on agarose gel. Excise the band corresponding to the tRNA-gRNA fragment from the gel and purify the DNA using a gel extraction kit.
5. Perform the ligation reaction with the linearized sgRNA vector (*see* **steps 4–5** in Subheading 3.4) in a molar ratio of 3:1 (insert/vector) using T4 DNA ligase (NEB) and the manufacturer's protocol.

3.6 Mutation Detection in Target Loci

3.6.1 DNA Extraction from Barley Plants (See Note 2)

1. Collect 2–3 cm fragments from as many leaves as possible and freeze them in liquid nitrogen. Store at—80 °C until use.
2. Grind the tissue in liquid nitrogen using mortar and pestle.
3. Transfer 100 mg of frozen tissue to a 2 mL Eppendorf tube.
4. Add 0.8 mL CTAB buffer and 5 μL of RNase A.
5. Incubate at 60 °C with shaking (500–700 rpm) for 30 min.
6. After incubation add 0.8 mL chloroform/isoamyl alcohol (24:1) and mix vigorously or vortex on high speed.
7. Centrifuge the samples at 10,000 × *g* for 10 min at room temperature.
8. Transfer the upper aqueous layer to a new 2 mL tube.
9. Add ¾ volume of isopropanol and 1/10 volume of sodium acetate (3 M) to precipitate nucleic acids.
10. Mix gently by inverting the tube several times and incubate for 10 min at room temperature.
11. Centrifuge the tubes at 12,000 × *g* for 10 min and gently pour off the supernatant.
12. To the tube with precipitate, add 1 mL of 70% ethanol and mix gently.

13. Centrifuge the tubes at 8,000 × *g* for 5 min and gently pour off the supernatant.
14. Open the tubes for 5 min to allow the precipitate to air-dry.
15. Resuspend the precipitated DNA in 50 μL of TE buffer.

3.6.2 PCR

1. Prepare PCR mixture in a final volume of 50 μL with the following composition: 1x reaction buffer, 1 μL of 10 mM dNTP mix, 4 μL of 5 μM forward and reverse primer (*see* **Note 11**), 3 μL of genomic DNA (60 ng/μL), 1–5 units of DNA polymerase, and nuclease-free water to a final volume of 50 μL.
2. Use the following program for PCR: initial denaturation step at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, (Ta of your primers) for 30 s, 72 °C for 30 s, and final extension at 72 °C for 120 s.
3. Following PCR, 25 μL of reaction mixture with loading buffer should be run on 1% agarose gel. The gel should be made with 1xTAE buffer supplemented with an appropriate amount of ethidium bromide or alternative dye. Run the electrophoresis in 1 x TAE buffer at 120 V for 45 min. The PCR should produce a single, strong band specific to your expected product length.

3.6.3 Restriction Analysis of PCR Products

RFLP (restriction fragment length polymorphism) is a method often used for the detection of mutations generated by Cas9 or other site-specific endonucleases. A key element of this method is a restriction enzyme that overlaps a DNA sequence in the Cas9 cleavage site, i.e., three bp upstream of the PAM motif in the target sequence (Fig. 1). The DNA repair mechanism introduces small indels (insertions/deletions) at the joint site, and as a result, the restriction site is disrupted, and the PCR product cannot be cut. The ability to use this method should be considered prior to the experiment, as it is sometimes impossible to find target sequences with a properly located restriction site.

1. Perform the PCR as described in Subheading 3.6.2. Take 10 μL of PCR mixture, and add 2 μL reaction buffer specific to the restriction enzyme, 1 μL (10 units) of restriction enzyme specific to the cleavage site in the sgRNA, and 17 μL of nuclease-free water.
2. Mix gently the reaction mixture, and incubate for 3 h in a water bath at the temperature appropriate for the restriction enzyme.
3. Run the reaction mixture in 1% agarose gel in 1 x TAE buffer at 120 V for 45 min.
4. Amplicons from wild-type plants should be cut completely by the restriction enzyme, giving two bands on the gel. Samples

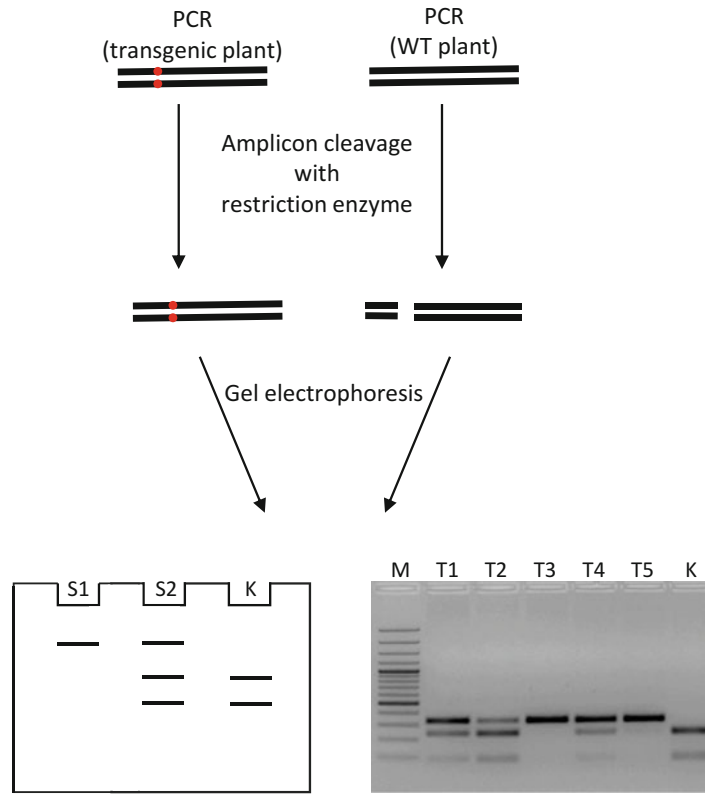

Fig. 5 Key steps of mutation detection in transgenic plants by restriction analysis of PCR products (PCR-RE). The restriction site was lost in target loci in which mutations occurred (indicated by red dots). PCR products from homozygous mutants are uncut (lanes S1, T3, and T5); samples from heterozygous mutants are a mix of cut and uncut amplicons (lanes S2, T1, T2, and T4); PCR products from wild-type plants are cut completely by the restriction enzyme (lane K)

from heterozygous mutant plants are mixture of cut and uncut amplicons that gives three bands on the gel, while samples from homozygous or biallelic mutant plants contain only uncut amplicons represented by a single band on the gel (Fig. 5).

3.6.4 T7 Endonuclease I Cleavage Method

T7 endonuclease I from bacteriophage T7 is one of the most commonly used enzymes in the enzyme mismatch cleavage (EMC) method, which is related to its wide commercial availability and relatively low price. In the EMC technique, the enzyme is used to digest heteroduplex DNA formed by annealing wild-type and mutant DNA strands (Fig. 6).

Hybridization of amplicon heteroduplexes—prepare the PCR for both transgenic and wild-type samples as described in Subheading 3.6.2.

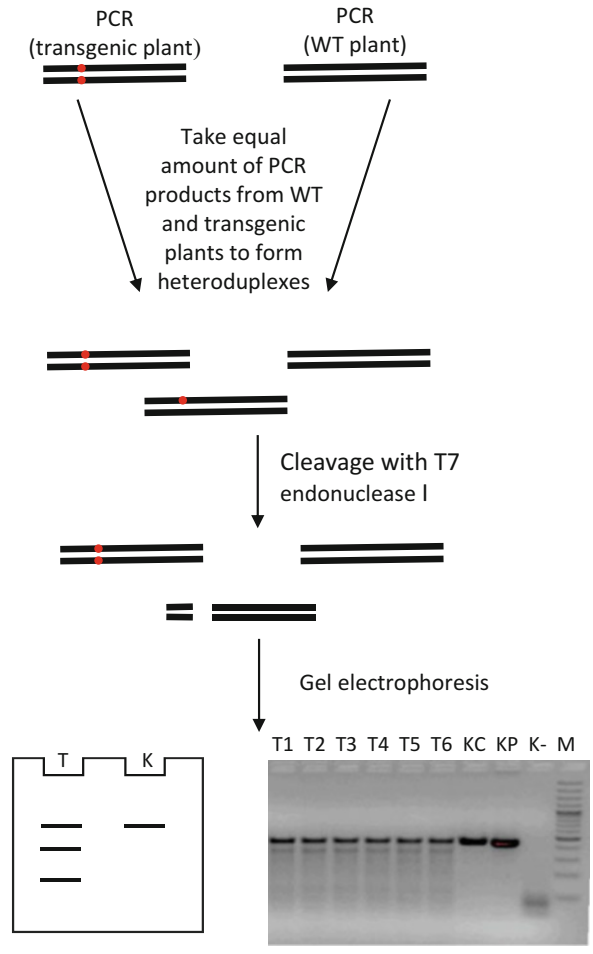

Fig. 6 Key steps of mutation detection in transgenic plants using T7 endonuclease. PCR products from mutant and wild-type plants are mixed in a 1:1 ratio to form heteroduplexes. T7 endonuclease cuts the heteroduplexes where unpaired nucleotides are located (indicated by red dots). T, T1–T6, samples from plants with detected mutation; K, KC, samples from the wild-type plants; KP, untreated control sample; K-, non-template control

1. Mix 5 μL of PCR mixture from the transgenic sample and 5 μL of PCR mixture from the wild-type sample (*see* **Note 12**).
2. Mix gently, and incubate in standard thermocycler with the following steps: 98 °C for 180 s, next between 98 °C and 80 °C with a temperature decrease of −2 °C per s, next between 80 °C and 30 °C with a temperature decrease of −0.1 °C per s, and then hold at 4 °C (*see* **Note 13**).
3. Enzymatic cleavage of heteroduplexes by T7 endonuclease I—to 10 μL of the heteroduplex mixture, add2 μL of T7 reaction buffer, 1 μL of T7 endonuclease I, and 7 μL of nuclease-free water.

4. Gently mix the reaction mixture and incubate for 15 min at 37 °C in a thermocycler.
5. After incubation, perform electrophoresis to visualize the reaction product. Use 1% agarose gel in 1 x TAE buffer at 120 V for 45 min.

3.6.5 qPCR with Dual Labeled Molecular Probes

The use of qPCR and dual labeled probes to detect mutations induced by CRISPR-Cas technology is a relatively new idea proposed by Peng et al. [26]. This method exploits two differentially labeled probes that are placed within one amplicon. The first probe is designed to be complementary to the target site where the potential mutation was induced, while the second probe is located outside the mutation site (Fig. 7a). The presence of mutations can be determined in real-time qPCR by reading the Cq ratio between the two probes. Moreover, the method is sufficiently sensitive to distinguish between heterozygous and homozygous mutations in transgenic plants.

1. Primers and probes can be designed using the OligoArchitect™ tool provided by Sigma-Aldrich (http://www.oligoarchitect.com/LoginServlet). The "mutant" probe should hybridize to the amplicon within the target site, and the "control" probe should be placed at some distance and hybridize within the sequence that is not affected by the mutation (Fig. 7a).
2. Prepare the qPCR mixture in a final volume of 20 μL with the following composition: 10 μL of Universal PCR Master Mix for probes, 0.8 μL of 10 μM forward and reverse primer, 0.4 μL of each molecular probe (10 μM), genomic DNA (60 ng/μL), and nuclease-free water to 20 μL.
3. Run the PCR with the following steps: initial denaturation step at 95 °C for 60 s followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at a temperature specific to your primers for 30 s, elongation at 72 °C for 30 s, and a final extension at 72 °C for 120 s. The readout of fluorescence increase on the HEX and 6FAM channels should occur in each cycle during the elongation step.
4. The presence of mutations in the tested samples can be determined by comparing the Cq values of the "mutant" and "control" probes. The signal coming from a "mutant" probe will be much lower than the signal from the control probe in the case of heterozygous plants or will not be detectable if the plant is a homozygous mutant (Fig. 7b). In wild-type samples, the amplification efficiency for both probes should be at the same level.
5. The relative amplification can be analyzed via the $2^{-\Delta\Delta CT}$ method using a "control" probe as an internal control

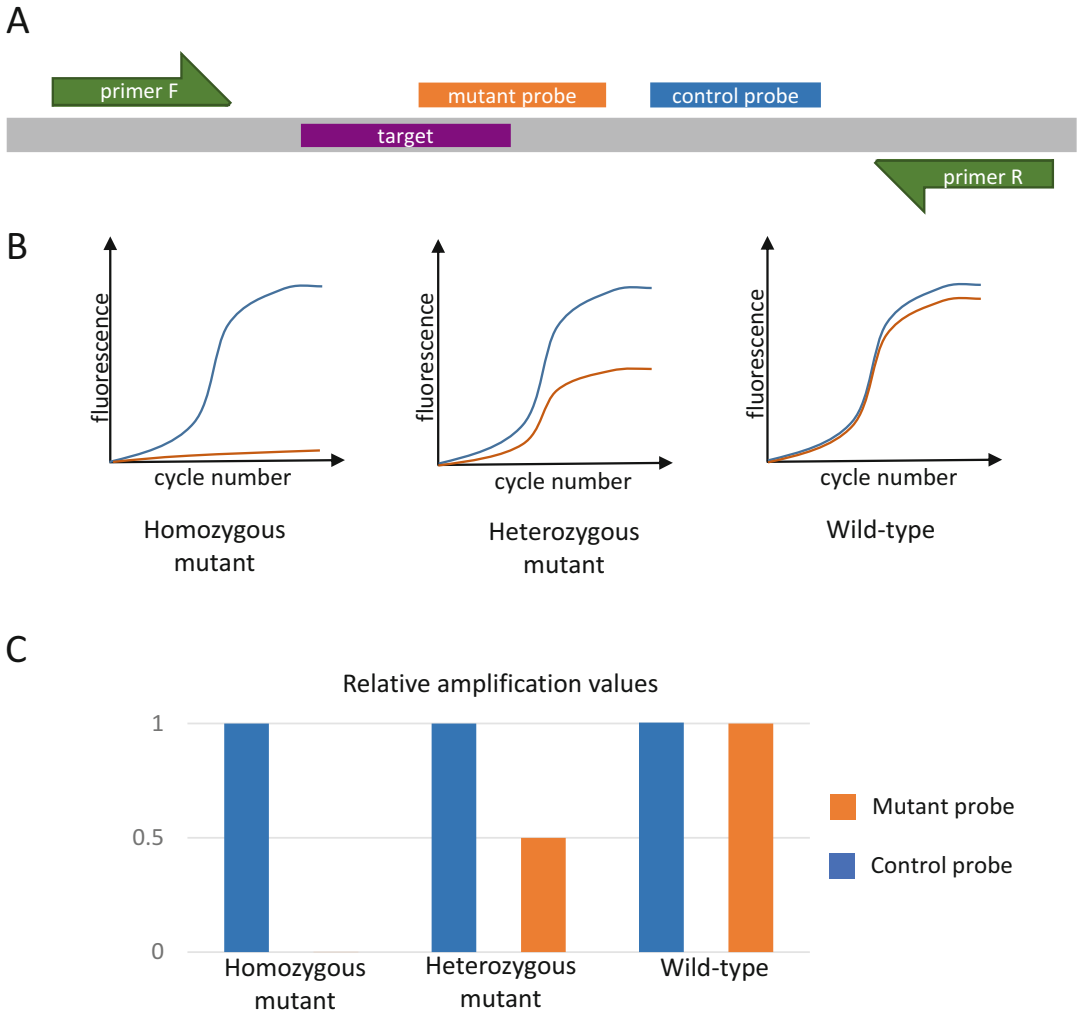

Fig. 7 Strategy of mutation detection in transgenic plants by qPCR with dual labeled probes. (**a**) Schematic structure of the target locus and strategy of designing molecular probes and PCR primers. The "mutant" probe partially overlaps the target sequence where the mutation is expected to be induced, while the "control" probe is located outside the target sequence. (**b**) Schematic presentation of the fluorescence increase of the "mutant" probe (orange line) and the "control" probe (blue line) during qPCR. (**c**) The relative expression levels of the mutant and control probes depending on the type of mutation

(normalizer) and wild-type templates as a calibrator sample with the expression value set to 1. The expression level of the tested samples was calculated as x-fold of 1 (Fig. 7c).

3.7 Identification of T_1 Transgene-Free Mutant Lines

1. T-DNA segregation in T_1 generations usually follows the Mendelian 3:1 ratio. However, it is difficult to predict the segregation pattern of induced mutations because it depends on the status of the T_0 mutant plant, which can be either homozygous, heterozygous, biallelic, or chimeric. For these reasons,

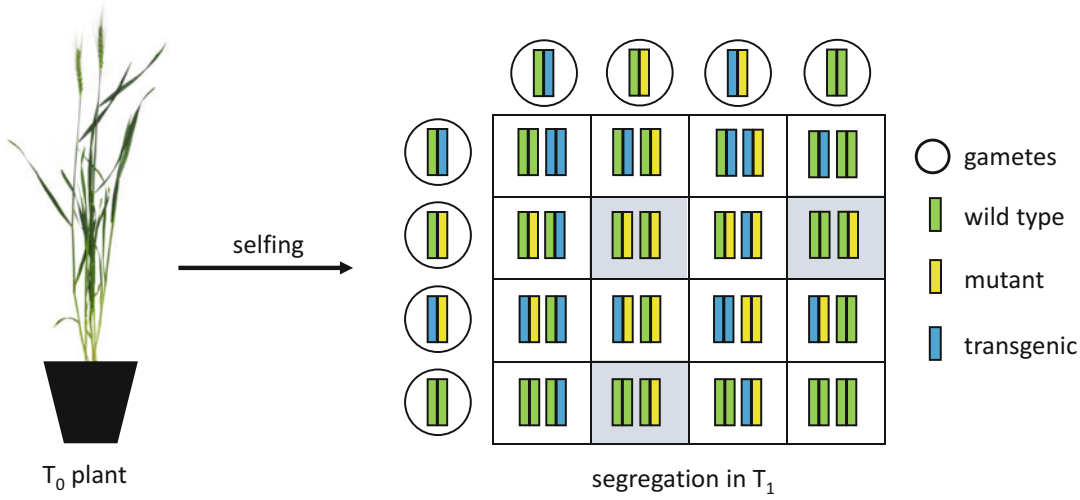

Fig. 8 Selection of transgene-free mutant plants. After selfing of previously selected T_0 mutants, the transgene-free homozygous mutant plants can be segregated in T_1 generation. The table presents the segregation of both transgene and target loci. Boxes representing transgene-free, genetically edited mutant plants were shaded

transgene-free, homozygous mutant lines can be selected only in T_1 progeny after selfing of T_0 plants (Fig. 8).

2. Select 10–20 T_0 plants with mutations detected at the target locus as described on Subheading 3.6. After maturity, from each T_0 plant, select approximately 20 healthy and well-developed seeds for germination. At least 12 T_1 seedlings from each T_0 plant should be screened.
3. Seeds can be germinated in 100 mm Petri dishes, either on filter paper or on glass beads. For uniform germination, the Petri dishes should be incubated first at 4 °C in the dark for 2 days and later at 24 °C for 4–5 days.
4. Transfer germinated seedlings to soil, and grow them until maturity under the following environmental conditions: temperature 18 °C day/12 °C night and a 16-h photoperiod with 350 μmol/m^2/s light intensity generated by fluorescent lamps (*see* **Note 14**).
5. Tissue samples for DNA extraction can usually be collected after 10–14 days when the first three leaves have emerged. Extract the DNA and screen the samples for mutation as described in Subheading 3.6.1.
6. To discriminate homozygous mutations from heterozygous or biallelic mutations and to identify the pattern of mutation (deletion or insertion, frameshift or not), the PCR product must be cloned and sequenced. For this purpose, convenient AT-cloning vectors, such as pGEM-t Easy, can be used.

7. Clean the PCR product using the PCR clean-up kit. For ligation with pGEM-T Easy, a 3:1 molar ratio of insert/vector is recommended, meaning that 0.15 pmol ends of insert are required for ligation with 1 μL of pGEM-T Easy vector. Perform the ligation reaction according to the manufacturer's protocol. Take 1 μL of reaction mixture for electroporation of *E. coli* cells (*see* Subheading 3.4, **step 3** for electroporation procedure).
8. The pGEM-T Easy vector allows white-blue colony screening. Prepare LB agar plates with the addition of 100 mg/L ampicillin. For the 80 mm plate, pipette 50 μL of X-Gal stock solution and 7 μL of IPTG stock solution, and spread it on the whole surface of the agar plate. Prewarm the plates at 37 °C and plate the transformed cells. Incubate the plates overnight at 37 °C. After this time, blue and white colonies should be easily distinguished; however, incubation at 4 °C for the next several hours should enhance the blue staining. Select only white colonies for plasmid isolation. Submit the extracted plasmid samples for Sanger sequencing using standard T7/SP6 primers: T7 5′-TAATACGACTCACTATAGG-3′, SP6 5′-ATTTAGGTGACACTATAG-3′.
9. To identify transgene-free mutants, the mutant plants must be PCR screened for the presence of the T-DNA. Primers specific to the selectable marker gene can be used. For the *hpt* gene, which confers resistance to hygromycin, we used the following primers: forward 5′-ATGACGCACAATCCCACTATCCT-3′, reverse 5′-AGTTCGGTTTCAGGCAGGTCTT-3′. Perform the reaction according to the specifications of your polymerase. The annealing temperature for the *hpt* primers is 68 °C, and the amplified product length is 405 bp.

4 Notes

1. For barley, we used the pBract211 vector, which is optimized for *Agrobacterium*-mediated transformation of monocot crops and gave us good transformation rates. However, this vector is available only in its native form (without the Cas9 gene) from the John Innes Centre (https://www.jic.ac.uk/technologies/genomic-services/bract/). The detailed procedure of how to adapt this vector for a Gateway-compatible Cas9 vector can be found here: [14].
2. Kits for plant DNA extraction can be used; however, for larger tissue samples, we recommend a standard CTAB protocol [27].
3. The designed primers should amplify a fragment long enough to allow the binding of two molecular probes within the amplicon. Usually, the optimal length of the amplicon for probes is 100–200 bp.

4. If the first defined nucleotide being G or A limits your options for choosing an optimal target sequence, you can choose target sequences that start with any other nucleotide. Just remember to include a G or A nucleotide in the sgRNA construct directly after the U6 or U3 promoter, respectively. We tested sgRNA with a 21 bp spacer and did not observe any negative effects on Cas9 performance.
5. The main advantage of this program is the ability to load any genome sequence derived from any organism and use the program on the user's computer independently from external servers.
6. For this purpose, we used a pCR8/GW/TOPO vector from Thermo Fisher Scientific (K250020). This vector is provided in linearized form ready for AT cloning, and it lacks the *BsaI* restriction site.
7. Usually, 1 μg of plasmid DNA should be cut completely by 1 unit of *Bsa*I enzyme in 1 h under optimal reaction conditions. However, it is safer to separate the plasmid on agarose gel to avoid potential false-positive colonies that may come from uncut residues of the plasmid. The linearized form of the plasmid migrates more slowly than the supercoiled form on agarose gel and appears as the upper band above the uncut product.
8. Many protocols recommend dephosphorylating the linearized vector and using phosphorylated oligonucleotides for ligation. Although it does no harm, it is not necessary in this case because the overhangs generated by *Bsa*I are not complementary, which prevents the vector from self-ligation. We achieved the best results after ligation of the phosphorylated vector with unphosphorylated oligonucleotides. However, if you still obtain false-positive colonies, you may add *Bsa*I enzyme to the ligation reaction.
9. If you cannot set the parameters mentioned in Table 3 in your thermocycler, you may boil the tube with the reaction mixture in a water bath for 5 min and then allow it to cool to room temperature. This alternative solution can be found in many protocols; however, it was not tested by us.
10. It is also possible to assemble the PTG construct by overlapping extension PCR or Gibson cloning. However, particular caution should be taken when designing the PCR primers to avoid incorrectly assembled constructs. To facilitate the process, one may use specialized tools for this purpose, such as SnapGene software or NEBuilder Assembly Tool (http://nebuilder.neb.com/#!/).
11. The primers should be designed such that the mutation is approximately 1/3 of the amplicon's length. We also

recommend amplicons of 400–600 bp in length for better separation of digested fragments in agarose gel.

12. Many T7 endonuclease I manufacturers recommend cleaning the reaction mixture after PCR. Based on our experience, this step is not necessary.
13. This is a modified protocol for heteroduplex formation by Guschin et al. [28]. There are many different protocols for heteroduplex formation, and based on our observations, the optimal conditions should be determined experimentally for each selected amplicon.
14. To save space in the greenhouse or growth chamber, we first put the seedlings into small pots (80 mm in diameter). After PCR screening, the negative plants are discarded, and the positives are transferred to larger pots (180–200 mm in diameter).

Acknowledgments

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Chapter 9

Genome Editing of Mammalian Cells Using CRISPR-Cas: From In Silico Designing to In-Culture Validation

Borhan Uddin, Patrick Partscht, and Taslima Nahar

Abstract

The RNA-guided clustered regularly interspaced short palindromic repeats (**C**RISPR)-**as**sociated (Cas) nuclease system has dramatically changed the field of cell and molecular biology. Any genomic sequence adjacent to a protospacer adjacent motif (PAM) site can potentially be edited by customizing the guide RNA (gRNA) that direct the Cas nuclease. Such versatility and flexibility have made the CRISPR-Cas system almost a default platform for genome editing nowadays. This system has gained widespread use also due to lower toxicity and simplicity of construction. Here, we describe a comprehensive protocol for gene deletion (knock-out) and tagging (knock-in) in mammalian cells, taking specific examples of Cas endonuclease systems and cell lines. However, the method can be adapted to edit genome employing other Cas plasmid systems as well as different human cell lines in a lab with standard cell and molecular biology facilities.

Key words Genome editing, CRISPR-Cas, Knock-out, Knock-in, Mammalian cell

1 Introduction

Analysis of the phenotypic effects of an interfered gene is the method of choice to decipher its physiological role. The function of a gene of interest can be altered by modifying its expression transiently or sequence permanently. Development in the field of transient RNA interference (RNAi) by small interfering RNA (siRNA) has transformed the field of reverse genetics. However, the efficiency of depletion and difficulties associated with complementation experiments have always raised the question about the reliability of RNAi [1]. Genome editing is a cleaner and more robust alternative to gene silencing. Recent advances in genome editing techniques are facilitating the manipulation of complex vertebrate genome with relative ease. Several strategies have evolved to program an endonuclease for binding a targeted site of interest in a sequence-specific manner. Zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and the

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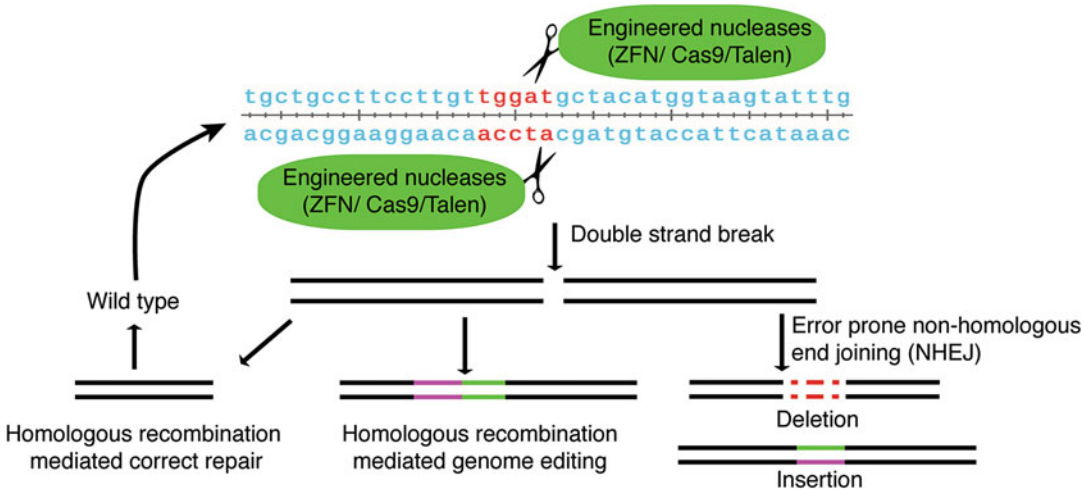

Fig. 1 Sequence-specific modification by nucleases. Zinc finger nucleases (ZFN), transcription activator-like effector nuclease (TALEN), and the RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease systems are engineered in such to cause DNA strand break (DSB) in a guided region of interest. The stand break can be repaired either by homologous recombination (HR) or by random nonhomologous end-joining (NHEJ) approaches. Homology-directed repair (HDR) can be employed to bring desired genetic changes, gene deletion, or tagging. In contrast, NHEJ has only the potential to disrupt gene function mediated by insertion or deletion of nucleotides of varying length (taken from [8])

RNA-guided clustered regularly interspaced short palindromic repeats (**C**RISPR)-associated (Cas) nuclease are the examples of such tools [2–7]. All these nucleases catalyze DNA strand break (DSB) upon binding and trigger high-fidelity homology-directed repair (HDR) and/or error-prone nonhomologous end joining (NHEJ) to repair this DSB. NHEJ causes random insertion or deletion of nucleotides which in turn disrupts gene function by the synthesis of altered protein due to frameshift and/or generation of truncated protein as a result of premature stop codon. Alternatively, HDR can be used to incorporate stop codon or desired tag for genome manipulation [8] (Fig. 1).

CRISPR-Cas system has evolved as a part of adaptive defense mechanism in bacteria and archaea in which short RNA is used to recognize and degrade foreign genetic materials [9]. This system consists of spacer-repeat array and the Cas genes. The variable spacer sequence in the spacer-repeat array is derived from the invading foreign genetic material in the immunization phase. This array is expressed as a long pre-CRISPR RNA (pre-crRNA) in the immunity phase and subsequently processed into small guiding crRNAs. The crRNAs then form a complex with Cas proteins and cleave the foreign genetic material complementary to the spacer sequence. The sequence recognition mechanism of CRISPR-Cas system is tweaked to recognize a desired sequence for specific genome alteration in human (Fig. 2).

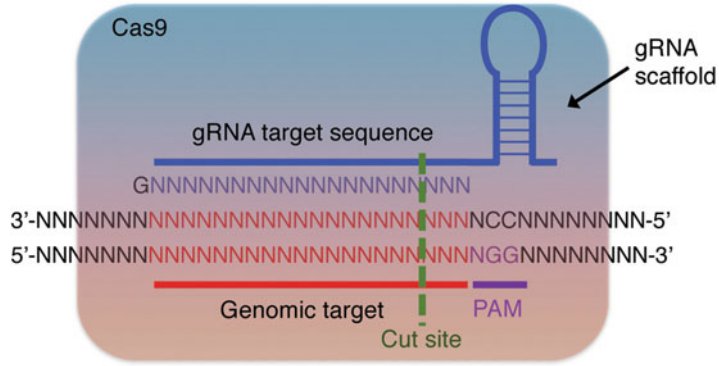

Fig. 2 Cas9-gRNA-mediated genome targeting. The Cas9 nuclease from *S. pyogenes* is targeted to the site complementary to the single guide gRNA (sgRNA) sequence and adjacent to the PAM (protospacer adjacent motif, NGG for Cas9) site. The sgRNA base pairs with the opposite strand and the highly likelihood of strand break is the three base pair upstream to the PAM site. A chimera of gRNA target sequence and gRNA scaffold, termed as sgRNA, is necessary for the complex formation with Cas9 protein. The codon of the Cas9 protein is optimized for human expression (adapted from [7])

In theory, any genomic sequence adjacent to a PAM site can be targeted by customizing the guide RNA (gRNA) that directs the Cas nuclease. Such versatility and flexibility have made this system almost a default platform for genome editing nowadays. CRISPR-Cas-mediated genome editing is at the forefront of all the available techniques also due to its lower toxicity in human cells as well as simplicity in construction [10]. Within the last few years, the Cas9 nuclease from *S. pyogenesis* was engineered to increase specificity [5, 11, 12]. A nickase variant of Cas9 is commonly being used to decrease off-target effects. This variant only cuts one strand and, consequently, a pair of gRNA is used to target the enzyme to cause a double-strand break [5]. Cas12a or Cpf1 (CRISPR from *Prevotella* and *Francisella 1*) is also a RNA-guided endonuclease of class II CRISPR-Cas system like Cas9. Nevertheless, it differs from Cas9 in many aspects such as T-rich PAM site, lack of tracrRNA, and staggered cut site distal from the PAM site. The staggered cut site makes this enzyme superior for knock-in experiments [13]. Here, we describe a comprehensive protocol for gene deletion (knock-out) and endogenous tagging (knock-in) in mammalian cells using CRISPR-Cas techniques. The protocol includes the details from in silico experiment designing to culture cell line validation taking specific examples of Cas endonuclease systems and cell lines. However, the mechanisms can be adapted to edit genome employing other Cas plasmid systems as well as different human cell lines. This protocol can be successfully performed in a lab with standard cell and molecular biology facilities.

2 Materials

2.1 Designing gRNA Sequence

1. Web tool such as GenBank for analyzing the gene sequence.
2. Sequence analysis software.
3. Web tools for designing gRNAs.

2.2 Synthesizing gRNA

1. Oligos: can be ordered from Sigma-Aldrich (https://www.sigmaaldrich.com) or IDT (https://sg.idtdna.com/pages).
2. Plasmids from Addgene (www.addgene.org).
3. DNase/RNase-free distilled water (Life Technologies).
4. Restriction endonuclease BbsI-HF® (New England BioLabs/NEB).
5. Antarctic Phosphatase (NEB).
6. CutSmart® buffer 10× (NEB).
7. T4 ligation buffer 10× (NEB).
8. T4 PNK (NEB).
9. Thermomixer (Eppendorf).
10. PCR cycler (Applied Biosystem, ThermoFisher).
11. Competent DH5-alpha *E. coli* cells.
12. Water bath for heat-shock transformation.
13. 1.5 mL microcentrifuge tubes (Eppendorf).
14. Sterilized glass beads for spreading *E. coli* cells.
15. LB agar: 1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride, 1.5% (w/v) agar.
16. LB broth: 1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride.
17. 10 mL bacterial culture tubes.
18. Ampicillin (100 μg/mL final concentration) (Sigma-Aldrich).
19. Incubator shaker (37 °C).
20. Qiagen® plasmid mini-, midi-, and maxiprep kit.
21. NanoDrop 2000 (NanoDrop).
22. Sanger sequencing facility.

2.3 Delivering Foreign DNA Inside Cells

1. Standard tissue culture reagents from Gibco® (ThermoFisher Scientific): Medium (DMEM GlutaMAX™, F-12, McCoy's 5A, Opti-MEM I reduced-serum medium, etc.), Dulbecco's PBS, trypsin-EDTA, FBS, penicillin-streptomycin.
2. Standard tissue culture flasks, plates and dishes, e.g., from TPP®.
3. Standard glasswares.

4. Pipetman Classic pipettes from Gilson®.
5. Lipofectamine™ 2000 kit (ThermoFisher Scientific).
6. Neon transfection system (ThermoFisher Scientific).

2.4 Enriching Transfected Cells

1. Flow cytometry instrument (BD biosciences, BD FACSCanto™ II).
2. Flow cytometry tubes (BD biosciences).
3. Puromycin (InvivoGen).

2.5 Evaluation of gRNA

1. QuickExtract™ DNA Extraction Solution (Lucigen®).
2. Q5® High-Fidelity DNA Polymerase (NEB).
3. dNTPs.
4. Primers for surveyor assay (Sigma-Aldrich).
5. QIAquick Spin Column (QIAGEN).
6. Surveyor assay kit (Transgenomic®).
7. 10% PAGE-TBE gel (polyacrylamide gel electrophoresis-Tris-Borate-EDTA).
8. Digital gel imaging system (Bio-Rad).
9. Image analysis software (ImageJ/Fiji).

2.6 Validating the Genome Editing

1. Standard setup for immunoblot experiment.
2. Standard setup for immunofluorescence experiment.

3 Methods

3.1 Choosing a Gene-Targeting Strategy

For any project, the choice of genome editing technique depends on the devised research question(s). Gene deletion is the method of choice if the physiological functions affected by the loss of gene under investigation are sought to reveal. Such gene knock-out experiments are generally followed by a bunch of relevant phenotypic studies to list the function(s)/pathway(s) that are affected as a result of loss of functions of that particular gene. However, if the localization and the molecular interactors/pathways of a gene are the primary foci, the endogenous tagging would be the strategy to employ. Endogenous tagging is usually done when suitable antibodies are not available for immunofluorescence/immunoblotting experiments and/or a researcher wants to track a protein over time in a live cell imaging experiment. Homology-directed point mutation can also be introduced by CRISPR-Cas-mediated genome editing. Such delicate gene/protein modifications are usually carried out to understand the impact of specific amino acids on the function of a particular gene/protein of interest. Sometimes the regulatory elements are also targeted to modulate the expression of

a particular gene. Nevertheless, in this chapter, we have focused on the two mostly used genome editing techniques in mammalian cells, e.g., gene knock-out and knock-in with greater details.

3.2 Making Knock-Out Cell Lines

3.2.1 Designing gRNA

For Cas9 nuclease, any 20 nt sequence upstream to the PAM sequence (NGG) can be used as gRNA to edit genome. Various web tools that design gRNA considering the likelihood of off-targets as well as RNA secondary structures are available. A host of such tools are listed in the website of Zhang lab (https://zlab.bio/guide-design-resources). CCTop (https://crispr.cos.uni-heidelberg.de/) is also a simple online tool for CRISPR-Cas9 gRNA prediction (*see* **Note 1**). The target sequence is designed depending on the purpose, knock-out/knock-in. For knock-in experiments, the tags are usually added to the N-terminal (1st exon) or C-terminal (last exon) of the protein. C-terminal site is preferable due to the presence of possible gene-regulatory regions in the N-terminal. Another advantage of tagging C-terminal is that the presence of expressed tag represents fusion protein rather than the expression of truncated tag-only product from the N-terminal. A tag can also be introduced elsewhere depending on the folding and conformation of the protein of interest. In case of knock-out experiments, the earlier the stop codon is introduced, the better for the later-on interpretation of phenotypes. Again, the targeted site should be decided by carefully analyzing the gene of interest. For example, the targeted site should always be upstream of the residues critical for the activity. So, the gene sequence should be downloaded from available online resources and analyzed with greater detail (https://www.ncbi.nlm.nih.gov/genbank/; https://genome.ucsc.edu/). In an ideal condition, gRNA should be chosen within a 50-base pair (bp) distance from the targeted sequence.

3.2.2 gRNA Synthesis and Cloning into the Delivery Vector

As delivery vector, pSpCas9(BB)-2A-GFP (pX458) or pSpCas9 (BB)-2A-Puro (pX459) plasmids are frequently used (http://www.addgene.org/crispr/) for mammalian cells. This plasmid system contains expression cassettes for sgRNA (under U6 promoter) and Cas9 (CBh promoter) in one plasmid (Fig. 3). So, the delivery of such gRNA-Cas9 containing single plasmid is sufficient for NHEJ-mediated gene deletion approach. For the HDR system, a separate plasmid containing the homology arms with desired sequence is needed to be delivered along with this plasmid (*see* **Note 2**). The delivery plasmid is digested with BbsI to insert gRNA after U6 promoter. Hence, the designed gRNAs are ordered as PCR (polymerase chain reaction) oligos keeping 5′-CACC-3′ overhang in forward and 5′-AAAC-3′ overhang in reverse oligos (Fig. 3).

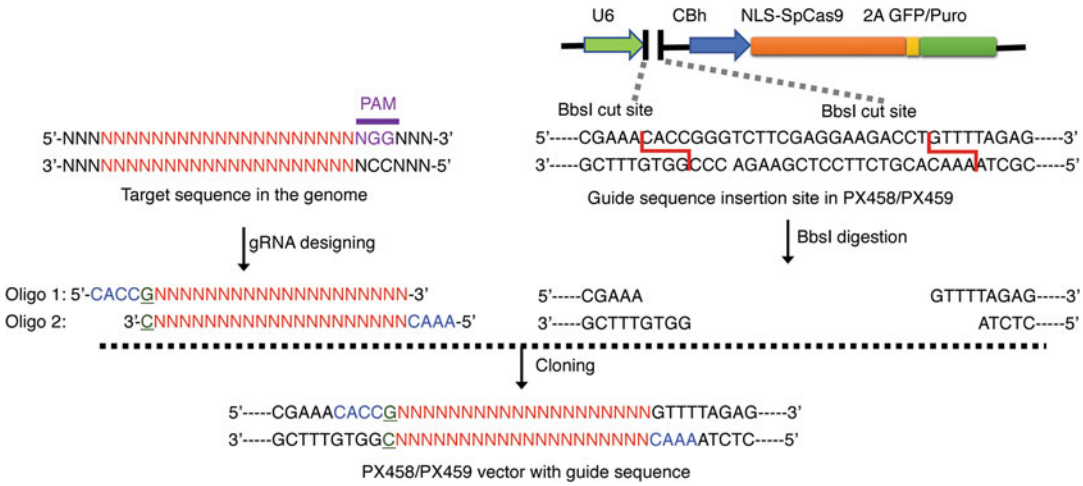

Fig. 3 Designing gRNA and cloning into delivery plasmid. To insert gRNA into delivery vector, 5'-CACC-3' overhang in the forward oligo and 5'-AAAC-3' overhang in the reverse oligo should be introduced. The oligos are annealed and cloned into BbsI-digested delivery vector (pX458/pX459). After transformation and purification of the vector, the sequencing should be carried out to confirm proper integration of gRNA

1. Digest 1 μg of pX458/pX459 with BbsI for 1 h at 37 °C:

	Amount
pX458 or pX459 (Addgene)	1 μg
BbsI-HF (NEB)	1 μL
Antarctic phosphatase (NEB)	1 μL
CutSmart® buffer 10× (NEB)	2 μL
ddH2O (Nuclease free)	X μL
Total	20 μL

2. Gel-purify the digested vector.
3. Phosphorylate and anneal oligos (sgRNAs):

	Amount (μL)
sgRNA top (100 mM)	1.0
sgRNA bottom (100 mM)	1.0
T4 ligation buffer 10× (NEB)	1.0
T4 PNK (NEB)	0.5
ddH_2O (Nuclease free)	6.5
Total	10.0

Anneal using a thermal cycler:

Temperature	Time
37 °C	30 min
95 °C	5 min
Ramp down to 25 °C	5 °C/min

4. Ligate the annealed oligos with digested gel-purified vector:

	Amount
Digested vector (50 ng) (from b)	X μL
Oligo duplex 1:200 dilution from c	1 μL
T4 ligation buffer 2× (NEB)	5 μL
ddH_2O (Nuclease free)	X μL
Subtotal	10 μL
T4 ligase (NEB)	1 μL
Total	11 μL

   - Incubate at 25 °C for 30 min
   - Heat inactivate at 65 °C for 10 min
   - Chill on ice
5. Transform 50 μL of competent DH5-alpha *E. coli* cells with 5 μL of the ligation reaction. Gently mix (don't vortex) the cells with ligation mixture and incubate on ice for 15–30 min. Heat shock the cells at 42 °C for exactly 1 min and immediately keep on ice for around 3 min. Spread the transformed cells on LB plate with proper antibiotic (ampicillin for pX458 and pX459) and allow to grow overnight at 37 °C.
6. Pick 8–10 colonies from LB plate and allow to grow overnight at 37 °C in 3–5 mL LB medium containing ampicillin.
7. Purify the plasmid through miniprep and restriction digest to pick the correct plasmid.
8. Send the plasmid for Sanger sequencing using U6 forward primer LKO. 1 5′ [14] (5′-GACTATCATATGCTTACCGT-3′) to check successful integration of gRNA.
9. Grow a midiprep/maxiprep culture of the correct clone and isolate plasmid DNA to have a concentration of 1–2 μg/μL for transfection.

3.2.3 Transient Transfection of the Delivery Vector

Delivery of foreign DNA to a mammalian cell greatly depends on the cell type. Please find the details about commonly used cell lines in various web resources such as ATCC (https://www.atcc.org/). Cancer cell lines such as HeLa, HCT116, and U2OS are easy to transfect, whereas primary cells such as fibroblast and epithelial cells are relatively difficult to transfect. Lipid or cationic polymer-based reagents are used to transfect the cancer cells. Primary cells can be successfully transfected by electroporation. All the cells should be cultured according to the protocol suggested by the global biological materials resource and standards organization ATCC (https://www.atcc.org/).

Lipid-Based Transfection for Cancer Cells (HCT116, HeLa, U2OS)

Lipofectamine™ 2000 (Invitrogen) can be used according to the supplier's guideline.

1. Seed cells and allow to grow to achieve 70–90% confluency in a 6-well plate during transfection.
2. Add fresh media (1800 μL) without antibiotics (penicillin/streptomycin) to the cells and keep it inside the incubator (37 °C).
3. Dilute Lipofectamine™ 2000 (4 μL) in 100 μL of Opti-MEM® medium without serum and gently mix. Incubate for 5 min at room temperature (RT).
4. Dilute vectors (1 μg) in 100 μL of Opti-MEM® medium without serum and gently mix and incubate it for 5 min at RT.
5. Gently mix the diluted Lipofectamine™ 2000 and diluted vector solutions. Incubate at RT for at least 20 min.
6. Add the mixture dropwise on top of the cells with fresh media.
7. Change media after 8–24 h and take measures to enrich transfected cells after 48 h (*see* **Note 3**).

Electroporation for Noncancerous Cells (RPE1)

Neon® transfection system is used according to the supplier's protocol:

1. Aspirate off the media from cells in 10 cm dish and wash once with PBS (5 mL).
2. Add 2–3 mL of trypsin-EDTA (just enough to cover the surface of the dish) and incubate at 37 °C in an incubator for ~5 min.
3. Add 8 mL of complete media to the dish and mix by pipetting gently (FBS in the media quenches trypsinization).
4. Transfer the cell suspension to a test tube, centrifuge (500 g) for 5 min, and remove the media.
5. Add 5 mL PBS and centrifuge again.

6. Count and prepare a suspension of 1×10^7 cells/mL with the resuspension buffer from the electroporation kit (Invitrogen™ Neon™ transfection system) (*see* **Note 4**).
7. Aliquot 100 μL or 1×10^6 cells in 1.5 mL Eppendorf tube for each transfection.
8. Add plasmids and gently mix (around 8 μg of gRNA-Cas9 plasmid and 2–5 μg of donor plasmid should be used). High concentration of plasmid is needed to avoid adding too much plasmid solution that alter the isotonic environment. Pipette carefully and avoid making bubbles. Mix around 15 μL DNA representing 10–15 μg of DNA with 100 μL cells.
9. Add 3 mL of E2 buffer to the electroporation tube (Neon® tube).
10. Load mix of harvested cells and plasmids into the Neon™ Pipette tip (*see* **Note 5**).
11. Plug the pipette into proper position in the Neon® transfection device and select appropriate protocol depending on the cell line and press Start (program for RPE1: pulse voltage (v)—1050; pulse width (ms)—30; pulse number—2).
12. Unplug the pipette and transfer the transfected cells into the 10 cm dish with pre-warmed media (*see* **Note 6**).

3.2.4 Enriching Transfected Cells

1. If pX458 plasmid is used, the cells are enriched by sorting (fluorescence-activated cell sorting/FACS) the cells that express GFP. In general, top 5–10% of GFP-expressing cells are sorted and propagated for further experiments.
2. If pX459 is used, add puromycin after 48–72 h of transfection and allow the selection process to be completed with 5–8 days. Cell confluency should be around 20–40% while starting antibiotic selection. A standard dose for puromycin is 2–5 μg/mL. However, the dose can vary according to the cell line and an antibiotic killing curve experiment should be carried out to determine the optimum dose for a specific cell line.
3. In both GFP and puromycin enrichment experiments, the blank control should be used to determine autofluorescence (in case of GFP) and efficiency of antibiotic (in case of puromycin).
4. Allow the cells to grow for 3–5 days and proceed to gRNA cutting efficiency measurement experiments.

3.2.5 Determining the Cutting Efficiency of gRNA

For targeting a specific gene, the use of multiple gRNAs is highly suggested for two reasons: (1) gRNA efficiency may vary depending on the chromosomal structure and (2) independent clones generated using different gRNAs will ensure the phenotypic findings in subsequent experiments. Nevertheless, it's always a

workload to maintain hundreds of clones from different gRNA experiments. So it's better to assay the gRNA cutting efficiency first and continue further experiments using the best two gRNAs (Fig. 4). Two methods are used nowadays to determine the cutting efficiency of gRNA: Surveyor assay and base sequence mix-read assay.

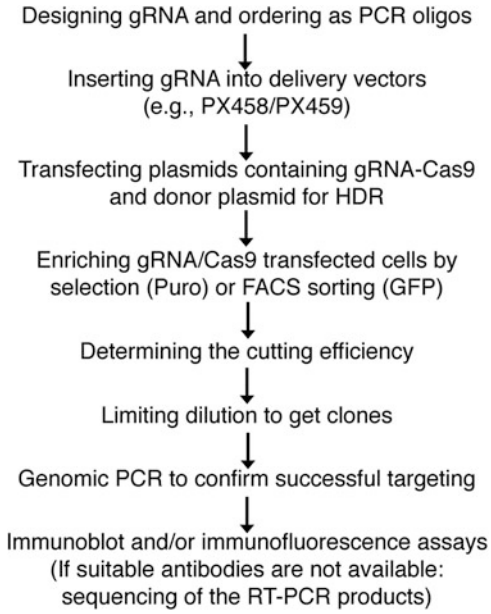

Fig. 4 Workflow for CRISPR-Cas-mediated generation of knock-out/knock-in cell lines. The target sequence should be determined after careful literature study of the gene of interest. Once the genomic locus is decided, at least three gRNAs should be designed using various available web tools (https:/zlab.bio/guide-design-resources). The gRNAs are synthesized and cloned into desired delivery plasmid as mentioned in Fig. 3. The delivery vector and the HDR vector (if specific changes are planned to be introduced) are transfected into the cells. Cas9-expressing cells are enriched by selection (Puro/pX459) or FACS (GFP/pX458). After sufficient expansion of cells (60–80% confluency in a 6-well plate), one third of the cells are harvested for genotyping PCR. The PCR primers should be designed in such to amplify 300–500 bp sequences, keeping the cut site in between. A different sequencing primer should be used to visualize the sequence surrounding the cut site. Base mix-read should be observed after cut site in case of NHEJ and desired sequence fusion should be observed in case of HDR. The most efficiently cut pool of cells should be subjected to limiting dilution for individual clones. Again, the genomic PCR should be carried out to confirm gene abruption (in case of NHEJ) or gene fusion (for HDR). The gene targeting should further be confirmed in protein label through immunoblot and immunofluorescence assays. If suitable antibodies are not available, base alterations should also be checked at RNA level through RT-PCR sequencing

Surveyor Assay/CEL-1 Assay

This system takes advantage of the in-del mutations caused by NHEJ after double-strand break. The target region is amplified using PCR primers flanking the targeted site. The amplicons should be a mixture of wild-type (WT) and NHEJ-mediated in-del products. The PCR products are denatured and reannealed to get hybrid of WT and NHEJ products. Such hybrid with DNA mismatch around the Cas nuclease cleavage site is recognized and cleaved by CEL-1/Surveyor mismatch endonuclease. The digested product is resolved by polyacrylamide gel electrophoresis (PAGE), and the ration of cleavage product to uncut WT product is directly proportional to the cutting efficiency of gRNA [15] (Fig. 5).

1. Extract genomic DNA using kit and manufacturer's protocol. QuickExtract™ DNA Extraction Solution (Lucigen®) provides a very simple and fast method for DNA purification.
2. Design PCR primer to amplify genomic region flanking the target site. In general, an amplicon size of 300–500 base pairs (bp) is preferred for efficient PCR amplification.
3. PCR amplification is carried out using Q5® High-Fidelity DNA Polymerase (NEB).

	Amount	Final conc.
ddH_2O (Nuclease free)	Adjustable	–
5× Q5 buffer	10.0 µL	1×
5× Q5 GC enhancer	10.0 µL	1×
2 mM dNTP	5.0 µL	200 µM
Forward primer (10 µM)	2.5 µL	0.5 µM
Reverse primer (10 µM)	2.5 µL	0.5 µM
Q5 HF DNA polymerase	0.5 µL	0.02 µ/µL
DNA template	0.2–0.5 µg	–
Total	50.0 µL	

Amplify using a thermal cycler:

Step	Temp. (°C)	Time	
Initial denaturation	98	30 s	
Denaturation	98	10 s	
Annealing[a]	50–72	30 s	30 cycles
Extension	72	30 s/kb	
Final extension	72	120 s	
Hold	10	–	

[a]Annealing temperature should be calculated using the web tool NEB Tm calculator (http://tmcalculator.neb.com/#!/main).

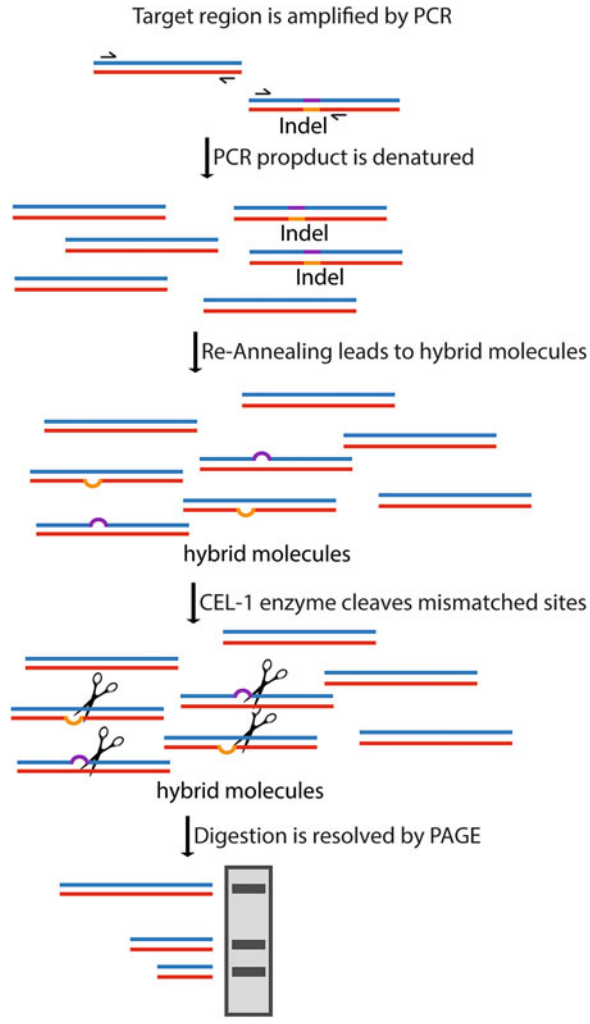

Fig. 5 Surveyor assay to determine gRNA cutting efficiency. Random insertion or deletion is the hallmark of NHEJ. The locus of interest is PCR amplified and the product is subjected to random heteroduplex formation. This leads to the hybrid DNA duplex with mismatch around the Cas nuclease cut site. Such mismatched DNA are cleaved by the CEL-I endonuclease. Upon resolving in PAGE, the ration between cleavage and parental bands represent the cutting efficiency of particular gRNA

4. Purify the PCR product using QIAquick Spin Column (QIAGEN).
5. Take 10 μL of PCR products and use the following protocol for heteroduplex formation:

Step	Temp. (°C)	Time (s)
Denaturation	95	10 min
Hybridization	95–85	−2 °C/min
	85–25	−0.1 °C/min
Hold	10	–

6. Add 1 μL of enhancer and 1 μL of Nuclease S (Transgenomic®) to the hybridized DNA samples and incubate at 42 °C for 60 min.
7. Run the digested samples on a 10% PAGE-TBE gel (polyacrylamide gel electrophoresis-Tris-Borate-EDTA).
8. Analyze the images with image analysis software such as ImageJ (https://imagej.nih.gov/ij/) or FIJI (https://fiji.sc/).

Base Sequence Mix-Read Assay

This is a very quick and simple qualitative test to determine gRNA cutting efficiency (thesis works from our lab [16, 17]). This method relies on the base mix-read in DNA sequencing. Aberrant in-del mutations that occurred during NHEJ causes the mixed population in DNA which can be read out as mixed-read. Like Surveyor assay, the targeted genomic region is amplified using PCR primers. The amplified product is purified and sent for sequencing using the same primer(s) used for amplification. The absence of WT sequence as well as degree of base mix-read indicated the extent of edited genome (Fig. 6).

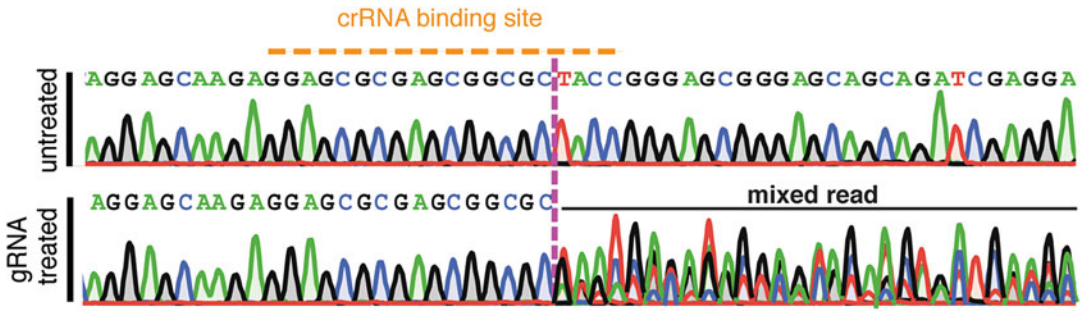

Fig. 6 Base mix-read assay to determine gRNA cutting efficiency. NHEJ-mediated aberrant in-del mutations cause a mixed population of bases around the cut site in a pool of cells. The PCR amplified products (similar to Surveyor assay, Fig. 5) from the targeted site is purified and sequenced. The mixed population of bases in the cell pool will eventually read out as base mix-read after/around the cut site. In a sequence analysis software, e.g., SnapGene, the histogram that indicates the quality of bases graphically shows severe drop in quality around the cut site. Such base mix-read indicates cutting efficiency of the gRNA

3.3 Making Knock-in Cell Lines by PCR Tagging

The availability of precise, programmable nucleases enables the insertion of any desired sequence into the genome. However, while knock-out studies using CRISPR-Cas9 have become more frequent, knock-in reports for applications such as gene tagging are not yet common and still remain challenging. Fueller et al. have developed a simple strategy to insert tags into endogenous mammalian genes using CRISPR-Cas12a without the need of time-consuming plasmid cloning and additional reagents like proteins, RNAs, or ssDNAs [18]. This technique, referred to as *mammalian PCR tagging*, only requires PCR to generate the gene-specific components for transfection and allows tagging efficiencies of up to 20% without selection. *PCR tagging* just demands a pre-designed plasmid (template cassette) bearing the desired tag and two unique primers (M1 and M2 oligo) containing 50–90 nucleotides-long homology arms of the target sequence, the variable crRNA sequence as well as an overlap with the template cassette. Simply by performing PCR, the template cassette and the M1/M2 oligos generate a gene-specific cassette (PCR cassette) that is able to integrate into the targeted sequence after co-transfecting it with a Cas12a (CPF1) helper plasmid (Fig. 7).

Since the designed oligos contain the variable crRNA and the template cassette provides the U6 Pol III promotor, the PCR cassette generates a crRNA gene enabling the recruitment of Cas12a. After induction of the double-strand break near the targeted site, the homology arms of the PCR cassette function as a template for the repair by HDR, thereby integrating the desired tag. Simultaneously, the crRNA binding site is destroyed upon integration of the tag, avoiding re-cleavage by Cas12a. Successful integration of a fluorescence tag can already be checked by fluorescence microscopy 3 days after transfection. However, some cell lines like HEK293T may show a diffuse cytoplasmic fluorescence which might be the result of a circularized PCR product of transient nature which is able to express the coded fluorophore driven by the U6 promotor. Removing the start codon of the fluorophore largely reduces the unwanted diffuse fluorescence potentially caused by cassette fusions; however, it can't be completely abolished since the homology arm or the crRNA sequence may provide a start codon. In case of occurrence of circularized fragments, the unspecific fluorescence will gradually fade while the specifically localized signal of the stable integrated fluorophore into the genome is constant. Cells can be enriched or clones can be generated by fluorescence-activated cell sorting (FACS).

3.3.1 Ordering Oligos and Necessary Plasmids

Design and order M1/M2 oligos using online design tool (www.pcr-tagging.com). Template plasmids with different tags (e.g., GFP) and optionally a selection marker (e.g., Zeocin) should be obtained from Addgene (www.addgene.org).

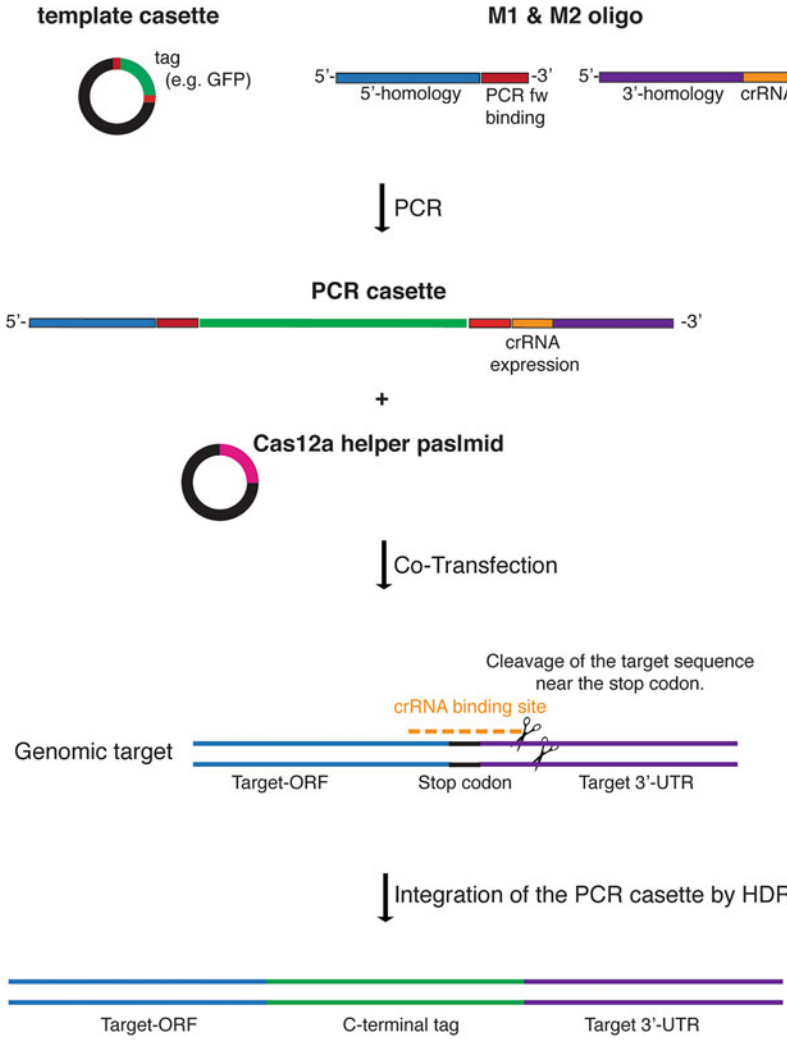

Fig. 7 Principle of mammalian PCR tagging. PCR cassette is generated by PCR using template cassette and designed M1/M2 oligos. Purified PCR cassette which provides gRNA gene is transfected together with a suitable Cas12a helper plasmid to induce double-strand break in the targeted sequence. Homology arms of the PCR cassette are used as template to repair cleavage, thereby integrating the c-terminal tag. Insertion of the PCR cassette destroys the crRNA binding site avoiding re-cleavage

3.3.2 Generating PCR Cassette

1. PCR cassette can be generated using high-fidelity (hi-fi) DNA polymerase. Commercial or self-purified hi-fi polymerases can be used. Please note that so far, only the Velocity polymerase (Bioline) was found to work in the buffer supplied by the manufacture. However, most polymerases work using the following buffer conditions and PCR settings:

	Amount
10× HiFi buffer[a]	5 μL
dNTPs (10 mM)	5 μL
$MgCl_2$ (50 mM)	2.5 μL
Betaine (5 M, Sigma)	5 μL
Template cassette	100 ng
M1 oligo (10 μM)	2.5 μL
M2 oligo (10 μM)	2.5 μL
ddH_2O (nuclease-free)	Adjustable
Velocity (Bioline), Phusion (T. Fisher) or self-purified DNA polymerase[b] (1 U/μL)	0.25 μL, 0.5 μL or 1 μL
Total	**50 μL**

[a]200 mM Tris–HCl, pH 8.8; 100 mM $(NH_4)2SO_4$, 500 mM KCl, 1% (v/v) Triton X-100, 1 mg/mL BSA, 20 mM $MgCl_2$
[b]Pfu-Sso7d [19]

Thermocycling conditions

Step	Temp. (°C)	Time	Cycle
Initial denaturation	95	3 min	
Denaturation	95	20 s	
Annealing	64	30 s	30
Extension	72	45 s/kb	
Final extension	72	5 min	
Hold	10		

2. Optional: Digest methylated template cassette after PCR. This step should be carried out when using a template cassette containing a selection marker.

	Amount (μL)
PCR reaction	50
DPN1 or FspEI	0.4
Enzyme activator	1.67

- Incubate at 37 °C for 1 h
- Heat inactivate at 80 °C for 20 min
- Chill on ice

Analyze PCR product by gel electrophoresis and purify nucleic acid by standard clean-up spin column.

3.3.3 Transfecting Cells

Transfect cells with the same amount of purified PCR cassette and corresponding Cas12a helper plasmid. Transfect HEK293T, HeLa, and U202 cells in a 24-well plate using a total amount of 1 μg DNA and Lipofectamine™ 2000 (Invitrogen) according to manufacturer's protocol (Subheading 3.2.3.1). For RPE-1, C2C12, and mESCs cells, electroporate 1×10^6 cells with a total amount of 10–15 μg DNA and seed into a 10 cm dish (according to Subheading 3.2.3.2). Evaluate targeting efficiency 3 days after transfection by fluorescence microscopy. If diffused fluorescence is detected, keep in culture until nonspecific signal is gone before proceeding. (Optional: Start selection using Zeocin (300–500 μg/mL) or puromycin (1–5 μg/mL) for approx. 2 weeks and stop when all WT control cells are dead.)

3.4 Validating the Genome Modification

The genome edited cells are allowed to grow and, after few days (3–5), subjected to clonal propagation through limiting dilution or FACS. Limiting dilution is a very simple method where cells are diluted to have around 2–3 cells/mL and then 200 μL of this suspension in a well of 96-well plate is added. FACS is a more precise method to plate 1 cell per well of 96-well plate. However, FACS itself can be stressful for certain cell types (*see* **Note 7**). The emerging clones in the 96-well plates are then expanded to grow enough for validation experiments. In general, for a relatively good gRNA, 72–96 clones per condition should be good enough to get 3–5 good clones. For a knock-out or knock-in experiment, the desired genetic changes can be confirmed at DNA, RNA, and protein levels. If proper antibody is available, validation at DNA and protein levels should be sufficient to confirm changes. However, if antibodies are not available, corresponding changes in DNA and RNA levels should be ensured by sequencing the PCR and RT-PCR (reverse transcription polymerase chain reaction) products. If antibodies permit, both immunoblot and immunofluorescence experiments should be carried out [20]. For knock-out experiments without donor construct, NHEJ is expected to introduce premature stop codon. In case of homology-mediated gene deletion, stop codon in all three reading frames are deliberately introduced along with the homologous sequences. For knock-in experiments using fluorescent tag, the validation is very straightforward. Fluorescence-expressing cells are sorted by flow cytometry (FACS) and genotyped to see monoallelic or biallelic genetic changes. If nonfluorescent tags are used such as FLAG, HA, or so on, immunoblot and immunofluorescence analysis should be carried out instead of FACS.

4 Notes

1. While designing gRNA, if there is no G at the beginning (5′-end), please add an extra G. This is critical for transcription under U6 promoter. Furthermore, such addition of base far from the PAM site does not affect specificity.
2. For HDR, the PAM site should be mutated in the donor cassette to avoid being targeted by Cas9 after homologous repair.
3. Cells are passaged in 3–4 days at a split ratio of 1:4 for primary cells (RPE1) or 1:8 for cancer cells (HeLa, HCT116, HEK293T, U2OS, etc.). Never allow cells to reach more than 80% confluency.
4. For counting cells, make sure that cells are dissociated properly and there is no clamp. Examining the cells under a microscope is highly suggested before making cell suspension for counting. Adjust the cell concentration in such to have better counting. Too much concentrated or diluted cells give erroneous count.
5. The cell and DNA mixture should be gently mixed and pipetted to avoid bubble formation. Bubble kills cells during electroporation.
6. Cells become fragile after electroporation. So disperse the cells in the plate by very mild shaking.
7. A lot of cells die after FACS. So, for single-cell dilution, grow enough cells, e.g., 4 96-well plates to get at least 96 clones.

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Chapter 10

Cloning-Free (DNA-Free) CRISPR-Cas9-Mediated Gene Editing in Human Liver Cell Line and Its Detection

Magdalena Śmiech, Paweł Leszczyński, Effi Haque, and Hiroaki Taniguchi

Abstract

The clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) system is a powerful tool for precise genome editing. The recent efforts of scientists allow to improve current genome engineering techniques, which are used to generate modified cell lines for functional studies. Here we describe, in detail, the protocol for DNA-free CRISPR-Cas9-mediated gene editing in a human liver cell line. Briefly, we used lipofection to deliver a ribonucleoprotein (RNP) complex consisting of Cas9 protein, crRNA, and tracrRNA to the cells. The crRNA quality was validated before transfection by performing an in vitro cleavage assay and visualized using an agarose gel. Afterward, we checked the efficiency of the target gene modification by Sanger sequencing and T7 endonuclease I digestion. Our goal is to provide a simple protocol for CRISPR-Cas9-mediated gene modification.

Key words CRISPR-Cas, Genome editing, Liver cell line, Sanger sequencing, T7 endonuclease I

1 Introduction

In recent years, genome editing techniques have developed and improved very intensively, allowing scientists to create new and more accurate models for further study. Rapid progress in genome modification methods has shed light on the successful application, among others, in the prevention and treatment of many diseases [1–3]. One of the latest developments in precise gene editing is the clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) system [4]. CRISPR is widely used and has been developed in a variety of fields, with a broad range of applications [5].

CRISPR-Cas9 was first discovered in a microbial immune system adapted for eukaryotic gene editing and contains three main elements: Cas9 endonuclease and two short RNAs: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) combined as a guide RNA (gRNA) [6]. Double-strand breaks (DSB), generated by CRISPR-Cas9 complex, activate the mechanisms of DNA repair.

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Nonhomologous end-joining (NHEJ) repair leads to insertion and deletions (indels) at the target site [7]. Delivery of these components into the cells can be achieved via electroporation, microinjection, or lipofection. Moreover, there are different distinguished methods for introducing CRISPR-Cas9 complex into the cells: DNA-based delivery (plasmid encoding the single guide RNA and Cas9), RNA-based delivery (mRNA for Cas9), or delivery as a ribonucleoprotein complex (RNP) [8]. Our protocol demonstrates a simple method for target gene editing using DNA-free (cloning-free), CRISPR-Cas9-mediated modifications in the human THLE-2 cell line derived from primary normal liver cells. In this protocol, we used purified Cas9 protein fused with nuclear localization signal (NLS). This approach reduces undesirable alterations in the genome and allows for gene editing with a high efficiency rate, simultaneously [8, 9].

Briefly, crRNA complementary to the target DNA sequence can be designed using convenient software. The efficiency of the RNP complex can be evaluated before practical application using an in vitro cleavage assay. The products of the reaction were observed on an agarose gel as two proper-size bands demonstrating the high activity of the designed CRISPR complex. Next, cells were transfected with RNP complex via lipofection and the target gene modification was checked after two days.

Crude cell lysate was used to run direct PCR. This method allows us to obtain rapid results without additional reagents used for DNA purification. CRISPR-Cas9-mediated modification was tested by the T7 Endonuclease I (T7EI) mismatch cleavage assay [10]. Additionally, Sanger sequencing was performed to confirm indels in the target site. This cloning-free procedure is quick, user-friendly, and efficient.

2 Materials

2.1 gRNA Design

To identify the target region for gene editing, crRNA was designed using DESKGEN [11]. crRNA (5′-TAGCTACAGTGAAATCTCG A-3′) complementary to the sequence located next to a protospacer adjacent motif (PAM) (5′-NGG for SpCas9) in the BRAF protein kinase motif (exon 15) was used in this study. Both crRNA and tracrRNA were purchased from Integrated DNA Technologies (IDT) and resuspended in Nuclease-Free Duplex Buffer (IDT) to the final concentration of 100 μM and stored at −20 °C.

2.2 Complete Medium for THLE-2 Cell Line

The complete culture medium was used to culture the THLE-2 cell line that consisted of LHC-8 medium (Gibco™), 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL of streptomycin, 5 ng/mL epidermal growth factor (EGF), and 70 ng/mL phosphoethanolamine.

Table 1
Components of lysis buffer

Component	Amount	Final concentration
1 M Tris–HCl, pH = 8.3	2 mL	200 mM
1 M KCl	2 mL	200 mM
Tween-20	90 μL	0.9%
Sterile water	5.91 mL	–
20 mg/mL Proteinase K (add before use)	6.25 μL per 1 mL of lysis buffer	125 μg/mL

2.3 Lysis Buffer

All components and concentrations for the lysis buffer are presented in Table 1.

3 Methods

3.1 In Vitro Cleavage Assay

This method was used to validate the activity of the RNP complex (*see* **Note 1**).

1. gRNA was prepared in a 0.2 mL tube by mixing 1 μL of crRNA (100 μM) and 1 μL of tracrRNA (100 μM) with 8 μL Nuclease-Free Duplex Buffer (IDT).
2. The tube was incubated at 95 °C for 5 min and cooled down to room temperature on the bench.
3. 10 μL of gRNA (10 μM) was combined with 1.6 μL of Cas9 (61 μM) and 88.4 μL of Opti-MEM (Gibco™) in a 0.5 mL tube.
4. The mixture was incubated at room temperature for 5 min to create the RNP complex.
5. 1.5 μL of the RNP complex (from **step 4**), 1 μL of PCR product of the target gene, and 12.5 μL of Opti-MEM were mixed in a 0.5 mL tube. The final volume was 15 μL.
6. The reaction was incubated overnight at 37 °C, followed by a 10-min incubation at 95 °C.

3.2 Agarose Gel Electrophoresis

Samples were loaded into the well of 1.5% agarose gel containing 1× Tris-borate-EDTA (TBE) buffer and stained with Simply-Safe™. Electrophoresis was run at 100 V for 45 min (Fig. 1).

3.3 Preparation of gRNA for Cell Transfection

1. 0.5 μL crRNA (100 μM), 0.5 μL tracrRNA (100 μM), and 49 μL of Nuclease-Free Duplex Buffer (IDT) were mixed in a 0.5 mL tube. The final concentration of the duplex was 1 μM.
2. The mixture was incubated at 95 °C for 5 min and cooled down to room temperature on the bench top.

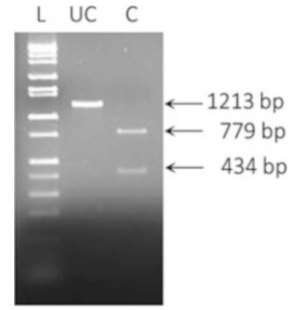

Fig. 1 Gel image of in vitro cleavage assay for determination of the activity of the RNP complex. UC (uncut) represents PCR product (input DNA). C represents PCR product treated with the RNP complex. Two bands at the expected size of 779 bp and 434 bp were observed on the agarose gel. DNA ladder (L) has a size of 50–10,000 bp

3.4 Creation of the RNP Complex for Cell Transfection

1. Cas9 (61 μM) was diluted to 1 μM in Opti-MEM.
2. 6 μL of 1 μM gRNA (from Subheading 3.3) was mixed with 6 μL of Cas9 (1 μM).
3. Next, 88 μL of Opti-MEM was added to the mixture from **step 2**. The final volume was 100 μL/well in a 24-well plate.
4. The components were mixed well and incubated at room temperature for 5 min (*see* **Notes 2** and **3**).

3.5 Preparation of THLE-2 Cells

1. Complete Medium was aspirated from the 25 cm^2 flask and the cells were washed with 2 mL of calcium and magnesium-free PBS.
2. 1 mL of 0.25% trypsin-EDTA was added to the flask and the cells were plated in a 37 °C with 5% CO_2 incubator for 4 min.
3. 8 mL of Complete Medium was added into the flask, mixed, and the cells were transferred to a 15 mL tube.
4. The cells were centrifuged at 200 × *g* for 5 min at room temperature. The supernatant was carefully removed.
5. 8 mL of PBS was added to the tube and the cells were washed by pipetting several times.
6. The cells were centrifuged at 200 × *g* for 5 min to pellet the cells and the supernatant was removed.
7. 2 mL of Complete Medium, without antibiotics, was added to the tube and the cells were resuspended.
8. The cell number and viability were determined using a cell counter.
9. The cells were diluted in Complete Medium (without antibiotics) to obtain 5 × 10^4 cells in 400 μL per well in a 24-well plate.

3.6 Reverse Transfect the RNP Complex

1. 100 μL of the RNP complex was mixed with 6 μL of Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen™) and 94 μL Opti-MEM. The final volume was 200 μL/well in a 24-well plate.
2. The components were mixed well and incubated at room temperature for 20 min.
3. 200 μL of reverse transfection complex was added to the well in a 24-well plate.
4. 5×10^4 cells in 400 μL per well in a 24-well plate were added.
5. The plate was placed into the incubator (37 °C and 5% CO_2) for 2 days.

3.7 Cell Lysis

1. The culture media was carefully aspirated from the culture well and cells were washed with 0.5 mL of PBS.
2. 100 μL of lysis buffer was added (Table 1) per well in a 24-well plate and the cells were detached with a cell scraper (*see* **Notes 4** and **5**).
3. The lysed cells were transferred to a 1.5 mL tube.
4. The samples were incubated at 56 °C for 30 min and the reaction was stopped at 95°C for 10 min.
5. PCR was performed immediately or the samples were stored at −20 °C.

3.8 Direct DNA Amplification

1. A master mix for DNA amplification was prepared by combining reagents listed in the Tables 2 and 3.
2. The reagents were mixed by pipetting up and down and centrifuging briefly.

Table 2
Master mix for direct DNA amplification

Reagent	Amount (μL)	Final concentration
2× Terra PCR Direct Buffer (with Mg^{2+}, dNTP)	12.5	1× Buffer; 2 mM Mg^{2+}; 400 μM dNTP each
10 μM forward primer (F)	1	400 nM
10 μM reverse primer (R)	1	400 nM
Terra PCR Direct polymerase mix	0.5	1.25 U
Sterile water	8.5	–
DNA (cell lysate)	1.5	–
Final volume per reaction	25	–

Table 3
Primer sequence used for PCR

Primer name	Sequence	Product length (bp)
Forward primer (F) Reverse primer (R)	CTGCAGCATCTTCATTCCAA GCTGCAATGCACACAAGTTT	1213

Table 4
Cycling conditions for direct PCR

Step	Temperature (°C)	Time (s)	Number of cycles
Initial denaturation Denaturation Annealing Extension	98 98 62 68	120 10 15 60	1 40
Final extension	68	300	1
Hold	4–12	∞	–

3. A program was set up on the thermal cycler (Table 4) and PCR was started.
4. Agarose electrophoresis was run to check PCR product.

3.9 T7 Endonuclease I Digestion

This method enables the determination of genome targeting efficiency, by the cleavage of non-perfectly matched DNA after the reannealing of DNA strands.

1. A reaction was prepared by combining 1 μL of PCR product (from Subheading 3.8) with 1 μL of 10× NEBuffer 2 (NEB, New England Biolabs) and 7.5 μL sterile water in a 0.2 mL tube.
2. Using a thermal cycler, the reaction was incubated at 95 °C for 5 min.
3. The tube was removed and allowed to cool down to room temperature on the bench.
4. 0.5 μL of T7 Endonuclease I (NEB) was added and the tube was incubated at 37 °C for 15 min (*see* **Note 6**). The reaction was inactivated at 95 °C for 10 min.
5. The samples were loaded along with input DNA as a control into the wells of the 1.5% agarose gel. Electrophoresis was run at 100 V for 45 min (Fig. 2a).
6. Densitometry analysis was performed using Image Lab™ Software or Image J [12, 13] (Fig. 2b) and calculated according to the following formula [15]:

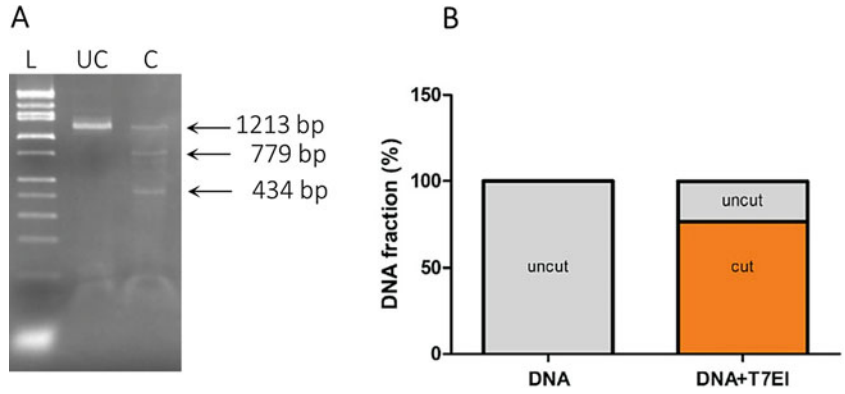

Fig. 2 CRISPR-Cas9 editing efficiency represented by gel image. (**a**) Result of T7EI-treated PCR products amplified from the target DNA sequence site of CRISPR-edited cells. UC (uncut) represents input PCR product. C (cut) represents PCR product after CRISPR-Cas9-mediated gene editing. DNA ladder (L) ranges from 50–10,000 bp (**b**) Comparison of uncut DNA sample (DNA) and DNA incubated with T7 Endonuclease I (DNA+T7EI)

The fraction cleaved is defined as:

$$\frac{\text{density of digested products}}{\text{density of digested products} + \text{undigested parental band}}$$

$$\%\text{DNA modification} = 100 \times [1 - (1 - \text{fraction cleaved})^{1/2}]$$

3.10 Sanger Sequencing

A 1213 bp *BRAF* gene fragment was sequenced using forward primer by a Sanger sequencing (Genomed S.A., Warsaw, Poland). Heterogeneous sequence (*see* **Note 7**) started from the cleavage site confirmed by CRISPR-derived gene editing (Fig. 3). The results were analyzed using FinchTV 1.4.0 software [14].

4 Notes

1. In vitro cleavage assay should be performed before practical application to validate the activity of the RNP complex.
2. The time required for the proper RNP complex formation should be no longer than 5–10 min.
3. The RNP complex should be stored at 4°C for up to 1 month or at -80°C for up to 6 months. We do not recommend to store RNP complex at -20°C due to its reduced stability.
4. Lysis buffer (without proteinase K) should be stored at room temperature. Adding proteinase K before use is mandatory.
5. The number of cells should not be less than 5 x10^4 and no more than 2 x10^6 cells in 100 μL of lysis buffer for sufficient PCR.

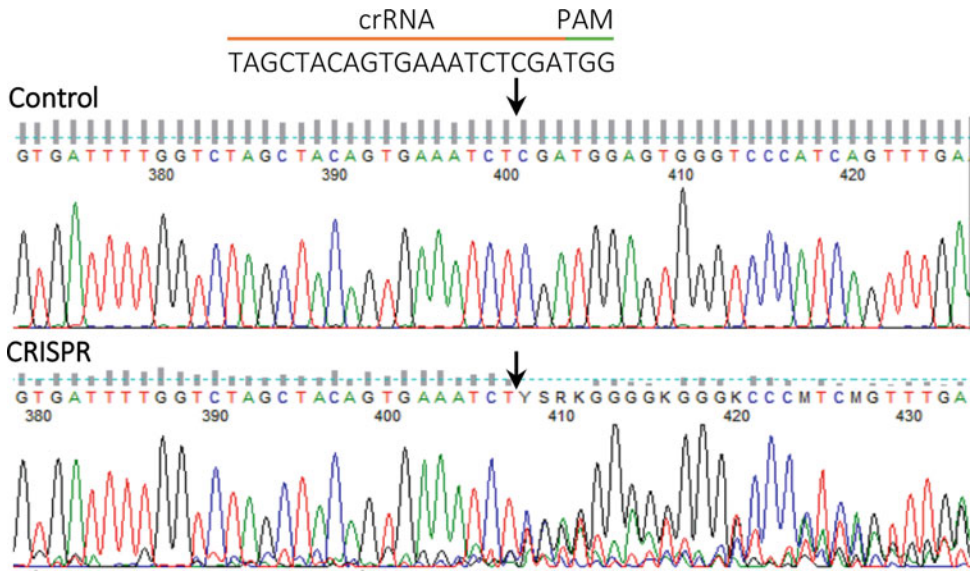

Fig. 3 Sanger sequencing analysis of *BRAF* sequence after CRISPR-Cas9-mediated gene editing in THLE-2 cell line. Control DNA (untreated cells) and DNA from CRISPR-edited cells were amplified and subjected to sequencing analysis. Black arrows point Cas9 cut sites

6. Optimal reaction temperature for T7 Endonuclease I activity is 37 °C.
7. This method generates a heterogeneous CRISPR-edited cell population. To obtain monoclonal cell line with desired gene modification, it is recommended to isolate single clones by serial limiting dilution or single cell sorting.

Acknowledgment

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Chapter 11

A Procedure to Design Guide RNA, Assemble Fragments, and Detect Mutation for Genome Editing in Flax

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Abstract

The game-changing molecular tool CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9) has recently been developed as an effective genome-editing tool. It is used for targeted mutagenesis, whereby Cas9 enzyme creates a DNA double-strand break (DSB), which is then repaired by mutation-prone non-homologous end joining (NHEJ) repair system; consequently, the resulting DNA sequence collects mutations either through insertion or deletion of nucleotides. As there are already substantial challenges in the agricultural production, we are in need of highly efficient molecular tool such as CRISPR-Cas9 with detailed information so that the tool can be embraced and applied routinely. Flax (*Linum usitatissimum* L.) is one of the economically important crops; in this chapter, we provide a guideline to design guide RNA, assembly of DNA fragments, and a detailed protocol for protoplast isolation, transfection, and mutation detection.

Key words CRISPR-Cas9, Genome editing, CRISPRdirect, Gibson assembly, Flax

1 Introduction

Molecular methods to edit plant genome sequence in vivo is a versatile tool not only to decode the function of an unknown gene fragment but it can potentially be a genetic solution to meet the needs of increasing global demands on food, medicine, timber, and clothing. Over the last decade, precise genome editing in higher organisms including plants has been achieved using nucleases such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)-associated RNA-guided DNA endonucleases (Cas) [1]. These methods introduce

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targeted mutagenesis; thereby the nucleases create double-strand DNA break (DSB), encouraging the cellular DNA repair system [2]. DNA repair takes place either by mutagenic non-homologous end joining (NHEJ) or more precise homology-directed (HD) DNA repair system [3]. While the NHEJ is quick and commonly observed, this route is highly error prone; thereby protein factors religate the DNA breaks directly or indirectly by introducing insertions or deletions of nucleotides [4–6], which potentially can lead to a targeted mutation causing a loss of function of a gene [2]. Alternatively, a homologous DNA fragment from a donor can be taken and replaced in the DNA break and repair the damage [7] and the process is known as a homologous-directed repair (HDR) system.

With the emergence of programmable nuclease, CRISPR-associated system (Cas), the targeted mutagenesis has become simple and quick in many plant species including both model and non-model species. This system has three components: (1) guide RNA (gRNA) sequence 20 bp, (2) gRNA scaffold, and (3) endonuclease Cas9 [8]. Upon introduction of the two components along with their regulators, gRNA binds to the complementary DNA segment, close to the protospacer adjacent motif (PAM) typically represented by "NGG" in the target gene [9]. This hybridization activates and directs the Cas9 protein to introduce a DSB, 3–4 bp upstream of the PAM motif. Thus, this simple mechanism has been applied to create targeted genome editing in a wide group of plant species [2].

The simplicity and flexibility of the CRISPR-Cas9 system has expanded its application in both animal and plant systems, leading to the development of transgenic animals and plants, identifying disease targets, finding the function of unknown genes, etc. [10]. While the targeted mutagenesis has been established over different crop species, this powerful technology is still difficult to apply in many plant species, including flax. Efficacy of the system has been a challenge, factors including Cas9 activity, delivery of the system, guide RNA design, off-target issues, etc. [10].

Here we describe the detailed procedure to execute CRISPR-Cas9-mediated genome editing using flax protoplast.

2 Materials and Methods

2.1 Identification of gRNA

Resources: Internet access, target gene, CRISPRdirect.

Protocol: There are two approaches to design gRNAs to target all copies of a gene. In the first strategy, using sequence alignment, gRNAs can be designed to target all the conserved regions [11]. In the second strategy, different gRNAs can be designed for each copy and co-transformed to target all the copies. The gRNA seed sequences can be selected according to the reference genome sequence using publicly available online tools either CRISPRdirect (https://crispr.dbcls.jp/) [12] or CRISPOR (http://crispor.tefor.net) [13]. A few requirements are needed for choosing the unique gRNA target sequence. Usually, we design 3–5 gRNAs for each gene.

1. The 20-bp target sequence should immediately precede the 5′-NGG PAM, which is essential for Cas9 to bind to the target DNA.
2. If the aim is to disrupt gene function, target sequences should be avoided at the 3′end of coding regions or intron. Gene disruptions in these positions may have a little or no effect on gene function. Off-target mutagenesis can be avoided by BLAST search using the reference genome database for the target gene.
3. The presence of an appropriate restriction enzyme site in the target sequence is optional. But if gRNA activity is to be detected by the PCR/RE assay, restriction enzyme sites within the target sequences will facilitate detection.
4. 5′-G is required for the stability of gRNA.

In this section, we describe steps to design flax CRISPR-Cas9 gRNA with reduced off-target sites using CRISPRdirect.

CRISPRdirect is a simple and reliable web-based tool for gRNA designing, which is freely available at http://crispr.dbcls.jp/. As we have already learned, the target sequence requirements are twofold:

1. First, the 5′-NGG protospacer adjacent motif (PAM) sequence must be located adjacent to the target sequence.
2. Second, the target sequence should be specific within the entire genome in order to avoid off-target editing.

The server (CRISPRdirect) currently incorporates the genomic sequences of over 200 species including flax (*Linum usitatissimum*) genome, v1.0.

The software searches target sequences of 20 nt adjacent to the PAM sequence (e.g., NGG, NRG) from both strands of the input sequence (Fig. 1a). Tables are generated showing a list of potential gRNAs with target position, target sequence, GC% of 20mer, restriction sites, number of potential target sites in the genome, etc. The software highlights the target positions indicating sequences that are highly specific and have fewer off-target hits (Figs. 1b and 2). It is recommended to avoid target sequences with "0" in "20mer + PAM" as such sequences may possibly span over exon-exon junctions. We should also avoid target sequences with TTTTs gRNA vectors with pol III promoter. The newly designed gRNA candidates can be synthesized and obtained from Integrate DNA Technologies (IDT) or any other local vendors.

2.2 Validation of gRNA

Although CRISPR-Cas9 has been shown as an effective tool in genome editing, success rate depends on the activity of gRNA as some gRNA may be proved as inactive or less active in inducing a DSB. Upon feeding a target sequence, different algorithms can generate large number of gRNAs; however, finding a functional

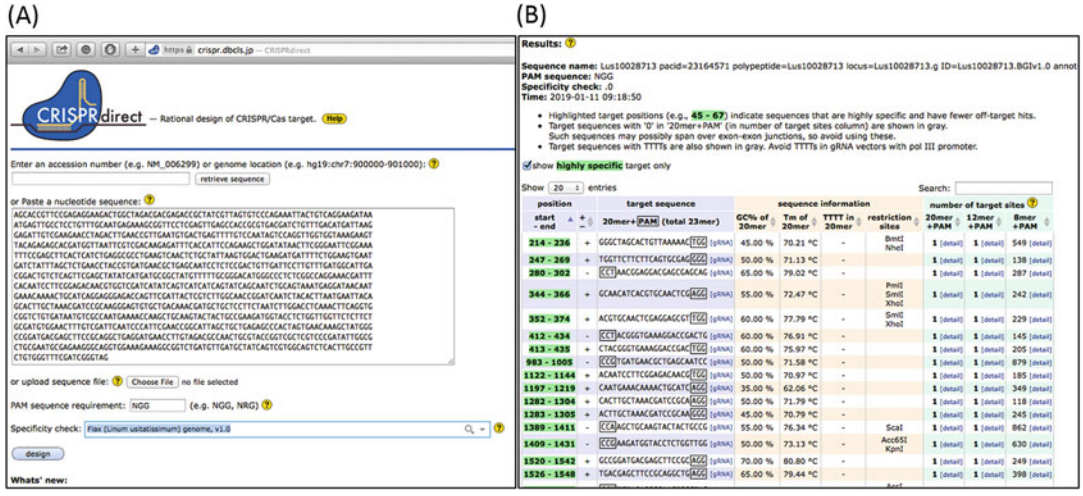

Fig. 1 Screen capture from the sequence entry page of the CRISPRdirect web-based tool. (**a**) First window on the server that shows the sequence input box and it accepts an accession number, a genome coordinate, or an arbitrary nucleotide sequence up to 10 kbp as input. (**b**) It shows the results with highly specific gRNA candidates. Green highlight indicates the high specificity

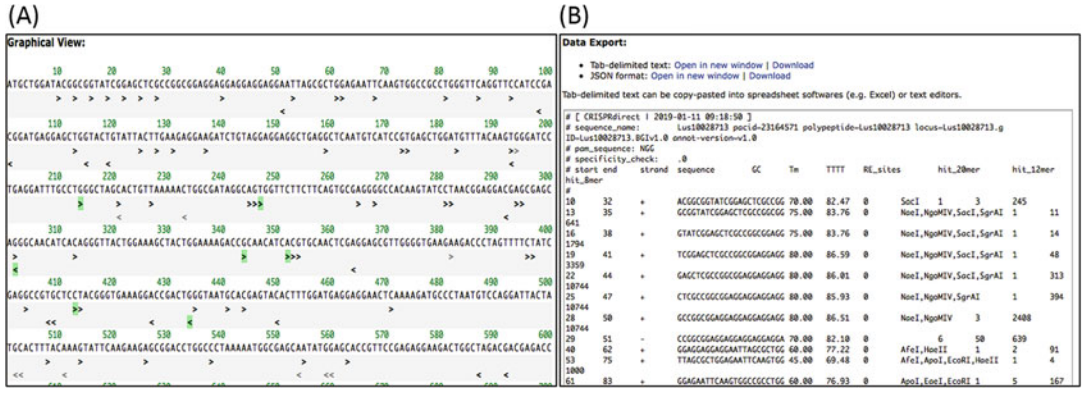

Fig. 2 Screen capture of the CRISPRdirect output window. (**a**) An output window that shows a graphical view of the target sites with positions and orientations. (**b**) The window with gRNA candidates that can also be exported in a different format including tab delimited and JSON format

gRNA is vital. Therefore, screening and validation of gRNAs in vitro is essential prior to proceeding to the construction of the CRISPR-Cas9 plasmid.

Resources: Cas9 Nuclease [New England Biolabs (NEB)], NEB Buffer 3.1, nuclease-free water, Proteinase K, gRNA fragment, amplified target DNA sequence, agarose, EZ-Vision DNA Dye, QIAquick PCR Purification, QIAprep Spin Miniprep Kit, target-specific primers, PCR reagents.

Protocol:

1. Use the target specific primer sequence to amplify the target fragment from your genomic DNA. Set up a 50 μL PCR reaction with the following reagents:

10× PCR buffer	5 μL
10 mM dNTPs	1 μL
10 mM forward primer	1 μL
10 mM reverse primer	1 μL
Template DNA	Variable (50–100 ng)
Taq DNA polymerase	0.25 μL
Nuclease-free water adjusted to	50 μL

2. Follow the PCR protocol to run the reaction, initial denaturation at 95 °C for 30 s, followed by 35 cycles (95 °C for 30 s, 45–62 °C for 30 s, 72 °C for 1 min/kb), final extension at 72 °C for 5 min, and hold at 4 °C (*see* **Note 1**).
3. Upon completion of the reaction, take 7 μL of the PCR product, add 1 μL of gel loading dye, and electrophorese for 20–30 min at 90 Volt to resolve the amplified product. Use a proper DNA ladder (100 bp) as a marker. Visualize the DNA under UV light (*see* **Note 2**).
4. Pool the triplicate samples that show a correct band size and proceed to the PCR purification step following the protocol of the QIAquick Purification Kit (Qiagen, Valencia, CA, USA).

2.2.1 Digestion Using Cas9 Nuclease

1. Using Cas9 nucleases, in vitro digestion of DNA can be performed following the manufacturer's protocol (New England Biolabs, MA, USA) with slight modifications. Prepare the reaction mixture at a room temperature in the following order.

Nuclease-free water	20 μL
NEB buffer 3.1	3 μL
300 nM gRNA	3 μL
1 μM Cas9 nuclease	1 μL

2. Incubate the reaction mixture for 10 min at 25 °C. After incubation, add 3 μL of 30 nM substrate DNA.
3. Gently mix the reaction mix and centrifuge for a brief moment to collect the mixture at the bottom of the tube.
4. Incubate the mixture at 37 °C for 20 min.

5. Add 1 μL of Proteinase K to the reaction mixture and gently mix. Centrifuge briefly to collect the mixture at the bottom of the tube.
6. Incubate the mixture at room temperature ~22 °C for 15 min.
7. Upon completion of the reaction, run the sample in 1.5% agarose gel and run in an electrophoresis machine for 20–30 min at 90 voltage and resolve the digested product. Use a proper DNA ladder (100 bp) as a marker. Visualize the DNA under UV light.

2.3 Target Fragment Synthesis

Assemble the gRNA, gRNA scaffold, promoter, and terminator sequences in silico; this helps visualization of sequence junctions. This step can best be done in a Word document by simply copying and pasting the desired fragment in a correct order. In the graphical representation (Fig. 3), the promoter sequence is followed by 20 bp gRNA and finally, the gRNA scaffold sequence is enveloped by a terminator sequence. The fragment is then synthesized using GenScript or any other vendors.

2.4 Assembly of Fragments Using Gibson Assembly

This step involves the assembly of gRNA components along with its regulatory elements into the vector plasmid containing Cas9.

2.4.1 Primers Designing

Resources: Internet access to Nebuilder.neb.com, Microsoft office or Notepad, synthesized DNA sequence (Fig. 3), full vector sequence (pRGE32; https://www.addgene.org/63159/).

Protocol: 1. Open the vector sequence and paste it in a Word file.

2. Paste the DNA insert in a right position in the vector; in this case, it is pasted in between the restriction sites (*Hind*III and *Sbf*I). For the specific site details, refer to the vector map and sequence (https://www.addgene.org/63159/).
3. Open https://nebuilder.neb.com.
4. Click on "New Fragment" button (https://nebuilder.neb.com/#!/add/).
5. Click on "Paste Sequence", and on the "Source Text" box, paste the second half of the vector sequence; in this case, sequence beginning from cctgcagg. . . (*Sbf*I) site all the way to the end (Fig. 4a).

Fig. 3 DNA fragments arranged in an order for synthesis. This fragment contains promoter sequence, gRNA, gRNA scaffold, and terminator sequence. The fragment can be synthesized using GenScript service or any other vendors

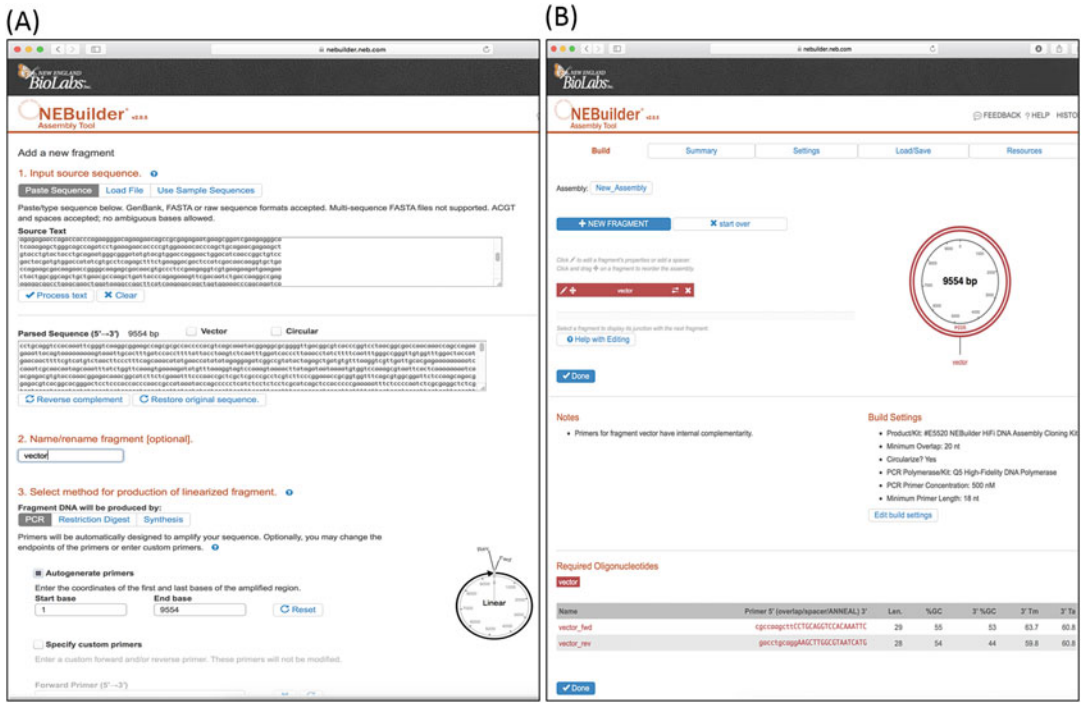

Fig. 4 Screen capture from the sequence entry page of the NEBuilder web-based tool. (**a**) First window on the server that shows the sequence input box. (**b**) An output window to input the vector fragment. The map shows the length of vector fragment. The blue highlighted tab "+New Fragment" shows the input button for the next DNA fragment to be combined with the vector fragment

6. Go back to your vector sequence page and copy the first half of the vector sequence; in this case, copy from cttgtacaa to aagctt (*Hind*III) and paste immediately after the portion of sequence in 5, in this case, acccagcttt. For the site details, refer to the vector map and sequence ((https://www.addgene.org/63159/).
7. Type the name of the fragment as "vector" in the "Name/rename fragment" (Fig. 4a).
8. Click on "Add" button.
9. A new page will open; now scroll up, locate "+ New Fragment", and click on it.
10. Paste the synthesized DNA fragment on the "Source Text" (Fig. 4b).
11. Upon clicking the "Add" button, you will be directed to a new page with two sets of primers that says "Required Oligonucleotides" (Fig. 5). These primer sequences can be synthesized/ordered for your PCR work to amplify the fragments for the Gibson Assembly (*see* **Note 3**).

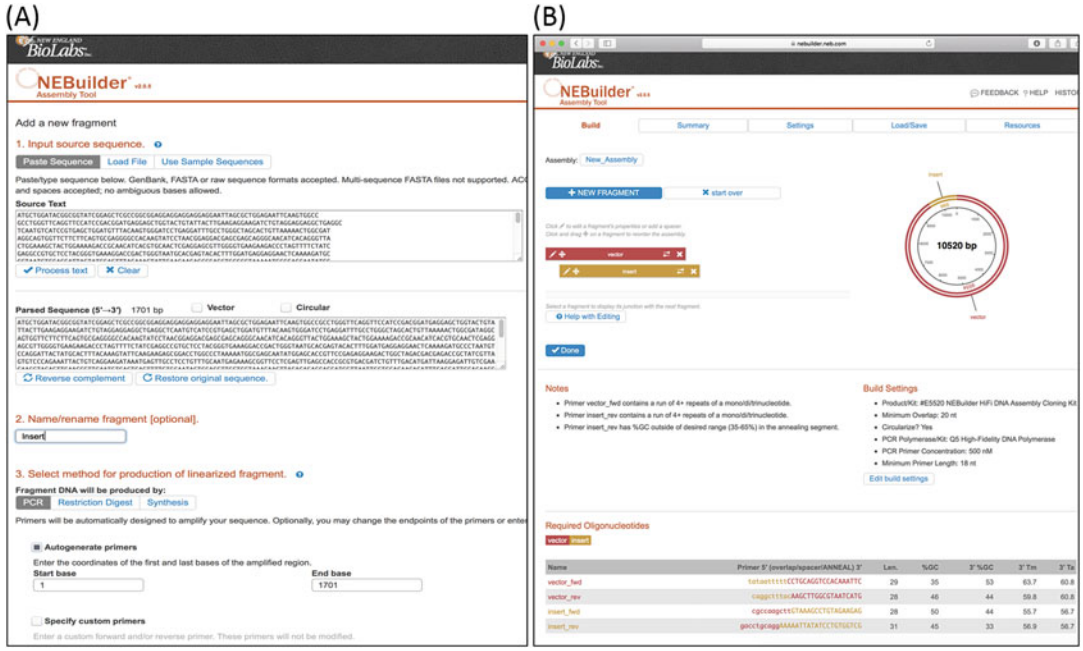

Fig. 5 Screen capture from the sequence entry page of the NEBuilder web-based tool. (**a**) The window shows the source text for the DNA fragment to be combined. (**b**) The final output window that shows both fragments (vector and insert) combined in the map. The four primers that are reported in this window are used in PCR to generate fragments for Gibson Assembly

2.4.2 *Gibson Assembly*

Resources: Plasmid pRGE32 (Addgene: https://www.addgene.org/63159/), newly synthesized sequence containing gRNA fragment along with its scaffold sequences, gRNA regulators (promoter and terminator sequence), restriction enzymes, *Hind*III and *Sbf*I, 10× CutSmart Buffer. QIAquick Gel Extraction Kit, Novel Juice DNA dye (GeneDirex, Nevada, USA), *Taq* DNA polymerase, BLooK LED transilluminator, (VWR Cat. No. BK001), Gibson Assembly Kit.

Protocol:

1. Digestion of pRGE32:
   Set up a double digest enzymatic reaction with *Hind*III-HF and *Sbf*I-HF enzymes following the manufacturer's protocol (New England Biolabs) along with the following considerations.

   Set up the reaction in the following order:

Gel purified DNA	1 μg
10× CutSmart buffer	5 μL (1×)
*Hind*III-HF	1.0 μL
*Sbf*I-HF	1.0 μL
Nuclease-free water	Variable (adjust to 50 μL total reaction volume)

2. Gently mix the reagents in the tube and briefly centrifuge to collect at the bottom. Incubate the reaction mix at 37 °C for 45 min (*see* **Note 4**).
3. Run the digested product in 1% agarose gel; you will see two bands, 549 bp and 9543 bp (*see* **Note 5**).
4. Before you load the sasmple in the gel, mix the sample and the Novel Juice (ratio, 5:1) in the following order.

   Sample 50 μL

   Novel Juice 5 μL (*see* **Note 6**).
5. Excise the 9543 bp gel band and purify the DNA using the QIAquick Gel Extraction Kit (Qiagen).
6. In a separate tube, using the primers developed from NEBuilder, perform PCR on the synthesized DNA fragment containing gRNA and plasmid vector containing Cas9. Use the following reaction mix for PCR.

5× LongAmp *Taq* Buffer	10 μL
10 mM dNTPs	1.50 μL
10 μM forward primer	3.0 μL (*see* **Note 7**)
10 μM reverse primer	3.0 μL (*see* **Note 7**)
Template DNA variable (5–15 ng for plasmid vector; 40–50 ng for synthesized DNA)	
LongAmp *Taq* polymerase	2.0 μL
Nuclease-free water adjusted to	50 μL

   (*See* **Note 8**).
7. Follow the PCR protocol to run your reaction, initial denaturation at 94 °C for 30 s, followed by 35 cycles (94 °C for 30 s, 45–62 °C for 30 s, 72 °C for 1 min/kb), and final extension at 72 °C for 10 min, and hold at 4 °C.
8. Subject the samples for PCR cleanup using QIAquick PCR Purification Kit (Qiagen).
9. Assemble the newly synthesized fragment using Gibson Assembly [14] following the PCR reaction protocol.

Amount of PCR purified vector	Variable (50–100 ng)
Amount of PCR purified insert	Variable (100–300 ng)
Gibson assembly master mix (2×)	10 μL
Deionized water	Add to make 20 μL

10. Incubate the samples at 50 °C for 15 min.

11. Following incubation, store samples at −20 °C until the next step.
12. Analyze the assembled reaction on 1% agarose gel. Correctly assembled product should produce plasmid of expected size (~10.5 kb).
13. Alternatively, it is also advisable to test the assembled product by performing PCR with primers that flank the synthesized fragment in the final vector sequence.

Alternative to Gibson Assembly: When synthesizing the target DNA fragments (*see* Subheading 2.3), restriction sites *Hind*III/*Sbf*1 can be included at the two ends of the sequence, corresponding to the restriction sites in the vector sequence. In this case, *Hind*III can be included close to the promoter sequence and *Sbf*1 on the opposite end close to the terminator sequence.

Any standard ligation protocol can be followed to assemble the synthesized insert and vector plasmid. Upon completion of the ligation, proceed to Subheading 2.4.3.

2.4.3 Chemically Competent Bacterial Cell Transformation

Resources: NEB 5-alpha competent *E. coli* cells, LB Broth, SOC Media, assembled DNA plasmid, Promega Plasmid Miniprep Kit.

Protocol: Day 1

1. Competent cells are frozen; thaw them on ice (*see* **Note 9**).
2. In a 1.5 mL microcentrifuge tube, put 50 μL 5-alpha competent cells and add 2 μL of assembled product from the earlier step. A gentle flick of 5–7 times with your finger is enough. Place the mixture on ice for 30 min (*see* **Note 10**).
3. Heat shock at 42 °C for 30 s (*see* **Note 11**).
4. Immediately, transfer the samples on ice for 2 min.
5. Gently, add 750 μL pre-warmed SOC media to the tubes with mixture.
6. Incubate the mixture at 37 °C for 1.5 h, shaking vigorously (250 rpm).
7. After 1.5 h, following aseptic technique, spread 100 μL of ampicillin (100 μg/mL) on the pre-warm bacterial culture plates and add 75 μL of the fresh culture. Spread evenly throughout the plate.
8. Incubate the plates at 37 °C overnight.

Day 2

1. Overnight culture should be enough to see the visible colonies.
2. Pick individual colony and culture in 3 mL LB broth overnight at 37 °C, shaking vigorously at 250 rpm.

Day 3

1. Isolate plasmid DNA containing Cas9 and gRNA from the bacterial culture from Day 2. Promega Plasmid Miniprep kit (Promega, WI, USA) can be used for the plasmid isolation step.
2. The plasmid can be kept at −20 °C until used for transfection.

2.5 Plant Material and Protoplast Isolation

In order to confirm if the targeted mutation was introduced, here we describe a simple and efficient method for protoplast isolation and transformation. These methods are adapted from published information in flax and related species [15–18]. We have chosen the PEG mediated transformation as a method to deliver target DNA to the plant cells. The steps for an efficient protoplast isolation and transformation usually include digesting the plant cell walls with enzymes such as cellulase and pectinase, washing the protoplasts with mannitol, incubating the protoplasts with the target DNA and PEG.

Resources: Bovine serum albumin (BSA), calcium chloride, Cellulase Onozuka, ethanol 70%, glucose, magnesium chloride, magnesium sulphate, mannitol, morpholinoethanesulfonic acid (MES), Murashige and Skoog (MS), Pectinase PY-23, PEG4000 (Polyethylene glycol 4000) phytoblend agar, potassium chloride, potassium nitrate, potassium phosphate monobasic, sodium chloride, sodium hypochlorite (commercial bleach), sterile deionized water, sucrose.

Chemical Compositions

Prepare all solutions with sterile deionized water and analytical grade reagents. Store all the reagents at room temperature, unless otherwise stated.

Flax seeds sterilization chemicals: Ethanol 70%, sodium hypochlorite 9.6%.

MS media: Sterile ½ strength MS medium, 1% sucrose, 0.7% phytoblend agar.

Salt solution: 1 mM Calcium chloride, 0.2 M potassium phosphate monobasic, 1 M potassium nitrate, 1 M magnesium sulphate, 0.5 M glucose, 1% (w/v) BSA; adjust the osmolality to 650 mOsm (kg H_2O)$^{-1}$.

Digestion solution: 1.2% (w/v) Cellulase Onozuka RS and 0.2% (w/v) Pectinase PY-23 in a salt solution.

Resuspension solution: 21% sucrose in salt solution.

W5 solution: 2 mM MES pH 5.7, 154 mM sodium chloride, 5 mM potassium chloride, 125 mM calcium chloride.

MMg solution: 4 mM MES, 0.6 M mannitol, 15 mM magnesium chloride.

PEG–$CaCl_2$ solution: 0.6 M Mannitol, 100 mM calcium chloride, 40% polyethylene glycol 4000.

WI solution: 4 mM MES pH 5.7, 0.6 M mannitol, 4 mM potassium chloride.

Protocol

2.5.1 Seed Sterilization and Germination In Vitro

Conduct all experiments in a sterile environment.

1. Sterilize the surface of flax seeds by adding 70% ethanol for 1 min. Wash three times with sterile deionized water. Immerse in 9.6% sodium hypochlorite for 5 min, and rinse thoroughly for 6 times in sterile deionized water. Dry the seeds on sterile filter paper or paper towel under a laminar flow hood.
2. Put the seeds in magenta boxes with solid MS media. No need of growth regulators at this stage. Place the boxes containing the seeds in a growth chamber under a continuous dim light (2.8 μmol m^2 s^{-1}, PAR) at 22 ± 1 °C [16].

2.5.2 Protoplast Isolation

1. Using a sharp scalpel, prepare 1–1.5 cm hypocotyl and leaf sections from 4–7 days old seedlings. Put a 2–3 mL of the digestion solution on a petri dish and make fragments on it; this way the sample does not dry (*see* **Note 12**).
2. Add 23 mL of the digestion solution to the petri dish.
3. Using a spatula, put sections of the hypocotyls and leaves in the petri dish containing the digestion solution.
4. Incubate the samples in dim light (2.8 μmol m^2 s^{-1}, PAR) at 24 ± 1 °C with gentle shaking (35 rpm) overnight (*see* **Note 13**).
5. Next day, very gently, swirl the petri dish and use a sealed glass Pasteur pipette to gently move the hypocotyl tissue in the digestion solution. This step helps release protoplasts (Fig. 6). Move the tissue to one corner and slowly filter the protoplast using a 60 μm pore nylon sieve (Sefar, Rüschlikon, Switzerland).
6. Divide the protoplast mixture into a 10 mL volume in a 15 mL test tube (*see* **Note 14**).
7. Spin the filtered suspension at 900 rpm for 10 min and remove the supernatant.
8. Resuspend the protoplasts very gently with 5 mL resuspension solution.
9. Add 5 mL of the salt solution on top.
10. Centrifuge the sample at 155 × *g* for 10 min without break in the centrifuge machine (Beckman GS-6 Centrifuge or equivalent).

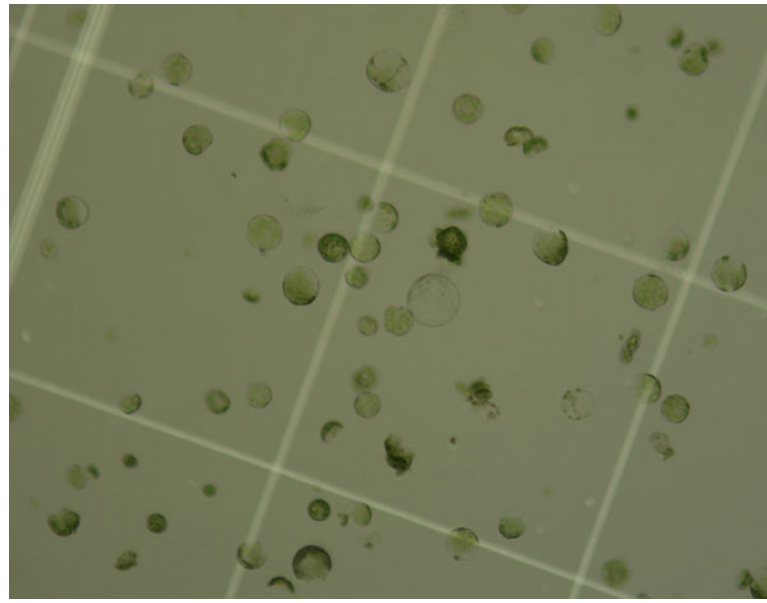

Fig. 6 Flax protoplasts, isolated from leaf and hypocotyl tissues

11. Take the protoplast layer at the boundary with a Pasteur pipette (*see* **Note 15**).
12. Repeat **steps 8–11** twice.
13. Verify the concentration of the protoplast using a hemocytometer (*see* **Note 16**).

2.5.3 Protoplast Transfection and Culture

1. Immediately before transfection, spin the protoplast suspension at 250 g for 5 min and suspend in MMg solution to a final concentration of 1×10^6 protoplasts/mL (*see* **Note 17**).
2. Pipette 0.3 mL protoplast suspensions into a 13 mL tube and add 15−20 μg of the CRISPR plasmid DNA (*see* **Note 18**).
3. Add 0.3 mL of the PEG-$CaCl_2$ solution. Gently flick the tube 3–4 times and mix the samples in the tube.
4. Incubate the mixture at room temperature for 20 min.
5. Add 2 volumes of the W5 solution to stop the transformation.
6. Collect the transformed protoplasts by centrifugation and resuspend in the WI solution.
7. Place the transformed protoplasts in 24-well culture plates.
8. Incubate the protoplast plates in the dark at 25 °C for up to 24 h–72 h.
9. Spin the protoplasts at 300 g for 2 min to collect the protoplasts.
10. Freeze the protoplasts in −80 °C until subsequent use.

Alternatively, transfected protoplasts can be further cultured for regeneration of organogenesis following the protocol outlined by [18].

2.6 Extraction of Genomic DNA from Protoplast

Resources: Cetrimonium bromide buffer (CTAB), chloroform, ethanol.

Protocol:

1. Add 100 μL of preheated CTAB buffer to the extracted protoplasts and incubate at 65 °C for 20 min and add 40 μL of chloroform (*see* **Note 19**).
2. Incubate the mixture at room temperature with agitation for 20 min (*see* **Note 20**).
3. Spin the mixture at 16000 g for 5 min.
4. Transfer the supernatant to a new tube and add 250 μL of ethanol and mix well.
5. Incubate the mixture on ice for 10 min.
6. Spin the mixture at 16000 g for 10 min at room temperature.
7. Wash the DNA pellet with 0.5 mL of 70% ethanol and air-dry it.
8. Elute the DNA with 50–100 μL of nuclease-free molecular-grade water.
9. Determine the DNA concentration using a NanoDrop or equivalent.

2.7 Detection of Induced Mutation Using T7E1 Assay and DNA Sequencing

In order to detect mutations via assays like T7 Exonuclease I (T7EI) assay, here we describe a protocol to successfully carry out the assay.

Resources: T7 Endonuclease I (T7E1) (NEB), purified DNA from transformed protoplast, purified target DNA from PCR product, 10× NEBuffer 2, Hot Start High-Fidelity 2× Master Mix, 0.25 M EDTA, target-specific primers, nuclease-free water, QIAquick PCR purification.

Protocol:

1. Set up a PCR reaction (in triplicates) following the guidelines outlined by the manufacturer (New England Biolabs) and also considering the modifications mentioned below.

Q5 Hot Start High-Fidelity 2× Master Mix	25 μL
10 μM forward primer	2.5 μL
10 μM reverse primer	2.5 μL
Template	Variable (50–100 ng)
Nuclease-free water	Add to 50 μL

2. Mix the reaction and briefly centrifuge to collect the mixture in the bottom.
3. Transfer the sample to the PCR machines and follow the cycling conditions, initial denaturation at 98 °C for 30 s, 35 cycles (98 °C for 5 s, 50–60 °C for 20 s, 72 °C for 20 s), and final extension at 72 °C for 2 min, and final hold at 4 °C (*see* **Note 21**).
4. Upon completion of the reaction, take 7 μL of the PCR product, add 1 μL of gel loading dye, and run in an electrophoresis machine for 20–30 min at 90 voltage and resolve the target product. Use a proper DNA ladder (100 bp) as a marker. Visualize the DNA under UV light.
5. Purify the PCR product using QIAquick PCR Purification (Qiagen).
6. Measure the concentration of purified DNA using Nanodrop or equivalent.

2.7.1 T7 Endonuclease I Digestion and Detection of Mutation

1. Set up a reaction in the following order:

PCR purified DNA	Variable (200 ng)
10× NEBuffer	2 μL
Nuclease-free water	Add 19 μL

2. Anneal the purified PCR products for the hybridization of DNA fragments.
3. Transfer the sample to the PCR machines and follow the parameters. Initial denaturation at 95 °C for 5 min and ramp down to 25 °C at the ramp rate of −0.1 °C/s; incubate at 25 °C for 30 more minutes.
4. Once the DNA fragments are annealed, add 1 μL of T7EI and gently mix the samples and incubate at 37 °C for 2 h.
5. Separate the digested product in 1% agarose gel electrophoresis.

2.7.2 Mutation Confirmation by Sequencing

1. Amplify the target with PCR primers flanking the CRISPR Cas9 target region (*see* **Note 22).**
2. Purify the PCR fragment using QIAquick PCR Purification Kit (Qiagen).
3. Sequence the amplicons using Sanger Sequencing (*see* **Note 23**).
4. Align the potential mutant sequenced fragment with the wild-type fragment. The enzyme Cas9 cleaves DNA within the target region approximately 3–4 nucleotides upstream of the PAM motif. There can be insertion or deletion of the nucleotides in the sequence.

3 Notes

1. It is recommended to use high-fidelity PCR *Taq* to avoid nucleotide error during PCR amplification. Also, perform the PCR reaction in triplicates and you can pool the samples to better yield DNA after PCR cleanup.
2. It is always recommended to wear gloves and proper personal equipment while performing post-PCR genotyping work including gel loading and using UV imaging.
3. NEBuilder designs primer creating 10 bp overhang fragment on each primer set; these extra nucleotide bases come from the other combining DNA fragments. It is recommended to check, manually, the location of primers in the sequence and you should see them in the junctions of the two DNA fragments.
4. Short incubation period may result in an incomplete digestion; these enzymes are safe to go longer period but overnight digestion may not require. Upon the completion of **step 2**, heat inactivation is not required because you will purify the sample using the QIAquick Gel Extraction Kit (Qiagen).
5. It is recommended to use LED-based gel transilluminator (VWR) rather than exposing to UV light.
6. Novel Juice (GeneDirex) stains DNA and it is easily visible using the LED-based transilluminator.
7. Run separate PCR reaction with the two primer sets, e.g., insert.fwd and insert.rev amplify the insert DNA and vector. fwd and vector.rev amplify vector DNA plasmid (Fig. 5b).
8. Gentle mixing is recommended; vigorous pipetting may destroy long DNA fragments. Prior to transfer of the reaction tubes to the thermocycler, preheat the lid at 94 °C. If preheat is not possible, overlay the sample with mineral oil.
9. Avoid multiple freeze thaw process.
10. Do *not* vortex.
11. Water bath works best.
12. Glass petri dish is best for this purpose.
13. Temperature must not rise above 25 °C.
14. Use dome-shaped test tube.
15. Usually, this is a thin layer you will see at the interface.
16. Maintain the protoplast concentration 5×10^6 per mL for transfection.
17. Do not leave in MMg solution for a long time.
18. DNA concentration should be >1 μg/μL concentration.

19. CTAB and chloroform are hazardous chemicals; please use proper personal protective equipment and proper work space while using these chemicals.
20. Put your samples in a fume hood.
21. Preheat the lid at 98 °C prior to putting the samples.
22. It is highly recommended to use high-fidelity DNA *Taq* polymerase because regular PCR *Taq* polymerase may introduce nucleotide errors during amplification, hence creating confusion with the expected mutation.
23. It is recommended to generate sequence from both forward and reverse primers to verify the mutation.

Conflicts of Interest: All authors have contributed and read the article and there is no conflict of interest.

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Chapter 12

Delivery of Cas9/sgRNA RNP into Wheat Microspores Using Synthetic CPP for Genome Editing and Gene Expression Modulation

Andriy Bilichak, Justin Luu, Fengying Jiang, and John Laurie

Abstract

Genome editing using sequence-specific nucleases has, undoubtedly, become one of the most important breakthroughs in biotechnology in recent years, allowing for rapid and efficient generation of crop varieties with novel or improved traits. Despite wide applicability of the technology, a challenge in bringing edited crops to market is the lack of regulatory alignment between jurisdictions (e.g., EU). Additionally, technical difficulties in efficient delivery of genome editing components into plant cells pose a problem for adoption of the technology by many groups, since transgenic pipelines and facilities need to be in place. Even with recent advancements in cell culture and plant regeneration, much work is still needed for reducing the burden of plant transformation. To address this, alternative methods for delivery of the genome editing machinery have been explored that potentially offer non-transgenic means to genome editing. In this chapter, we describe a novel method for delivery of in vitro purified Cas9/sgRNA complexes into wheat microspores for generation of homozygous edited plants. We provide guidelines for purification of Cas9 protein from bacterial culture, in vitro transcription of sgRNA, and delivery of the ribonuclear complex into the cells using cell-penetrating peptides. Additionally, we demonstrate utility of the technology through non-transgenic modulation of gene activity using the chimeric activators dCas9-VP64. With further optimization, the method has the potential to accelerate the development of cereals with novel traits by speeding up the editing process and by producing germplasm that potentially avoids regulation.

Key words Genome editing, Cas9/sgRNA, dCas9-VP64, Cell-penetrating peptides, Wheat microspores

1 Introduction

Genome editing (GE) using sequence-specific nucleases (SSN) is a tool that has become routine for both functional genomic studies and for creation of gain-of-function mutants with desirable phenotypes. In this regard, GE is now widely used as a novel breeding method to speed up development of new varieties of staple crops. Conventional breeding practice of the small grain cereals relies on efficient production of fully homozygous plants with fixed

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beneficial traits. Some of the methods available to breeders include single seed descent (SSD) [1] and doubled haploid (DH) production through either pollination with alien species followed by embryo rescue or immature microspore culture (IMC) [2, 3]. While the SSD method does not result in generation of completely homozygous lines even after recurrent self-pollination for many generations, the DH methods offer production of lines with 100% homozygosity in a single generation [4]. Mature haploid green plants either spontaneously double their chromosomes or need to eventually be treated with a chromosome doubling agent to produce doubled haploid lines. The ability to work with haploid cells/tissues is desirable for biotechnology applications since it significantly reduces time and cost required for generation of complete homozygous lines with altered genomes. Application of these methods for bread wheat (*Triticum aestivum* L.) breeding is especially important considering that the plant is allohexaploid (AABBDD, $2n = 6x = 42$) and requires modification of six copies of a gene in somatic tissues if full homozygosity for a gene is desired.

Microspores are predecessors of male gametes that under certain conditions can undergo embryogenesis in vitro to produce green plants. Being single cells and haploid in nature, microspores provide an opportunity for production of genome edited homozygous plants in a way that potentially avoids germline chimeras. In comparison to the conventional breeding practices, the approach significantly reduces time and resources required for bringing plants with novel traits to the market [4]. Combining the IMC method together with GE provides extra flexibility and power for crop breeding programs. Unfortunately, there exists uncertainty in how genome edited crops will trade in the international market, since regulations do not align between countries and regions (e.g., EU) [5]. It is believed that delivery of designed endonucleases (DE) in the form of protein into plant cells can potentially alleviate the concerns regarding transgenic plants. The applicability of such approaches for generation of transgene-free genome edited crops was demonstrated in the elegant studies on lettuce, corn, and wheat. In these experiments, purified Cas9 protein complexed with sgRNA as a ribonucleoprotein (RNP) complex was delivered into plant cells/tissues with subsequent regeneration of edited plants [6–8]. In all of these studies, diploid somatic cells/tissues (protoplasts and callus) were used for the introduction of edits at the target site using either PEG-mediated delivery or particle bombardment. Here we describe an alternative method for delivery of proteins into microspores and plant cells by using cell penetrating peptides (CPPs) [9]. CPPs typically possess basic amino acids that allow for their translocation through the plasma membrane and accumulation in the cytoplasm and nucleus [10]. Additionally, a number of other peptides with amphipathic and hydrophobic

amino acids also possess cell penetration properties. Depending on the origin, CPPs can be classified as protein-derived, synthetic and chimeric [11]. Similar to other methods of delivering the Cas9/sgRNA RNP complex, CPP technology can be used for intracellular transport of deficient Cas9 fused to VP64 transcriptional activator (dCas9-VP64) for the purpose of activation of embryogenesis-related genes in microspores or modulation of gene activities in somatic tissues. In this chapter, we describe a protocol for overexpression and purification of functional Cas9 and dCas9-VP64 proteins from bacterial culture, complexing these proteins with in vitro transcribed sgRNA and delivery into wheat microspores for downstream analysis of either edits at the target sites or alterations in gene activity, respectively. Although the method still requires user optimization of transfection paraments to increase efficiency of RNP complex delivery into viable microspores, the technique offers an alternative and cost-efficient method for transgene-free genome editing in crops amenable to IMC.

2 Materials

2.1 Plasmids

1. pET-NLS-Cas9-6×His and pET-dCas9-VP64-6×His plasmids are available from Addgene for overexpression and purification of NLS-Cas9-6×HIS (Cas9 hereafter) and NLS-dCas9-VP64-6×HIS (dCas9-VP64 hereafter) from bacterial culture: cat# 62934 and 62935, respectively.
2. sgRNA seeding sequence is cloned into pT7-sgRNA that contains sgRNA backbone and can be acquired from Addgene as well, cat # 46759.

2.2 Components Required for Cloning and Gene Expression Analysis

1. 100 μM each of two complementary oligos for the sgRNA seeding sequence.
2. Cloning buffers: 10× NEB3 buffer (NEB, cat # B7003S) and 10 × T4 ligase buffer (NEB, cat # B0202S).
3. Enzymes: BsmBI (NEB, cat # R0580S), BglII (NEB, cat # R0144S), SalI (NEB, cat # R3138S), BamHI (NEB, cat # R0136S), T4 DNA ligase (NEB, cat # M0202S), and Proteinase K (NEB, cat # P8107S).
4. *E. coli* competent cells: NEB 5-alpha chemical competent cells (NEB, cat # C2987I) and BL21 (DE3) (NEB, cat # C2527I).
5. Primers for qPCR expression analysis of the target gene and up to three reference genes.
6. Plasmid miniprep kit (NEB, cat # T1010S).
7. HiScribe T7 High Yield RNA Synthesis Kit (NEB, cat # E2040S).

8. mirVana miRNA Isolation Kit (Thermo Fisher Scientific, cat # AM1560).
9. DNeasy Plant Mini Kit (Qiagen, cat # 69104).
10. RNeasy Plant Mini Kit (Qiagen, cat # 74903).
11. SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, cat # 11754050).
12. PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, cat # A25741).

2.3 Materials Required for Protein Purification and Analysis

1. LB liquid and solid medium supplemented with 100 μg/mL carbenicillin.
2. HisTrap HP column, 1 mL (GE, cat # 17524701).
3. 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG).
4. His-Tag buffer A (20 mM HEPES, 0.3 M NaCl, 5% glycerol and 40 mM imidazole, pH 7.5) supplemented with EDTA-free protease inhibitor cocktail (Sigma, cat #11873580001), His-Tag buffer B (20 mM HEPES, 0.3 M NaCl, 5% glycerol and 500 mM imidazole, pH 7.5).
5. Sephadex G-25 in PD-10 desalting columns (GE, cat # 17085101).
6. 100 K Amicon Ultra-15 spin concentrators (EMD Millipore, cat # UFC910008).
7. HiLoad 16/60, Superdex 200 prep grade size exclusion column (SEC, GE Healthcare Life Sciences), SEC buffer (20 mM HEPES, 0.15 M NaCl, pH 7.5).
8. 8% SDS PAGE, 10× SDS PAGE buffer (250 mM Tris, 1.92 M glycine and 1% SDS, pH 8.3).
9. Pre-stained protein ladder (GeneDirex, cat # PM008–0500).
10. Commercial or self-made protein loading buffer.

2.4 Materials Required for Microspores Isolation

1. Tillers of preferred wheat cultivar harvested at the mid-to-late uninucleate stage of microspores (*see* **Note 1**).
2. 10% (v/v) bleach (5.25% sodium hypochlorite).
3. Filter-sterilized microspore extraction solution (14 mM KNO_3, 1.76 mM $(NH_4)_2SO_4$, 1.47 mM KH_2PO_4, 0.56 mM $CaCl_2$, 0.38 mM $MgSO_4$, 0.1 mM $FeSO_4$, 0.1 mM Na_2EDTA, 0.9 mM MES and 400 mM mannitol, pH 6.5).
4. Sterile 20% maltose solution.
5. 100 μm Sterile mesh (VWR International).

2.5 Cell-Penetrating Peptide and Protein Labeling

1. Cys (Npys)-(D-Arg)$_9$ (R_9 hereafter, AnaSpec, cat # AS-61206).
2. Alexa Fluor 647 (AlF-647) protein labeling kit (Thermo Fisher Scientific, cat # A20173).

2.6 Equipment

1. PCR thermocycler.
2. QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific).
3. PAGE and agarose electrophoresis tanks.
4. 110 mL Warring blender cup (VWR International).
5. Refrigerated centrifuge with a swinging bucket rotor.
6. FPLC system (AKTA purifier, GE Healthcare Life Sciences).
7. Sonicator (Qsonica, model Q55).
8. Confocal laser scanning microscope Olympus, FV1000 with 594/633 laser line.
9. 28 °C incubator.
10. Refrigerated orbital shaker.

3 Methods

The purification process for obtaining Cas9 and dCas9-VP64 proteins from bacterial cultures takes around 4 days. An additional day should be allocated for labeling of proteins with fluorescent dye to monitor efficiency of cells transfection. Production of sgRNA through transcription and purification will take an additional day to complete. When all components for transfection are in place, microspores isolation and transfection should be performed on the same day, so not to compromise microspores viability. Isolation and transfection of microspores is done using aseptic techniques in laminar flow hood. All buffers and materials should be sterilized as described in appropriate sections.

3.1 Overexpression and Purification of Cas9 and dCas9-VP64 Proteins from Bacterial Culture Using FPLC System

1. Transform either pET-NLS-Cas9-6×His or pET-dCas9-VP64-6×His plasmid into chemically competent *E. coli* cells BL21 (DE3) according to the manufacturer's instructions and select single colonies on LB agar plates supplemented with 100 μg/mL of carbenicillin.
2. Add a single colony to 20 mL of LB medium in the presence of 100 μg/mL of carbenicillin and grow overnight at 37 °C with shaking at 250 rpm.
3. The following day, inoculate 500 mL of LB medium supplemented with 100 μg/mL of carbenicillin with 20 mL of the overnight culture and incubate at 37 °C with shaking at 250 rpm until an optical density of 0.5 at 600 nm (OD600).
4. Induce protein expression from the introduced plasmids by supplementing the bacterial culture with 1 mM IPTG and continue incubation at 22 °C with shaking for ~16 h.

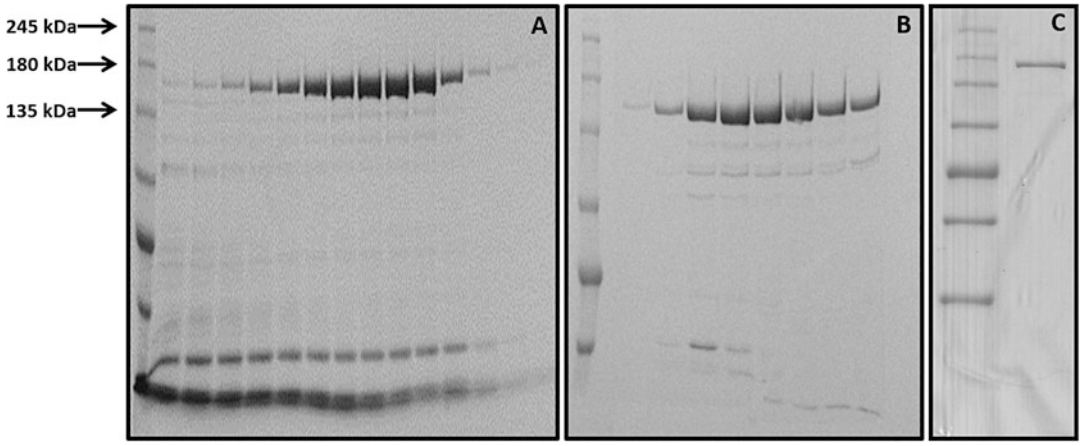

Fig. 1 SDS-PAGE analysis of Cas9 FPLC fractions following purification with either (**a**) His-Trap column or (**b**) size exclusion column. (**c**) SDS-PAGE of purified dCas9-VP64. Molecular weights of Cas9-6×His and dCas9-VP64-6×His proteins are 160 and 169 kDa, respectively. Analysis was done using 8% SDS-PAGE

5. Harvest the bacterial culture by centrifugation at 8000 × *g* for 10 min at 4 °C and resuspend the pellet in 50 mL of chilled His-Tag buffer A supplemented with EDTA-free protease inhibitor cocktail.
6. Lyse the cells using sonication (50% intensity, 30 s on, 1 min off time, four cycles) and remove the insoluble debris by centrifugation (18,500 × *g*, 1 h at 4 °C).
7. Apply clarified supernatant directly onto 1 mL His-Trap column equilibrated with the native lysis buffer using an FPLC system.
8. Elute bound protein using a linear gradient of His-Tag buffer B (20 mM HEPES, 300 mM NaCl, 100 μM $ZnCl_2$, 5% glycerol and 500 mM imidazole, pH 7.5) from 0 to 100%.
9. Analyze fractions on the 8% PAGE and combine those containing more than 90% pure protein together (*see* **Note 2**, Fig. 1).
10. In case if a higher purity of proteins is required, proceed with size exclusion purification of concentrated protein solution using HiLoad 16/60, Superdex 200 prep grade size exclusion column attached to FPLC system (*see* **Note 3**).

3.2 Isolation of Wheat Microspores

1. Isolation of microspores is done from tillers of healthy wheat plants grown in chamber. The stage of microspores in tiller is verified using a median floret (*see* **Note 1**).
2. Sterilize 12 spikes per extraction using 10% (v/v) bleach (5.25% sodium hypochlorite) for 3 min followed by three washes with sterile double distilled water with constant agitation.

3. Dissect florets aseptically and transfer to a sterile and refrigerated 110 mL Warring blender cup containing 50 mL of filter sterilized extraction solution at 4 °C.
4. Blend the florets twice for 7 s at low speed.
5. Filter the extract through 100 μm sterile mesh and pellet the cells by centrifugation (100 × *g* for 5 min at 4 °C) using a swinging bucket rotor (Eppendorf AG, Hamburg, Germany).
6. Remove the supernatant, resuspend the pellet in 16 mL of ice-cold extraction solution, and spin again at 100 × *g* for 5 min at 4 °C.
7. Following a second spin, resuspend the microspore cells in 6 mL of pre-chilled, sterile 20% maltose solution and carefully add 1 mL of extraction medium on top.
8. Centrifuge content at 100 × *g* for 13 min at 4 °C and remove the viable microspore cells from the interphase and transfer to the fresh 15 mL tube.
9. Wash the microspores again with 15 mL of extraction solution and resuspended to the final concentration of 1000 cells/μL.

3.3 In Vitro Transcription of sgRNA and Testing Activity of Cas9/sgRNA RNP

1. Search sgRNA target sites in the endogenous sequences using bioinformatic tool of your choice. Typically, we use CHOP-CHOP v3 software for this purpose [12] (*see* **Note 4**).
2. Following selection of targets with the highest ranking, design the following oligos: forward, $TAGGN_{18}$, and reverse, $AAACN_{18}$, where N_{18} is the target sequence in forward and reverse complementary orientation, respectively (*see* **Note 5**).
3. Anneal the two oligos (2 μL of 100 μM stock each in a 20 μL 1× NEB3 buffer solution) by incubating the mixture at 95 °C for 5 min, ramping down to 50 °C at 0.1 °C/s, incubating at 50 °C for 10 min, and chilling to 4 °C at normal ramp speed (1 °C/s).
4. Ligate the annealed oligos to the pT7-sgRNA vector by mixing the following components: 1 μL of annealed oligos, 400 ng of the vector, 1 μL of 10× NEBuffer 3, 1 μL of 10 × T4 ligase Buffer, 0.5 μL of BsmBI, 0.3 μL of BglII, 0.3 μL of SalI, 0.5 μL of T4 DNA ligase, and water to a total of 10 μL. Perform digestion and ligation in a single step in a thermal cycler with the following program: 3 cycles of 20 min at 37 °C/15 min at 16 °C, followed by 10 min at 37 °C, 15 min at 55 °C, and 15 min at 80 °C (optional).
5. Use 2 μL of the ligation product and plate 10% of the transformants onto a $LB/Carb_{100}$ plate.

6. Verify integrity of cloned construct with sequencing and purify enough of the plasmid for in vitro transcription (*see* **Notes 6** and **7**).
7. Linearize up to 5 μg of the plasmid with BamHI restriction enzyme and purify using phenol/chloroform extraction as described in the manual for HiScribe T7 High Yield RNA Synthesis Kit.
8. Use 1 μg of linearized plasmid for transcription of the sgRNA following the manufacturer's instructions for reactions with short RNA transcripts (<0.3 kb).
9. Purify the sgRNA with mirVana miRNA Isolation Kit. sgRNA can be further concentrated by ethanol precipitation if desired.
10. PCR amplify an endogenous fragment that contains sgRNA target site and clone it into high copy number plasmid to use as a template for testing activity of Cas9/sgRNA RNP complex in vitro (*see* **Note 8**).
11. Set up the following reaction to test the activity and specificity of Cas9/sgRNA complex: 300 ng of linearized plasmid with a target site, 1 μg of Cas9 protein, 1 μg of sgRNA, and 1× NEB3 buffer in a total volume of 20 μL. Incubate reaction for 1 h at 37 °C, add loading dye, and load straight on 1% agarose gel (Fig. 2a). Distinct bands of specific size should be visible on the gel as a result of specific cleavage activity of Cas9/sgRNA complex.

3.4 Labeling of Cas9 Protein with the Fluorophore and Transfection of Microspores with RNP-CPP Complex

1. To assess CPP-mediated delivery of either Cas9 or dCas9-VP64 protein into microspores, label the proteins with Alexa Fluor 647 (AlF-647) using the labeling kit according to the manufacturer's instructions.
2. Purify labeled protein using Sephadex G-25 gel filtration column, followed by concentration using Amicon Ultra-15 100 K centrifugal filter units. The last step also serves as a quality control to verify the absence of any free and unbound dye following purification.
3. Conjugate either Cas9 or dCas9-VP64 protein with R_9 CPP by combining 5 μg of protein aliquots with different amounts of CPP. Adjust volume of reaction mixture to 100 μL using microspore extraction solution. Incubate the mixture for 2 h at room temperature. Typical weight ratios of protein to CPP that we use in our experiments is from 20:1 to 5:1, respectively. Following conjugation step, proceed with transfection of microspores (*see* **Note 9**).
4. Combine AlF647-Cas9-CPP conjugate together with 100 μL of microspore solution (1000 cells/μL) and keep for 48 h in the dark at 28 °C.

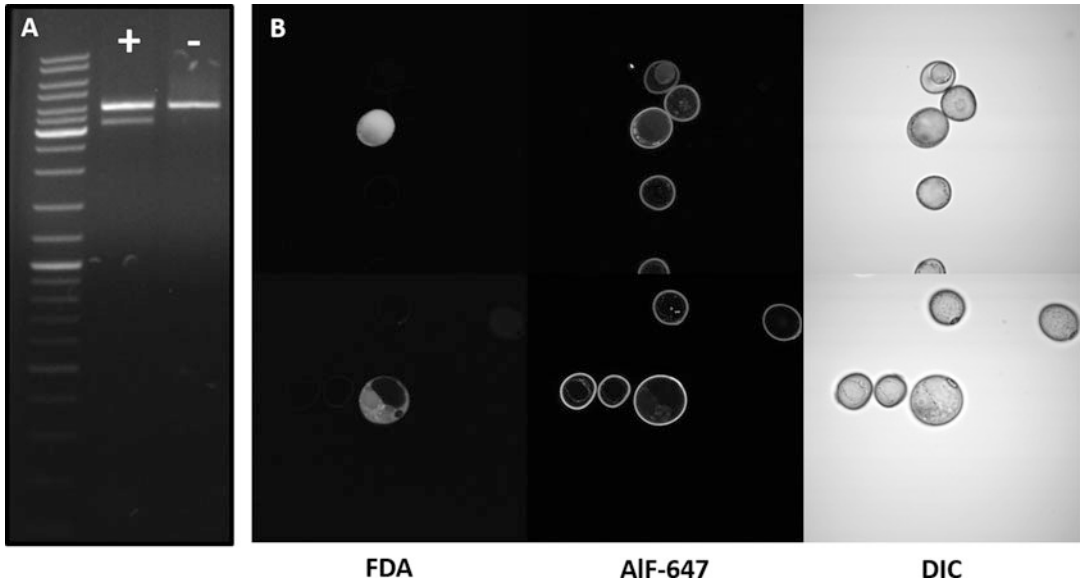

Fig. 2 (**a**) In vitro analysis of cleavage activity of purified Cas9 using plasmid with the cloned target site. 300 ng of linearized plasmid with a target site was combined with 1 μg of Cas9 protein, 1 μg of sgRNA and 1× NEB3 buffer in a total volume of 20 μL. The reaction was incubated for 1 h at 37 °C and analyzed on 1% agarose gel. "+" reaction with Cas9/sgRNA complex and "-" undigested plasmid. (**b**) Transfection of wheat microspores with Cas9 protein labeled with Alexa Fluor 647. Transfection of microspores (1000 cells/μL) was done with 5 μg of labeled Cas9 combined with 2 μg of R9 CPP in a total volume of 100 μL. The mixture was incubated for 48 h. Upper panel, DIC image of microspores, and lower panel, AlF-647, 594/633 laser line. Transfection of microspores is evident from localization of Cas9-AlF-647 in cytoplasm and nucleus. Negatively charged microspore exine traps positively charged R9-Cas9 complex resulting in a strong signal from the outer surface of the non-transfected cells. FDA—fluorescein diacetate was used as a viability stain. Image acquisition was done using confocal laser scanning microscope Olympus, FV1000 with 594/633 laser line for AlF-647 and GFP emission filter for FDA

5. Wash the microspores by spinning the cells at 100 × *g* for 5 min and completely remove the supernatant. Resuspend the cells in 2 mL of microspore extraction solution. Repeat this procedure 2 more times.
6. Perform visual examination of transfected microspores using confocal laser scanning microscope with 594/633 laser line (Fig. 2b).
7. For transfection of complete Cas9/sgRNA complex, combine equal amounts of either Cas9 or dCas9-VP64 protein and sgRNA in a tube and incubate for 5 min at room temperature (*see* **Note 10**).
8. Add optimized amount of R_9 CPP to the Cas9/sgRNA mixture, bring volume to 100 μL with microspore extraction solution, and incubate the mixture for 2 h at room temperature.

9. Add 100 µL of microspore solution (1000 cells/µL) to R_9-Cas9/sgRNA mixture, transfer solution into 2 mL tubes, and perform transfection for a minimum of 48 h.
10. Following transfection, collect the cells by centrifugation, remove all the supernatant, and proceed to either gDNA or RNA isolation. Alternatively, cells can be stored at −80 °C for later processing.
11. In case if Cas9/sgRNA complex was delivered into microspores, indels at the target site should be analyzed using Illumina sequencing (*see* **Note 11**).
12. To analyze activation of gene expression following delivery of dCas9-VP64/sgRNA complex, isolate total RNA from treated microspores or untreated controls. Perform cDNA synthesis and quantify expression of the target gene against at least two reference genes from treated and untreated microspores (Fig. 3, **Note 12**).

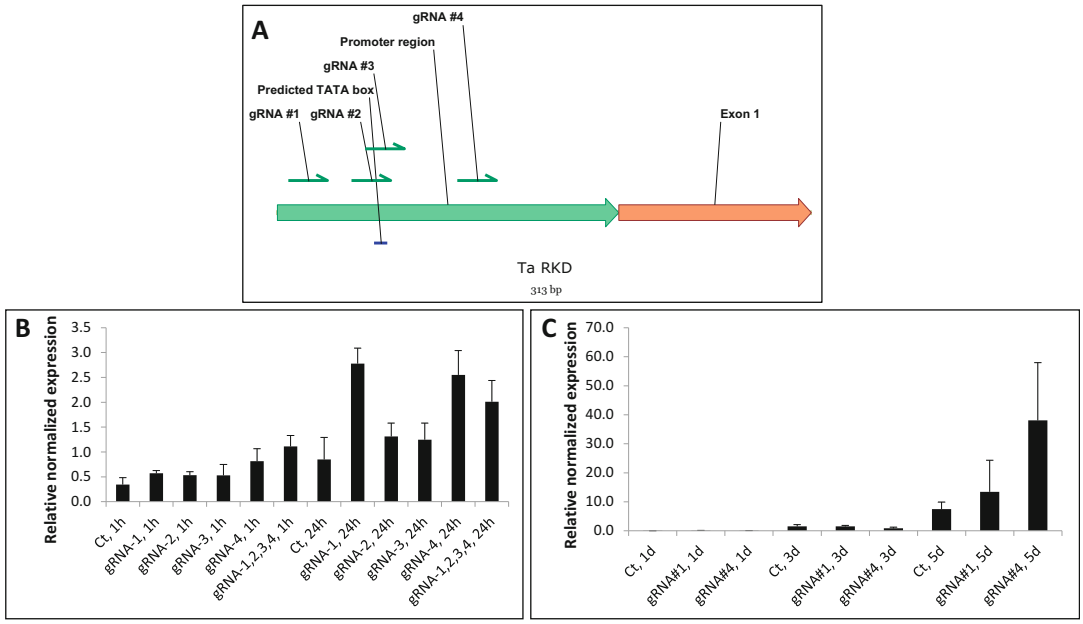

Fig. 3 (**a**) Schematic positions of sgRNA targets at *TaRKD* promoter region. Note that sgRNAs 2 and 3 are placed at the TATA box region whereas sgRNAs 1 and 4 are at the 5′ and 3′ regions in relation to the TATA box, respectively. (**b** and **c**) RT-qPCR expression analysis of *TaRKD* gene following transfection of wheat microspores with R_9-dCas9-VP64/sgRNA complex. Transfection was done for different time periods either in the presence of single sgRNA or in combination of all four. Note that only sgRNAs around the TATA box resulted in the highest activation of gene expression as compared to control. Normalization of gene expression was done using ΔΔCt method against *TaGAPDH* and *TaEF1α*

4 Notes

1. To determine stage of microspores, remove anthers from the middle floret, place anthers on the microscope slide, and add drop of water. Cover with the cover slip to squeeze the anthers, so the microspores will come out from the anthers. Most of the cells should contain large single or fragmented vacuole and a single nucleus at the periphery. In our experiments, we typically use cultivar AC Andrew.
2. Molecular weights of Cas9-6×His and dCas9-VP64-6×His proteins are 160 and 169 kDa, respectively.
3. Purification of proteins using size exclusion column is optional and required only if more than 90% protein purity is required. Combine the purest fractions from His-Trap purification and concentrate them to 500 μL using 100 K Amicon Ultra-15 spin concentrators. Load the entire volume onto HiLoad 16/60, Superdex 200 prep-grade size exclusion column using 1 mL sample loop. Collect elution fractions (2 mL each) and monitor elution of the proteins at 280 nm. Due to relatively high molecular weight of Cas9 protein, it should elute as the first major pick on chromatogram. Analyze every fraction containing the protein on the 8% PAGE and combine the purest fractions together.
4. When using CHOPCHOP v3 online tool, select targets with the highest score and those containing unique restriction sites at the cut site or near it. In our experience, sgRNAs for dCas9-VP64 activator should be selected around TATA box, but not at the TATA box itself.
5. Make sure not to include PAM region into the oligos sequence.
6. Typically, cloning efficiency is extremely high and only two to four colonies are needed to be screened for the presence of insert.
7. Using 1 μg of linearized plasmid in transcription reaction can generate over 100 μg of short RNA.
8. In our experiments, we typically clone a 500–1000 bp fragment containing target site in the middle into pUC18 plasmid.
9. R9 CPP demonstrates cytotoxicity at high concentrations. We observed that adding more than 100 ng/μL of R9 CPP per 100,000 of microspores is lethal for the cells.
10. We typically use from 5 to 15 μg of Cas9 protein with an equal amount of sgRNA per single transfection reaction.
11. We usually generate 200 bp amplicons containing a target site in the middle using primers with unique barcodes for multiplexing, if several different samples are to be analyzed.

12. We routinely use *TaGAPDH* (TRIAE_CS42_6DL_TGACv1_526670_AA1689480.1) and *TaEF1α* (TRIAE_CS42_U_TGACv1_641551_AA2097790) genes as the references for gene expression studies.

Acknowledgment

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Chapter 13

CRISPR-Cas9-Mediated Gene Editing in Wheat: A Step-by-Step Protocol

Pankaj K. Bhowmik and M. Tofazzal Islam

Abstract

The CRISPR-Cas9-mediated gene editing has sparked a new revolution in biological and agricultural research. This innovative technology is not only valuable for understanding the function of genes but also offers an efficient approach for genetic manipulation and crop improvement. However, the efficiency of gene editing in wheat (*Triticum aestivum* L.) is highly variable because of the genetic complexity of this plant species including 17 gigabases of genetic code, hexaploid genome, 80% genome with repetitive DNA, and large gene families. Delivery of gene editing reagents into wheat relies on optimization of transformation and plant regeneration methods. Currently available systems of wheat transformation are *Agrobacterium*-mediated transformation and biolistic bombardment. Recently, potential application of haploid microspore-based gene editing system in wheat has also been described. Here, we provide a step-by-step protocol for the implementation of CRISPR-mediated wheat gene editing and trait improvement. We begin with gRNA designing using online tools, followed by assembling CRISPR constructs or ribonucleoprotein (RNP) complex and in vitro and in vivo gRNA validation using wheat mesophyll protoplasts. Furthermore, we provide details of the tissue culture protocols for a stable wheat transformation using the most efficient gRNA for editing the gene of interest. Plant selection and regeneration protocols are also provided. We conclude this chapter with the mutation detection methods before phenotyping for the desired traits.

Key words CRISPR-Cas9, gRNA, Wheat, Protoplast

1 Introduction

Wheat (*Triticum aestivum* L.) is one of the major sources of food for much of the world. It provides about a fifth of the total calories consumed by humans and contributes more protein than any other food source [1, 2]. Success in classical breeding and genetic engineering efforts are limited due to insufficient knowledge and understanding of wheat biology and the molecular basis of central agronomic traits. Improvement of wheat for tolerance to emerging biotic such as wheat blast [3] and abiotic stresses triggered by global climate change are badly needed for ensuring food and nutritional security of increasing population of the world [4]. To

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provide a foundation for improvement through molecular breeding, using recent advances in sequencing, the International Wheat Genome Sequencing Consortium presents an annotated reference genome with a detailed analysis of gene content among subgenomes and the structural organization for all the chromosomes [5]. The decoding of wheat genome offers new opportunity for CRISPR (clustered regularly interspaced short palindromic repeats)-based genome modification to show the potential for using this genome in agricultural research and breeding [4]. The CRISPR-Cas technology becomes a revolutionary technology for the rapid and precise improvement of crop plants including wheat [6, 7]. However, the transformation procedure of the hexaploid wheat is recalcitrant and thus success in genetic engineering including genome editing technology is limited [8–14]. We developed a protocol for CRISPR-Cas9-based targeted mutagenesis in wheat microspores [9]. This chapter describes and illustrates (Figs. 1, 2, 3, 4, 5, 6, and 7) the step-by-step procedure of our newly developed protocol.

2 Designing of Guide RNA

There are two approaches to design sgRNAs to target all copies of a gene in a polyploid species. In the first strategy, using sequence alignment, sgRNAs can be designed to target all the conserved regions [9]. In the second strategy, different sgRNAs can be designed for each copy and co-transformed to target all the copies. The guide RNA seed sequences are selected according to the reference genome sequence by using publicly available online tools (WheatCRISPR, https://crispr.bioinfo.nrc.ca; CRISPR Direct, https://crispr.dbcls.jp/). A few requirements are needed for choosing the unique gRNA target sequence. Usually we design 3–5 gRNAs for each gene.

1. The 20 bp target sequence should immediately precede the 5′-NGG PAM, which is essential for Cas9 binding to the target DNA.
2. If the aim is to disrupt gene function, target sequences at the 3′end of coding regions or introns should be avoided.
3. Gene disruptions in these positions may have little or no effect on gene function. Off-target mutagenesis could be avoided by BLAST (basic local alignment search tool); search the reference genome database.
4. Appropriate restriction enzyme site present in the target sequence is optional. But if gRNA activity is to be detected by the PCR/RE assay, restriction enzyme sites within the target sequences will facilitate detection.

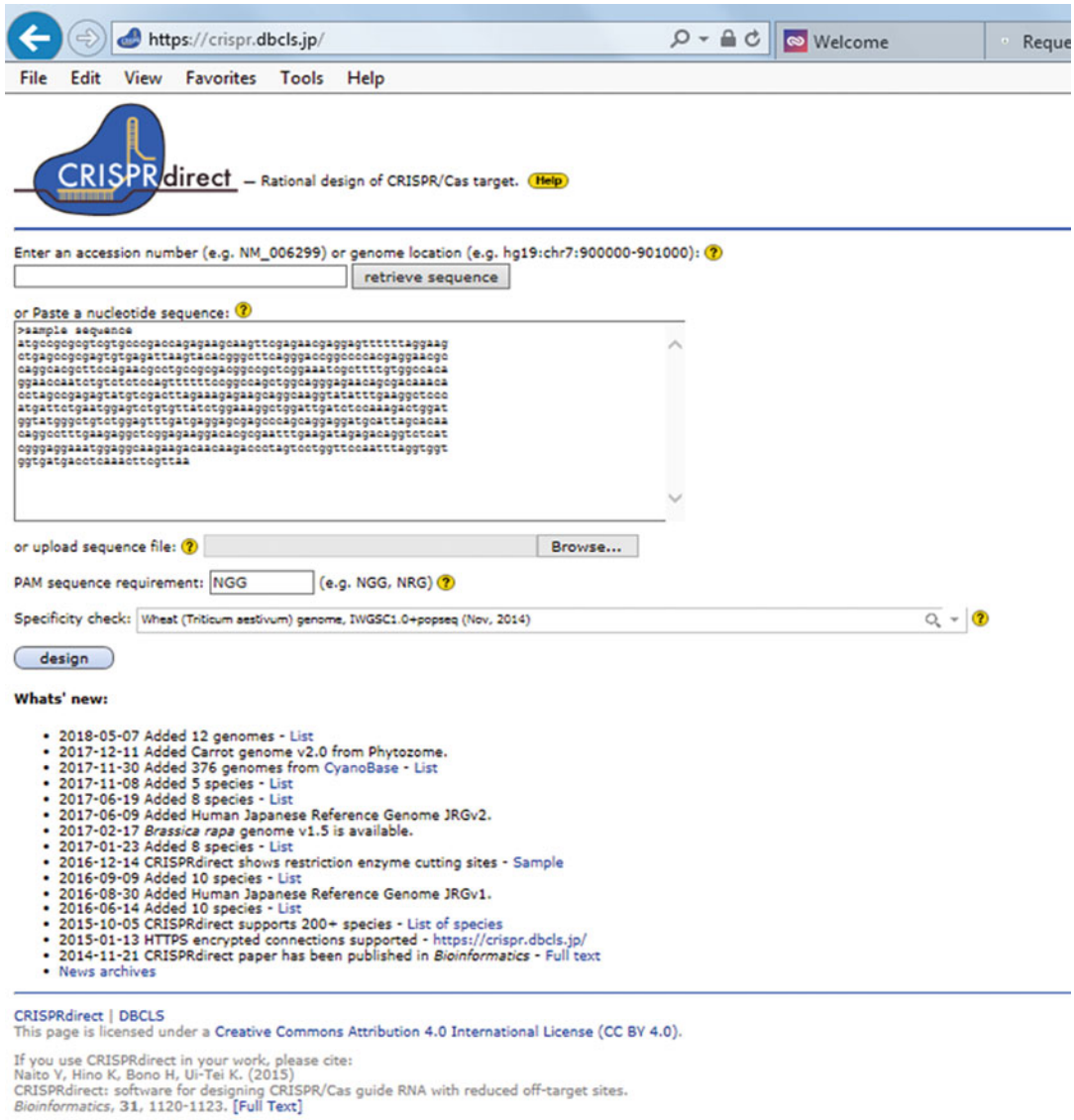

Fig. 1 Screen capture from the sequence entry page of the CRISPR direct web based tool

5. 5′-G is required for the stability of gRNA as the polymerase III promoter used to express gRNA is most effective with transcripts that starts with guanine.

The following is an example of one of the best publicly available online tools for designing sgRNAs for wheat:

design

Results:

Sequence name: sample sequence
PAM sequence: NGG
Specificity check: Human (Homo sapiens) genome, GRCh37/hg19 (Feb, 2009)
Time: 2019-11-03 21:42:52

- Highlighted target positions (e.g., **45 - 67**) indicate sequences that are highly specific and have fewer off-target hits.
- Target sequences with '0' in '20mer+PAM' (in number of target sites column) are shown in gray. Such sequences may possibly span over exon-exon junctions, so avoid using these.
- Target sequences with TTTTs are also shown in gray. Avoid TTTTs in gRNA vectors with pol III promoter.

☐ show **highly specific** target only

Show 20 entries Search:

position		target sequence	sequence information				number of target sites		
start - end	+ −	20mer+PAM (total 23mer)	GC% of 20mer	Tm of 20mer	TTTT in 20mer	restriction sites	20mer +PAM	12mer +PAM	8mer +PAM
4 - 26	-	ccg cgcgtcgtgcccgaccagag [gRNA]	75.00 %	82.34 °C	-	BmeI580I	1 [detail]	3 [detail]	103 [detail]
16 - 38	-	ccc gaccagagaagcaagttcga [gRNA]	50.00 %	71.17 °C	-		1 [detail]	26 [detail]	4354 [detail]
17 - 39	-	ccg accagagaagcaagttcgag [gRNA]	50.00 %	71.53 °C	-		1 [detail]	32 [detail]	5910 [detail]
21 - 43	-	cca gagaagcaagttcgagaacg [gRNA]	50.00 %	69.25 °C	-		1 [detail]	24 [detail]	8744 [detail]
24 - 46	+	gagaagcaagttcgagaacg agg [gRNA]	50.00 %	69.25 °C	-		1 [detail]	2 [detail]	306 [detail]
35 - 57	+	tcgagaacgaggagtttttt agg [gRNA]	40.00 %	66.49 °C	+		1 [detail]	25 [detail]	9901 [detail]
66 - 88	-	ccg cgagtgtgagattaagtaca [gRNA]	40.00 %	65.70 °C	-		0 [detail]	3 [detail]	1097 [detail]
69 - 91	+	cgagtgtgagattaagtaca cgg [gRNA]	40.00 %	65.70 °C	-		0 [detail]	26 [detail]	3790 [detail]
70 - 92	+	gagtgtgagattaagtacac ggg [gRNA]	40.00 %	65.27 °C	-		0 [detail]	3 [detail]	3604 [detail]
77 - 99	+	agattaagtacacgggcttc agg [gRNA]	45.00 %	70.40 °C	-		1 [detail]	6 [detail]	1306 [detail]
78 - 100	+	gattaagtacacgggcttca ggg [gRNA]	45.00 %	70.01 °C	-		1 [detail]	9 [detail]	6616 [detail]
83 - 105	+	agtacacgggcttcagggac cgg [gRNA]	60.00 %	78.32 °C	-		1 [detail]	63 [detail]	6397 [detail]
93 - 115	+	cttcagggaccggccccacg agg [gRNA]	75.00 %	84.21 °C	-		1 [detail]	7 [detail]	2568 [detail]
102 - 124	+	ccggccccacgaggaacgcc agg [gRNA]	80.00 %	86.67 °C	-		1 [detail]	1 [detail]	1244 [detail]
102 - 124	-	ccg gccccacgaggaacgccagg [gRNA]	75.00 %	84.31 °C	-		1 [detail]	19 [detail]	3777 [detail]
106 - 128	-	ccc cacgaggaacgccaggcacg [gRNA]	70.00 %	80.55 °C	-		1 [detail]	1 [detail]	1098 [detail]
107 - 129	-	ccc acgaggaacgccaggcacgc [gRNA]	70.00 %	82.51 °C	-		1 [detail]	2 [detail]	1116 [detail]
108 - 130	-	cca cgaggaacgccaggcacgct [gRNA]	70.00 %	82.52 °C	-		1 [detail]	2 [detail]	1011 [detail]
120 - 142	-	cca ggcacgcttccagaacgcct [gRNA]	65.00 %	80.61 °C	-		1 [detail]	6 [detail]	1510 [detail]
130 - 152	+	ttccagaacgcctgccgcga cgg [gRNA]	65.00 %	80.58 °C	-		1 [detail]	4 [detail]	289 [detail]

Showing 1 to 20 of 77 entries First Previous 1 2 3 4 Next Last

Fig. 2 Screen capture of the CRISPR direct [12] output window showing list of CRISPR-Cas target candidates for designing gRNAs

CRISPRdirect:

Software for designing wheat CRISPR-Cas guide RNA with reduced off-target sites

CRISPRdirect [12] is a very simple and easy-to-use web-based tool for gRNA designing which is freely available at http://crispr.dbcls.jp/. As we have already learned, the target sequence requirements are twofold:

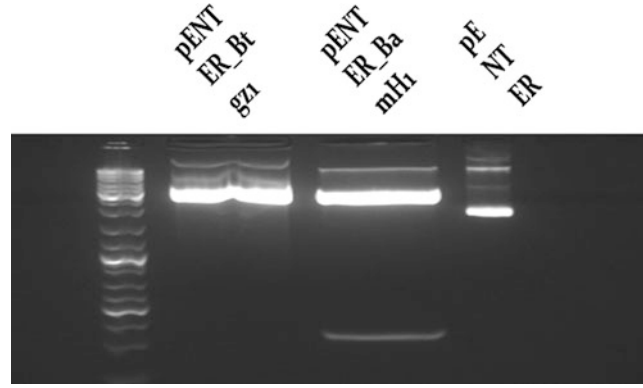

Fig. 3 Gel Electrophoresis of pENTR plasmid digested using two different restriction enzymes. Lane 1, BtgZI digest; lane 2, Bam HI digest lane 3, uncut pENTR plasmid

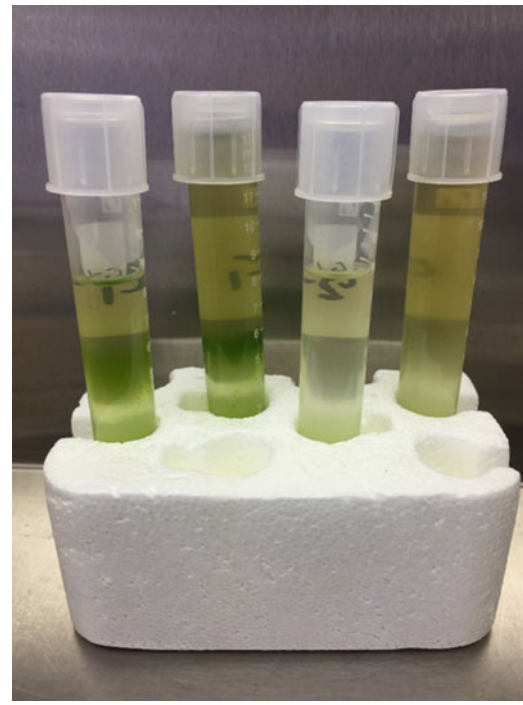

Fig. 4 Protoplast solution after sucrose gradient separation

1. First, the 5′-NGG protospacer adjacent motif (PAM) sequence must be located adjacent to the target sequence.
2. Second, the target sequence should be specific within the entire genome in order to avoid off-target editing.

The server currently incorporates the genomic sequences of bread wheat generated by the International Wheat Genome

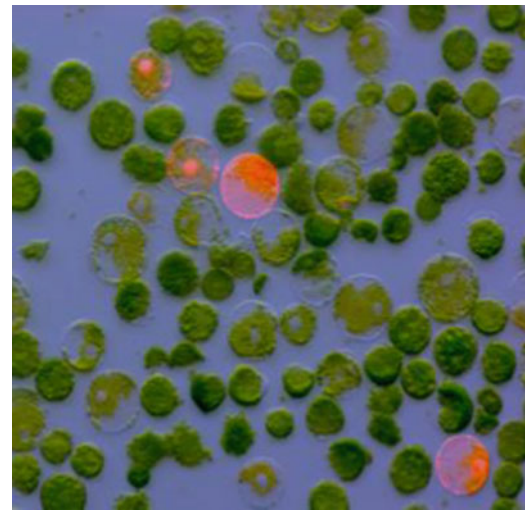

Fig. 5 Transformed wheat protoplast expressing Cas9-dsRed plasmid DNA

Sequencing Consortium (IWGSC). The IWGSC, with more than 1100 members in 55 countries, is an international, collaborative consortium, established in 2005 by a group of wheat growers, plant scientists, and public and private breeders. The CRISPR direct web server accepts an accession number, a genome coordinate, or an arbitrary nucleotide sequence up to 10 kbp as input (Fig. 1). After giving inputs for "PAM sequence requirement" and "specificity check" from the drop-down selection, the software will give a list of CRISPR-Cas target candidates for designing gRNAs (Fig. 2).

The CRISPR direct searches target sequences of 20nt adjacent to the PAM sequence (e.g., NGG and NRG) from both strands of the input sequence. The table and list of potential gRNAs contains target position, target sequence, additional information on the sequence and the number of target sites in the genome. The additional information on the sequences such as GC content and calculated melting temperature™ are also provided. The software highlights the target positions (e.g., 45–67) indicating sequences that are highly specific and have fewer off-target hits. It is recommended to avoid target sequences with "0" in "20mer+PAM" as such sequences may possibly span over exon-exon junctions. We should also avoid target sequences with TTTTs gRNA vectors with pol III promoter (Figs. 8 and 9).

3 Cloning and Reassembling Constructs

Depending on the gRNA scaffold vectors, only the ~20-nt target sequence of the sgRNAs needs to be replaced to target a different genomic site; the remainder of the sgRNAs—the "scaffold"—

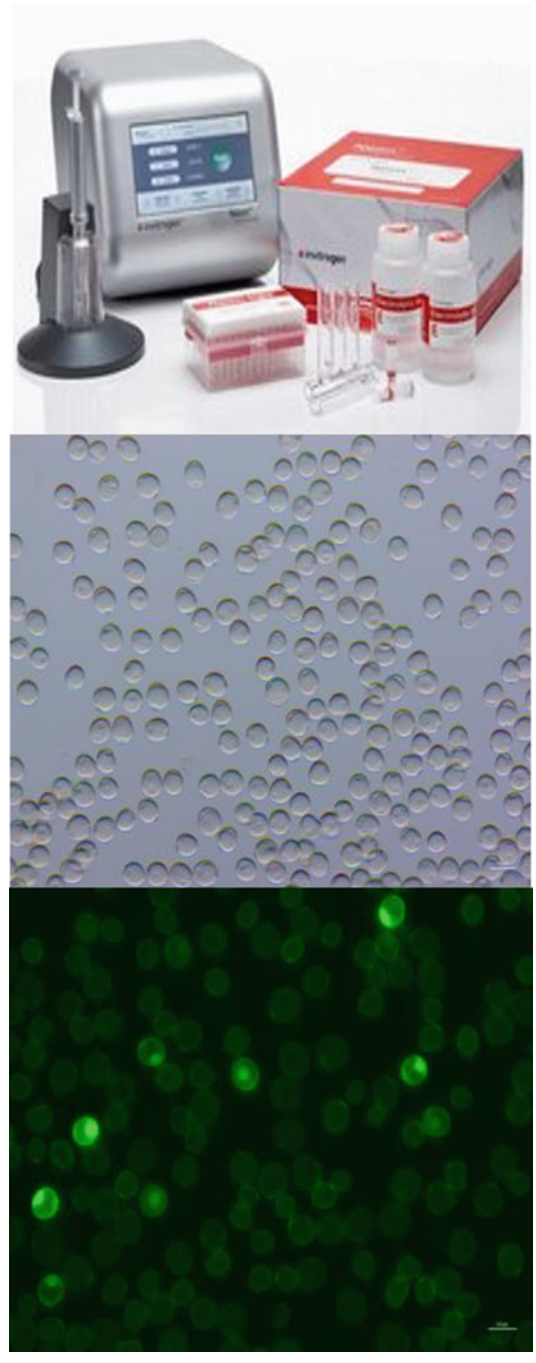

Fig. 6 Neon Electroporation system and microspores before and after electroporation

Fig. 7 The PDS-1000/He system with coated gold on macrocarriers and isolated scuttella ready for bombardment

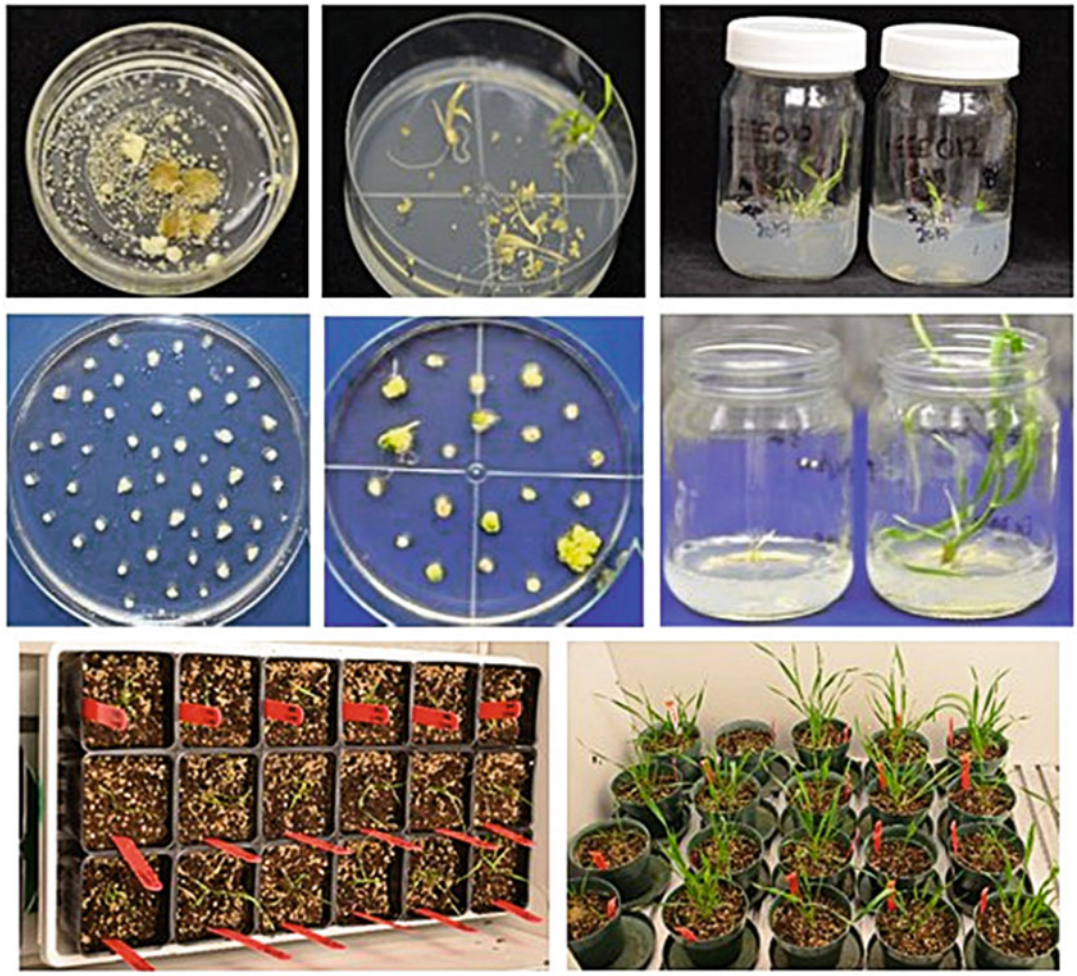

Fig. 8 Regeneration of embryo and green plants after microspore electroporation and bombardment of scuttella

remains the same. Two methods for cloning and reassembling CRISPR constructs are described below using PENTR4-gRNA1 vector as an example. This protocol can be adapted for any other CRISPR vector purchased from Addgene (https://www.addgene.org/).

Example 1 using PENTR4-gRNA1 vector: To design the sgRNAs oligos, two complementary oligonucleotides (21–25 nt) with sense strand consisting of 4-nt overhang of TGTT at the 5′ end and antisense strand consisting of extra AAAC at the 5′ end for BtgZI restriction site could be synthesized. Similarly, another two complementary oligonucleotides are designed with sense strand containing a 4-nt overhang of GTGT at the 5′ end and antisense strand containing AAAC at the 5′ end for *Bsa*I restriction site. To construct the gRNA gene targeting the genomic locus of interest,

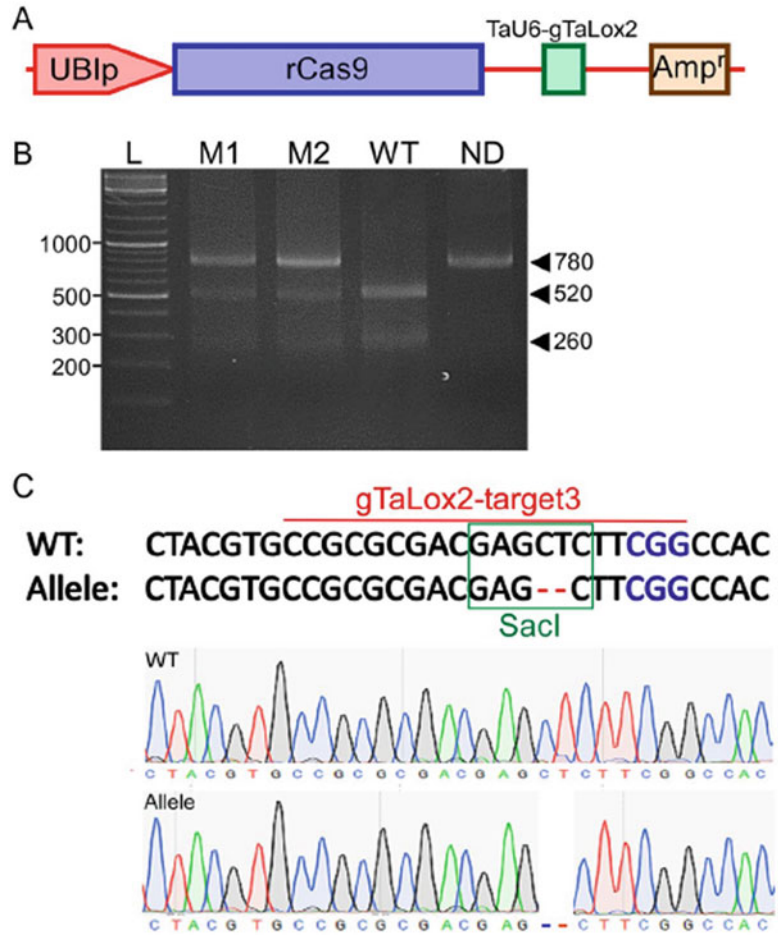

Fig. 9 Example of mutation detection (after [9]). (**a**) Schematic diagram showing the target construct used for CRISPR editing in wheat microspores. (**b**) PCR/restriction enzyme assay to detect mutations in the *TaLox2* gene induced by Cas9/gTaLox2. (**c**) A 2 bp deletion detected at the cleavage site in the sequenced product of *TaLox2* from microspores transfected with the construct with Sanger sequencing electropherograms shown below

two complementary oligonucleotides are first annealed and then ligated into the BtgZI-digested pENTR4-gRNA1 vector. After the cloning step, the positive candidate clone is sequenced to confirm the accuracy of seed sequence in the gRNA scaffold.

Reagents:	Restriction enzymes and CutSmart Buffer (New England Biolabs)
	AmpliTaq DNA Polymerase with all the components (Applied Biosystems)
	ATP, 10 mM; T4 DNA Ligase (New England Biolabs)
	LR Recombinant Clonase (Invitrogen)

(continued)

	QIAquick Gel Extraction Kit (QIAGEN)
	QIAquick PCR Purification Kit (QIAGEN)
Equipment:	Gel electrophoresis and imaging units
	Freezers (−20 °C)
	Incubators (28 °C, 37 °C)
	Benchtop Microcentrifuge
	Thermocycler

Step 1: Digest 1μg of pENTR4-gRNA1 vector with BtgZI restriction enzyme for 3 h at 37 °C. In a PCR tube, mix the following components and make the final volume 20 μL with nuclease-free water.

PENTR4-gRNA1	1.0 μg
BtgZI (NEB)	1.0 μL
RNAase A	0.2 μL
10× CutSmart Buffer	2.0 μL

Step 2: After digestion, gel-purify using QIAquick Gel Extraction Kit.

Step 3: Phosphorylate and anneal each pair of primers ordered from IDT corresponding to each designed gRNA. In a PCR tube, mix the following components and make the final volume 20 μL with nuclease-free water. Annealing could be done by heating a beaker of water to boiling point and cooling down to room temperature in a PCR tube.

Primer/Oligo 1 (10 pM)	2.0 μL
Primer/Oligo 1 (10 pM)	2.0 μL
PNK buffer (Fisher)	2.0 μL
T4 PNK (Fisher)	2.0 μL
ATP	1.0 μL

Step 4: Ligate and clone the annealed dsOligos from **step 3** into BtgZI-digested pENTR4-gRNA1 vector from **step 1**. Using a PCR tube, set up the 10 μL ligation reaction with the following components.

Digested pENTR4-gRNA1	50.00 ng
Annealed dsOligos	5.0 μL
T4 DNA ligase (NEB)	2.0 μL
10× ligation buffer	1.0 μL

Incubate the ligation reaction overnight at 4 °C and transform *Escherichia coli DH5α* competent cells with half of the ligation reaction.

Example 2 using PTaU6-sgRNA vector: This vector contains a unique AarI restriction site, into which annealed target oligos can be cloned. The oligo pairs should be Fwd: 5′-CTTGN (20)-3′; Rev: 5′-AAACN (20)-3. To obtain efficient sgRNAs and avoid repeating experiments, we design and construct at least three sgRNAs per target locus and validate efficiencies of sgRNAs using mesophyll protoplast system.

4 Guide RNA Validation

4.1 Mesophyll Protoplast System

To validate and assess the activity of sgRNAs rapidly, we isolate mesophyll protoplast from 10 to 14-days-old seedlings with the cell wall-dissolving enzyme solution. Polyethylene glycol 4000 (PEG4000)-mediated transformation is used to transform the constructs into protoplasts. After 48 h, the protoplasts are collected to extract DNA for PCR-RE assay.

Reagents:	Mannitol Onozuka R-10 (Bioworld) Macerozyme (Bioworld) PEG4000 (Sigma-Aldrich) BSA (Sigma-Aldrich) 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.2 M, pH 5.7 (Sigma-Aldrich) Mannitol, 0.8 M (Sigma-Aldrich) $CaCl_2$, 1 M (Sigma-Aldrich) KCl, 2 M (Sigma-Aldrich, cat) $MgCl_2$, 2 M (Sigma-Aldrich) Murashige and Skoog (MS) Basal Medium (Sigma-Aldrich)
Equipment:	Razor blade (Fisher Scientific) Tissue culture plate – six-well (VWR) Round-bottom centrifuge tube (VWR) Lab shaker (Thermo Fisher Scientific) Falcon cell strainer 40 μm (Fisher Scientific) Incubators (28 °C, 37 °C) Benchtop Microcentrifuge Fluorescence microscope

Step 1: *Isolation of wheat protoplasts*—Prepare fresh enzyme solution before isolation of protoplasts. Adjust pH 5.7–5.8, make final volume 200 mL, and sterilize using 0.2 μm filter. With a razor blade, chop into small pieces the central part of the leaves of 10–14-days-old plantlets. Incubate chopped leaves on a shaker after adding 15 mL of the enzyme solution.

Enzyme solution	
Mannitol (FW 182.17) 0.45 M	16.40 g
MES, 20 mM	20.00 mL
Cellulase Onozuka R-10 (1%)	2.0 g
Macerozyme (0.25%)	0.5 g
MS Basal Medium (1×)	100 mL
BSA (0.1%)	0.2 g

Filter the digested protoplast solution through 40 μm Falcon cell strainer by pipetting with a 30 mL pipette very slowly and add 2 mL of buffer W5 to the filter. Prepare two 15 mL Falcon tubes with 8 mL 0.55 M sucrose solution and slowly add filtered protoplasts solution to each tube. Centrifuge at 200 × *g* for 5 min at 4 °C (sucrose-gradient separation, Fig. 4). After centrifuging carefully, collect protoplasts solution from the intermediate layer and add to a fresh 15 mL Falcon tube. Add 10 mL W5 solution very carefully to the wall of the tube to resuspend the protoplasts. Centrifuge at 40 × *g* for 2 min at room temperature. Discard supernatant and wash twice with 5 mL W5 solution. Count protoplasts using a hemocytometer and adjust cell count to 10^6 cells/mL, resuspending in MMG buffer.

W5 solution (500 mL)	
MES (pH 5.7) 2 M	5.0 mL
NaCl (0.2 M)	30.8 mL
$CaCl_2$ (2.5 M)	25 mL
KCl (0.5 M)	5.0 mL

MMG buffer (200 mL)	
Mannitol, 0.4 M	14.57 g
MES (pH 5.7) 4 mM	4.0 mL
$MgCl_2$ (15 mM)	6.0 mL

Step 2: *PEG-mediated protoplast transformation*—In a 2.0 mL microcentrifuge tube, take 20–40 μL (1 μg/μL) plasmid DNA from Cas9 and gRNA constructs cloned and reassembled previously (*see* Chapter 2). Pipette 200 μL of protoplast solution (10^6 cells/mL) to each tube containing 40 μL of the DNA for transformation. Mix gently by hitting the tube with the finger. Add equal volume (240 μL) of freshly prepared 40% PEG to the tube

and mix gently. Incubate at room temperature for 20–30 min and add approximately 900 μL of buffer W5 to stop the transformation. Centrifuge for 5 min at 40 × *g* at room temperature. Discard supernatant and wash twice with 5 mL W5 solution. After washing, transfer transformed protoplasts to a six-well culture plate and incubate at 25 °C for 48 h. If you have a fluorescence reporter in your constructs, you can confirm transformation efficiency by observing under a fluorescence stereo microscope.

PEG $CaCl_2$ solution (200 mL)	
PEG 4000, 40%	80.00 g
Mannitol, 0.2 M	7.29 g
$CaCl_2$, 100 mM	8.0 mL

Step 3: *Molecular screening for CRISPR edits and gRNA validation in protoplasts* – After 48 h of incubation, collect pulled protoplasts by centrifuging at 2,000 × *g* for 5 min at 4 °C. Extract DNA using QIAGEN DNeasy plant mini kit. PCR-amplify the targeted genomic region for PCR-RE assay. Make sure that the restriction enzyme site in the sgRNA target is the only one in the amplicon. The PCR error and nonspecific amplification can be avoided by using high-fidelity Taq polymerase. Digest the PCR product with restriction enzyme and run all 50 μL digestion products on a 2% agarose gel in TAE buffer using standard protocols. If editing occurs, the digestion of the PCR product will generate an uncleaved band but the control DNA will be cleaved. Purify the uncleaved band (DNA) from the gel using QIAGEN gel extraction kit, clone, and sequence using Sanger sequencing method. If the gRNA does not contain appropriate restriction site, a T7E1 assay could be used for mutation detection.

5 Wheat Transformation

Depending on wheat explants, the transformation systems could be optimized for the wheat genotype of interest. Both electroporation and biolistic mediated delivery methods could be used for efficient stable transformation. A minimum of 75,000 cells using 10–20 μg DNA and a pulsing voltage of 500 is optimal for microspore transfection using the Neon transfection system [9]. Biolistic bombardment of wheat embryos is performed using a PDS1000/He particle bombardment system (Bio-Rad) with a target distance of 6.0 cm from the stopping plate at helium pressure of 600 psi [10]. After bombardment, embryos are transferred to callus induction medium. In the third week, all calli are transferred to regeneration medium. After 3–5 weeks, green shoots appeared on the

surface of the calli. These are transferred to a rooting medium, and a large number of T_0 seedlings can be obtained about 1–2 weeks later.

5.1 Microspore Transformation Using

Step 1: *Growing donor plants and microspore isolation*—Grow desired wheat genotype in a growth cabinet with a photoperiod of 19 h per day (intensity 300 μE m^{-2} s^{-1}) and temperature 18/15 °C day/night with a relative humidity at about 70% according to Eudes and Amundsen [11]. Apply fertilizer every 2 weeks (20-20-20, 200 ppm) with water. Collect tillers when the spike is at 1–3 cm of emergence from the boot. Store harvested tillers in the refrigerator (4 °C) for 21 days before microspore isolation with their bases in distilled water and their heads wrapped in aluminum foil. Leave some tillers on the plants for collecting ovaries later.

In order to isolate microspores, check the stored spikes after 18 ± 3 days after collection. The mid to late uninucleate microspore stage is preferred for efficient transformation and regeneration. In a laminar flow hood, remove awns from heads with scissors and sterilize eight spikes for microspore isolation and four spikes for ovaries using 10% bleach (5.25% sodium hypochlorite) for 3 min and rinsing four times for 1 min with sterile double distilled water with constant agitation. Dissect anther spikes aseptically and transfer to a sterile and refrigerated 110 mL Warring blender cup (VWR international, # 58983-093) containing 50 mL filter sterilized NPB99 liquid medium on ice. Blend anthers twice for 7 s at low speed (3,600 × *g*). Pour suspension through 100 μm sterile mesh (VWR International, # CA21008-950) into two 50 mL centrifuge tubes (25 mL each).

Reagents:	2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM, pH 5.7 (Sigma-Aldrich) Mannitol, 0.4 M (Sigma-Aldrich) Fe-EDTA, 100 μM (Sigma-Aldrich) Maltose, 90 g/L (Sigma-Aldrich) 2, 4-D, 0.2 mg/L (Sigma-Aldrich, cat) Kinetin 0.2 mg/L (Sigma-Aldrich, cat) Glutathione, 0.615 mg/L (Sigma-Aldrich) Phenyl acetic acid, 1 mg/L (Sigma-Aldrich) Larcoll, 40 mg/L (Sigma-Aldrich) Cefotaxime, 100 mg/L (Sigma-Aldrich) Murashige and Skoog (MS) Basal Medium (Sigma-Aldrich)
Equipment:	Razor blade (Fisher Scientific) Tissue culture plate—six-well (VWR) Falcon cell strainer 100 μm (Fisher Scientific) Incubators (28 °C, 37 °C) Benchtop Microcentrifuge Fluorescence microscope

Collect microspores by centrifugation at 200 × *g* for 5 min at 4 °C using a swinging bucket rotor. Discard supernatant and collect the microspores pellets from both tubes together in one 15 mL tube. Wash twice with NPB99 using centrifugation in the same condition as before. Discard supernatant and resuspend the pellet in 5–6 mL of 20% maltose, and then layer 1 mL of NPB99 on top and centrifuge at 200 × *g* for 15 min for gradient purification to separate from nonviable microspores and debris. Collect healthy microspore in a new 15 mL tube from the intermediate band at the interface. Fill up the tube with NPB99 and centrifuge again at 200 × *g* for 5 min. Discard supernatant and resuspend the microspores with NPB99 in a total volume of 1.4 mL. Count isolated microspores using hemocytometer and adjust to 1×10^5 cells per mL.

Step 2: *Microspore transformation*—Prepare aliquots of 25,000–200,000 isolated microspores per 1.5 mL centrifuge tube for electroporation. Remove the NPB99 media from tube and resuspend the microspores in Neon electroporation buffer. Add 20–30 μg of DNA for Cas9-gRNA constructs to each tube containing microspores and mix by gently flicking the tube. Perform electroporation in 100 μL volumes using at least three biological replicates for each treatment following protocol as described in the Neon transfection manual (https://www.thermofisher.com/order/catalog/product/MPK5000).

Set up a Neon® Tube with 3 mL electrolytic buffer E2 into the Neon® Pipette Station. Set pulse conditions at 500 voltage with 20 pulse width and number of pulses as 3. Take 100 μL of the microspore-DNA mixture from centrifuge tube into the Neon® Pipette avoiding air bubbles. Air bubbles during pipetting will cause arcing during electroporation leading to lowered or failed transfection. Then press **Start** on the touchscreen. After electroporation, slowly remove the Neon® Pipette from the Neon® Pipette Station and immediately transfer the microspores from the Neon® Tip into the culture plate containing 3 mL of the NPB99 culture medium. Observe sample under microscope for viability and transfection efficiency and continue incubating at 28 °C for regeneration.

6 Transformation of Wheat Embryos Using Biolistic Gen Gun

Step 1: *Growing donor plants and isolation of scuttella*—Germinate seeds of the desired wheat genotype on wet filter paper and plant five seeds per 6-inch pots. Grow in a growth cabinet at 22 °C with a 20 h/4 h light dark cycle. When the plants begin to flower, check the plants daily and tag the heads with the date on which the first anther appears. Collect the heads 10 or 11 days after the first anther. Pick the green seeds out of the collected heads into a

50 mL Falcon. Sterilize by wetting with 70% ethanol for 30 s. Pour off the alcohol and fill the tube with 30% Javex containing a drop of Tween 20. Agitate gently but frequently for 15 min. Discard the Javex and rinse with sterile water for three times. Place the embryos in a 100 mm Petri dish containing a bit of water. Under a dissecting microscope, turn the seed with the groove down. Hold the seed with one pair of fine forceps and use the other to tear open the seed coat at the embryo end. Pick up the embryo and place it scutellum up on callus induction medium. Seal the plates and incubate overnight at 22 °C in darkness.

Reagents:	Murashige and Skoog salts and vitamins (Sigma-Aldrich) Sucrose, 3% (Sigma-Aldrich) Thiamine.HCL, 4 mg/L (Sigma-Aldrich) $CuSO_4 \cdot 5H_2O$, 2 mg/L (Sigma-Aldrich) 2, 4-D, 2 mg/L (Sigma-Aldrich) MES, pH 5.8, 500 mg/L (Sigma-Aldrich) Phytagel, 0.2% (Sigma-Aldrich) L-PPT (Sigma-Aldrich)
Equipment:	Light microscope Laminar flow hood Bunsen burner Forceps

Step 2: *Biolistic Transformation* – Prepare gold particles and coat DNA and gold on macro carriers following the detailed protocol of the Bio-Rad PDS particle delivery system (http://www.bio-rad.com/en-bd/product/pds-1000-he-hepta-systems?ID=1730e08d-f43a-46ea-b7f3-7b35c04c36eb). Arrange the isolated scuttella facing upward in the center of a plate of osmotic medium to form a target area. Place the stopping screen in the macrocarrier assembly followed by the macrocarrier with the dried DNA coated gold on it. Then place the rupture disk in its holder and tighten it. Turn on the helium tank and set to 200 PSI above the rupture disk. Also turn on the gene gun and vacuum pump. Close the chamber door and create a vacuum and hold it at 25–27 mmHg pressure. Perform bombardment by pressing the shoot button until the disk ruptures. Release vacuum and take out the plate with scuttella, seal, and incubate about 4 h in darkness. Observe under fluorescence microscope for evidence of transformation.

7 Regeneration of Transformants

7.1 Microspore Regeneration After Electroporation

Plate microspores immediately after electroporation on glass bottom Petri dishes containing 3 mL of NPB99 liquid media. Add 3–4 ovaries per plate and seal with Parafilm and incubate at 28 °C in the

dark for embryoid development. After 20–30 days, remove embryos larger than 0.5 mm from the Petri dishes and plate onto GEM culture medium. Seal Petri dishes with Parafilm and place 30 cm beneath Sylvania Pentron 4100 K spectrum bulbs (21 W) delivering 125 μmol m^{-2} s^{-1} lights (16 h light period) at 25 °C constant temperature. Once the embryos turned green, aseptically transfer them onto 50 mL rooting media in magenta vessels under the same conditions.

7.2 Embryo Regeneration After Bombardment

After incubating bombarded scutella, transfer them to Callus Induction Media (CIM) at 35–40 per plate and incubate as before for 2 weeks. After 2 weeks, transfer again to fresh CIM and keep for a further 2 weeks. Transfer the embryos to Shoot Induction Medium (SIM) with PPT selection agent at 20 per plate, and place it in the tissue culture room at 25 °C, with 100–120 μE illumination and 16 h light/8 h dark. In 2–3 weeks, transfer the green shoots to fresh SIM at 7 or 8 per plate (Fig. 8).

Callus Induction Medium (CIM): Murashige and Skoog salts and vitamins

3% Sucrose

4 mg/L Thiamine.HCL

2 mg/L $CuSO_4 \cdot 5H_2O$

2 mg/L 2,4-D

500 mg/L MES, pH 5.8

0.2% Phytagel

Autoclave, cool, and add 100 mg/L of glutamine and 0.05 mg/L of thidiazuron.

Osmotic Medium: Murashige and Skoog salts and vitamins

3% Sucrose

4 mg/L Thiamine.HCL

2 mg/L $CuSO_4 \cdot 5H_2O$

2 mg/L 2, 4-D

500 mg/L MES

36.44 g/L Mannitol

36.44 g Sorbitol, pH 5.8

0.2% Phytagel

Autoclave and cool. Before pouring, add 0.05 mg/L of thidiazuron.

Shoot Induction Medium (SIM): ½ Strength Murashige and Skoog salts and vitamins

3% Sucrose

250 mg/L MES, pH 5.8

0.2% Phytagel

Autoclave, cool, and add selection agent. Pour in deep (25 mm × 100 mm) Petri dishes.

Root Induction Medium (RIM): ½ Strength Murashige and Skoog salts and vitamins

1% Sucrose

250 mg/L MES, pH 5.8

0.8% Phytagar

Autoclave and add selection agent after cooling.

When shoots have elongated enough to have a recognizable stem, transfer the green shoots to Root Induction Media (RIM) containing 5 mg/L PPT selection agent. After another 2–3 weeks when enough roots are formed, transfer them to soil.

8 Mutation Detection

In order to confirm plasmid DNA delivery, genomic DNA can be extracted from pooled putative transformed microspores and individual regenerated plantlets (Fig. 9). Amplify the targeted gene fragments using gene-specific primers. Run all the digested products on a 2% agarose gel in TAE buffer. Purify PCR fragments using a QIAGEN kit and digest with restriction enzymes assuming that if an edit had occurred and NHEJ erroneously repaired the cut, the restriction site should have been destroyed. Gel-extract the undigested band which corresponds to mutant gDNA and clone into a TOPO-Blunt plasmid and sequence for mutation detection.

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Chapter 14

CRISPR-Cas9-Based Genome Editing of Banana

Leena Tripathi, Valentine Otang Ntui, and Jaindra Nath Tripathi

Abstract

Genome editing is an emerging powerful new breeding tool, which can be applied for genetic improvement of banana for important agronomic traits such as resistance to biotic stresses, adaptation to climate change, and high yielding. Banana is an important staple food and cash crop, feeding millions of people in tropical and subtropical countries. Recently, CRISPR-Cas9-based genome editing system has been established for banana in a few laboratories. Here, we describe the procedures for generation of genome-edited events of banana, detection of targeted and potential off-target mutations, and phenotyping for important traits such as disease resistance. This chapter will provide readers strategy for applying CRISPR-Cas9-based genome editing for improvement of banana.

Key words Banana, Genome editing, CRISPR-Cas9, gRNA

1 Introduction

Banana (*Musa* spp.) is one of the world's most important staple food crops cultivated in tropics and subtropics with an annual production of 145 million tons [1]. One-third of its global production is from Africa, with East Africa being the largest banana-growing and banana-consuming region. They are of different types such as dessert, cooking, roasting, and brewing types, providing food to millions of people. Banana production is seriously affected by several factors, particularly biotic and abiotic stresses. Improved varieties of banana with resistance to diseases and pests, tolerance to abiotic stresses, and higher yields can be developed more precisely using new breeding tools like genome editing.

Genome editing technologies using specific nucleases have been developed as effective genetic engineering methods to target and digest DNA at specific locations in the genome of an organism [2]. The most commonly used tools for targeted genome editing in plants are zinc finger nucleases (ZFNs) [3, 4], TAL effector proteins (TALENs) [5–7], and RNA-guided endonucleases (RGENs)

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or CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) [8–10]. These nucleases respond in a target-specific manner and induce a double-strand break (DSB) in the gene sequence [11]. The DSB is repaired either by homologous recombination (HR) or error-prone nonhomologous end-joining (NHEJ) mechanisms, which may lead to mutations disrupting gene function [12].

CRISPR-Cas9 system has emerged as a potent genome editing tool due to its simplicity, design flexibility, and high efficiency and has been successfully applied in many organisms, including several plant species [13]. It is based on the type II CRISPR-Cas immune system in bacteria that protects against invading DNA viruses and/or plasmids. The CRISPR-Cas9 molecular immunity system comprises the Cas9 endonuclease of *Streptococcus pyogenes* and a synthetic single guide RNA (gRNA), which directs the Cas9 endonuclease to a target sequence complementary to the 20 nucleotides preceding the protospacer adjacent motif (PAM), which is required for Cas9 activity [8, 14]. Multiplexing of CRISPR-Cas9 system, by integrating two or more gRNAs, which are a distance apart, is useful in generating large deletions and knocking out multiple genes in plants [15].

Genome editing using CRISPR-Cas9 technology has been recently developed for banana [16–18]. The steps involved in the genome editing of banana [18] are illustrated in Fig. 1. This chapter presents protocols routinely used in the authors' laboratory for genome editing of banana for knocking out the genes and characterization of edited events.

2 Materials

2.1 Reference Banana Genome

All the banana cultivars including plantains are polyploid clones derived from *Musa accuminata* (A genome) or/and *Musa balbisiana* (B genome). The cultivars can be grouped as diploids (AA, BB, AB), triploids (AAA, AAB, ABB), and tetraploids (AAAA, AAAB, AABB, ABBB) based on genomes. The wholegenome sequence of *Musa accuminata* (DH Pahang) and *Musa balbisiana* (Pisang Klutuk Wulung) are publicly available at the Banana Genome Hub database (http://bananagenome-hub.southgreen.fr/) [19]. These genome sequences can be used as reference genome for editing of the potential targets in banana.

2.2 Tools for Designing gRNA

Many tools are available to design gRNA and most of them provide information on the location and efficiency of the gRNA as well as off-target effects. Researchers have their preference for using the tool. Some of the commonly used tools for gRNA design are listed below:

http://zifit.partners.org/ZiFiT/ChoiceMenu.aspx

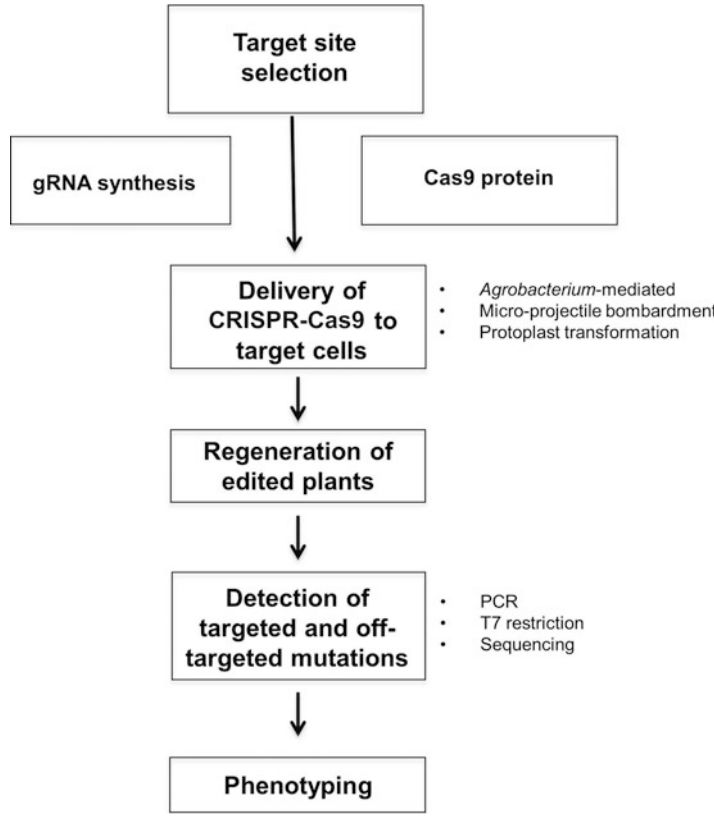

Fig. 1 Illustration of various steps involved in genome editing of banana

https://chopchop.rc.fas.harvard.edu/

http://www.genome-engineering.org/crispr/

http://crispor.tefor.net/

https://benchling.com/crispr

https://www.deskgen.com

Alt-R Custom Cas9 crRNA Design Tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM).

ATUM CRISPR DNA Design Tool (https://www.atum.bio).

In our laboratory, we routinely use ATUM CRISPR DNA Design Tool or Alt-R Custom Cas9 crRNA Design Tool from IDT for editing of banana. These tools provide us with highly efficient gRNAs with minimal off-target effects.

2.3 Plasmid Constructs

For preparing CRISPR-Cas9 constructs for editing of banana, four different plasmid vectors are used for cloning [20]. Each plasmid contains a complete expression cassette for gRNA and is regulated by the OsU6 promoter. The first plasmid is the Golden Gate entry vector. This plasmid also contains tetracycline efflux protein, which

confers resistance to tetracycline and enables the selection of positive clones. The second plasmid is the Golden Gate recipient vector. In this plasmid, the LacZ gene is readily replaced by gRNA expression cassettes via Golden Gate reactions. It contains the Gateway recombination sites to facilitate the integration of the gRNAs and *Cas9* gene cassette into the binary plasmid. It also contains amino glycoside adenylyltransferase, which confers resistance to spectinomycin and streptomycin. Only two gRNAs at a time can be integrated into this plasmid. The third plasmid is the vector containing the *Cas9* gene cassette. This is a Gateway-compatible plasmid. This plasmid contains a plant codon-optimized *Cas9* gene with high GC content and aminoglycoside adenylyltransferase that confers resistance to spectinomycin and streptomycin. The fourth plasmid is the binary vector such as pMDC32, which is also a Gateway-compatible plasmid. It contains *nptII* gene, which confers resistance to kanamycin and enables selection of positive clones. This plasmid also contains a *hpt* selection marker for *in planta* selection.

2.4 Banana Cell Suspension

An ideal explant for delivering CRISPR-Cas9 plasmid constructs to the banana genome is embryogenic cell suspensions. These are single cells with the potential to regenerate into complete plantlets in 7–9 months. However, the generation of embryogenic cell suspensions is a lengthy process, laborious, and cultivar-dependent [21, 22]. Therefore, the availability of embryogenic cell suspensions of different cultivars is very critical. The generation of embryogenic cell suspension of banana has been reported from various explants, especially male flowers and multiple buds [21, 23–27].

Embryogenic cell suspensions of several cultivars such as Gonja Manjaya, Dwarf Cavendish, Sukali Ndiizi, Zebrina, Gross Michel, Agbagba, Orishele, and Obino l'Ewai available at the International Institute of Tropical Agriculture (IITA), Nairobi, Kenya, can be used as starting material for genome editing.

2.5 Banana Protoplast

Protoplast is required for delivering CRISPR-Cas9 ribonucleoprotein (RNPs) for DNA-free editing of banana. The protoplast of banana can be isolated using the protocol developed earlier [28]. The protoplasts are isolated from embryogenic cell suspensions of banana. The complete plantlets can be regenerated from viable protoplast cultured on regeneration medium [29]. The accomplishments with protoplast culture are still limited, and plant regeneration from protoplast is an inefficient technique with a majority of banana cultivars due to lack of fine embryogenic cells.

3 Methods

3.1 Design of gRNA

Once a target gene for editing identified, the gene sequences are downloaded from genome A or/and B, depending upon the genome composition of the cultivar. The sequences are then aligned to identify conserved regions as target sites for editing. Primers specific to the gene, based on the reference genome, are designed, and the gene is re-sequenced from the interested cultivar to get the exact sequences without any changes. Single or multiple gRNAs can be designed for the target site in the gene using the tools listed above.

Multiplexing of gRNA can enhance mutation efficiency and create large mutations if Cas9 cleaves at two target sites simultaneously and also can knock out multiple genes at once [18]. The gRNA tool design several potential gRNAs from which the gRNAs with higher efficiency and having minimal or no potential off-target effect should be selected.

3.2 Plasmid Construct Preparation

In order to clone the gRNA into the expression vector, adaptors are added to the 5′ end of the gRNAs and the corresponding reverse sequences. The gRNA sequences are then synthesized as oligos and used for cloning through Golden Gate and Gateway techniques [18, 20]. The gRNA expression vectors are usually linearized to produce overhang; this facilitates the cloning of gRNAs. Forward and reverse oligos of each gRNA are phosphorylated and annealed and ligated to the gRNA expression vectors using T4 ligase. The ligated product is then sequenced to confirm the insert.

The two gRNAs are then assembled into the Golden Gate recipient and Gateway vector by digestion with the appropriate enzyme(s) depending on the vector and ligation with T_4 DNA ligase. The Golden Gate assembly reaction together with a *Cas9* entry plasmid are inserted into the Gateway binary plasmid. The plasmid is then mobilized to *Agrobacterium tumefaciens* through electroporation [30]. The various steps involved in the cloning of gRNA(s) and *Cas9* gene into the binary plasmid are summarized in Fig. 2.

3.3 Preparation of CRISPR-Cas9 Ribonucleoproteins (RNPs)

Preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) can also be delivered directly into the plant cells [31–34]. These RNA-guided endonucleases (RGENs)-RNPs direct genome editing of the target sites immediately after transfection and are degraded rapidly by endogenous proteases in cells, thus reducing off-target effects and leaving no traces of foreign DNA elements [31, 35].

RNPs can be assembled following the protocol from IDT (www.idtdna.com). Each RNA oligo (crRNA and tracrRNA) is suspended in nuclease-free TE buffer to a final concentration of

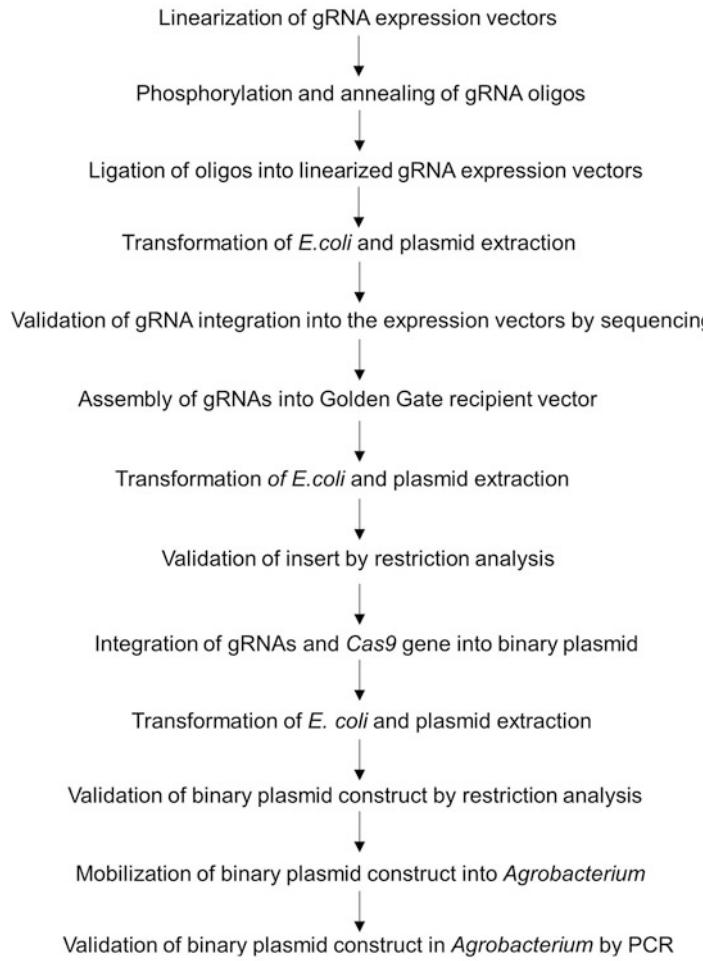

Fig. 2 Flow diagram showing steps for producing the CRIPSR-Cas9 plasmid construct

100 μM. The two RNA oligos are then mixed in a PCR tube to a final duplex concentration of 3 μM (3 μL of each oligo and 94 μL of nuclease-free duplex buffer). Using a thermal cycler, the mixture is heated at 95 ° C for 5 min and cooled to 25 ° C. The Cas9 protein is also diluted to a working concentration of 5 μM in the Cas9 working buffer (20 mM HEPES, 150 mM KCI, 5% glycerol and 1 mM DTT, pH 7.5). To assemble the RNP complex, 1.5 pmol of duplexed RNA oligos are mixed with 1.5 pmol of Cas9 protein in Opti-MEM media to a final volume of 12.5 μL. The mixture is incubated at room temperature for 5 min. The RNP complex can then be delivered to plant cells.

3.4 Delivery of Plasmid Construct into Banana Cells and Generation of Complete Plants

Highly efficient transformation and regeneration system is required for gene editing in banana [36]. The regeneration of plant cells into complete plants after delivering the CRISPR-Cas9 plasmid is the biggest challenge in the application of genome editing in several crops [37]. The CRISPR-Cas9 plasmid construct can be delivered to embryogenic cells of bananas through either *Agrobacterium*-mediated transformation or microprojectile bombardment. Protocols for microprojectile bombardment technique are available for modifying banana [38, 39]. *Agrobacterium*-mediated transformation protocols have also been established for many cultivars of banana and plantains and routinely used in several laboratories to deliver plasmid construct into banana cells [18, 21, 22, 40–43].

Embryogenic cells of banana can be transformed with *Agrobacterium tumefaciens* harboring CRISPR-Cas9 plasmid construct following the protocol described by [18] (Fig. 3). A single colony of *Agrobacterium* containing the CRISPR-Cas9 construct is grown with shaking for 48 h at 200 rpm in liquid LB medium containing 50 mg/L kanamycin and 50 mg/L rifampicin. Two hundred microliters of the liquid culture is refreshed in the same medium and grown for 24 h at the same conditions. The fresh culture is centrifuged at 3,200 × *g* for 10 min, and the pellet is resuspended in 25 mL bacterial suspension medium containing 200 μM of acetosyringone. The bacterial suspension is incubated at room temperature for 2 h at 60 rpm to an OD_{600} of 0.5–0.6. Embryogenic cell suspensions of banana are cocultivated with the *Agrobacterium tumefaciens* for 3 days in the dark at 22 °C in cocultivation media containing 200 μM of acetosyringone. After 3 days, *Agrobacterium*-infected cell suspensions are washed with liquid callus induction medium (MS containing 1 mg/l 2,4-D and 300 mg/L cefotaxime) and transferred to embryo development medium without selection antibiotic for 1 week. After 1 week, the cells are transferred to embryo regeneration medium supplemented with 300 mg/L cefotaxime and 25 mg/L hygromycin for 8 weeks

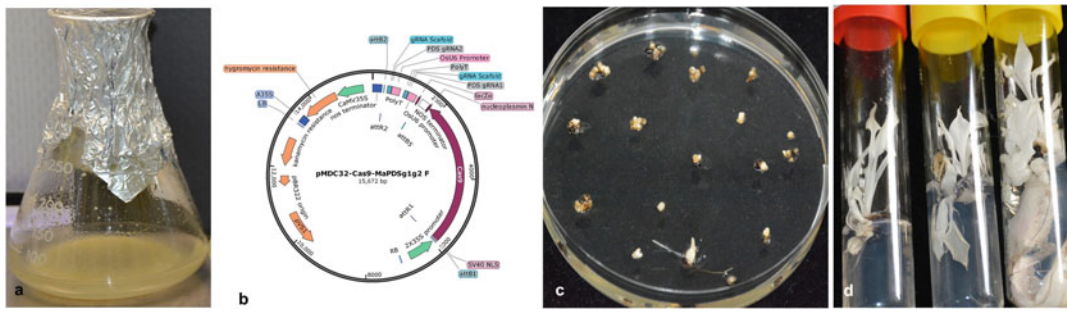

Fig. 3 Generation of genome-edited banana plants through *Agrobacterium*-mediated delivery of CRISPR-Cas9 plasmid. (**a**) Embryogenic cell suspension, (**b**) plasmid construct containing gRNAs targeting *PDS* gene and *Cas9* gene, (**c**) *Agro*-infected embryos regenerating on selective medium, and (**d**) plants with edits in *PDS* gene showing albino phenotype

in the dark. The medium is refreshed every 2 weeks. Developed embryos are then transferred to hormone-free medium for 4–6 weeks in the dark for embryo maturation. Mature embryos are transferred to embryo germination medium and exposed to light at 26–28 °C and a photoperiod of 16 h/8 h (day/night) for 4 weeks. Embryo development medium, maturation medium, and germination medium are supplemented with 300 mg/L cefotaxime and 25 mg/L hygromycin. Finally, regenerated shoots are transferred to the proliferation medium for maintenance and multiplication with subculturing every 4–6 weeks. All media apart from the liquid callus induction medium are solidified with 3 g/L gelrite. Figure 3 describes the generation of genome-edited banana plants targeting the knockout of *phytoene desaturase (PDS)* gene as a marker.

3.5 Delivery of CRISPR-Cas9 Ribonucleoprotein (RNP) Complexes into Banana Protoplast and Regeneration of Complete Plants

Direct delivery of RNPs into plant cells could be achieved by electroporation, particle bombardment, and protoplast transfection by polyethylene glycol (PEG) cell penetrating peptides or mesoporous silica nanoparticle (MSN)-mediated direct protein delivery. Transient expression of a gene has been reported in banana after electroporation or PEG-mediated delivery of DNA into the protoplast [39, 44]. However, the transformation efficiency was quite low.

The PEG-mediated delivery of RNPs into cells is most commonly reported for several crop species. However, there is no protocol reported for the regeneration of complete plantlets after PEG-mediated transfection of banana protoplast. Therefore, the knowledge generated from other crops can be applied for the transfection of RNPs into banana protoplasts and then regeneration of complete plants. The protocol for transfection described here is according to [31, 45]. For the transfection experiment, 5×10^5 of plant protoplasts are resuspended in 200 μL of MMG solution (4 mM MES pH 5.7, 0.4 M mannitol, 15 mM $MgCl_2$). The protoplast suspension is mixed with the 5–20 μL of RNP complex prepared as above. An equal volume (5–20 μL) of freshly prepared PEG solution (40% [w/v] PEG 4000, Sigma no. 95904, 0.2 M mannitol, and 0.1 M $CaCl_2$) is added to the protoplast suspension and mixed gently. The mixture is incubated at room temperature in the dark for 15 min. After incubation, 950 μL of W5 solution (2 mM MES pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl) is added, mixed well by inverting, and centrifuged at 320 × *g* for 3 min. The supernatant is discarded, and the pellet is resuspended gently in 1 mL WI solution (0.5 M mannitol, 20 mM KCl, and 4 mM MES pH 5.7) and incubated in the dark at 25 °C for 24–48 h. After 48 h, protoplasts are collected and regenerated on the regeneration medium to complete plants.

3.6 Molecular Characterization

3.6.1 DNA Extraction

Total DNA is extracted from the leaves of wild-type and putative edited events using modified cetyltrimethylammonium bromide (CTAB). About 100 mg of fresh leaves sample is ground to a fine powder using mortar and pestle. The ground tissue is transferred to 2 mL Eppendorf tubes and 500 μL of extraction buffer (100 mM Tris–HCl, pH 8.0; 20 mM EDTA (pH 8.0); 1.4 M NaCl; 2% PVP-10; 0.8% cetyltrimethylammonium bromide and 0.001% mercaptoethanol) is added. The samples are vortexed vigorously, incubated at 65 °C for 30 min, and then centrifuged for 10 min at 30,000 × *g*. The supernatant is subsequently mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and the mixture centrifuged at 30,000 x *g* for 10 min. After a second chloroform-isoamyl alcohol extraction, the supernatant is mixed with an equal volume of isopropanol and incubated at room temperature for 10 min. Nucleic acids are pelleted by centrifugation as described above. The pellets are washed with 70% ethanol, air-dried for 30 min, and resuspended in 50 μL of TE buffer containing RNase. The samples are stored at −20 °C. The concentration and quality of the DNA is checked with Nanodrop.

3.6.2 PCR Analysis to Confirm Integration of Cas9 Gene

The integration of the *Cas9* gene should be confirmed in the events generated through *Agrobacterium*-mediated transformation using plasmid constructs. PCR analysis is performed with genomic DNA using primers specific to the *Cas9* gene (Fig. 4a). The reaction is set up in a 20 μL reaction volume containing 1 μL genomic DNA (100 ng/μL), 10 μL of HotStarTaq master mix, 1 μL of 10 μM of each primer, and 7 μL nuclease-free water. PCR amplification conditions will depend upon the primers used. After amplification, 10 μL of PCR product is resolved on 1% agarose gel stained with gel red.

3.6.3 PCR Analysis to Detect Band Shift

If the edited events are generated using two gRNAs and Cas9 cuts simultaneously at both target sites, a large fragment gets deleted, which can be detected by resolution of amplicons on agarose gel as band shift (Fig. 4b). Primers are designed flanking the gRNAs using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The targets are amplified using these primers, resolved on 1% agarose gel, stained with gel red, and checked for shorter band in comparison to wild-type non-edited control.

3.6.4 T7 Analysis

T7 endonuclease I (T7E1) assay should be performed to detect the smaller indels (Fig. 4c). The DNA fragments containing the targeted sites are amplified by PCR using a pair of primers flanking the gRNAs and Phusion High-Fidelity DNA Polymerase (NEB). The PCR product is then denatured-annealed at 95 °C for 5 min, ramped down to 25 °C at 0.1 C s^{-1}, and incubated at 25 °C for another 30 min. The annealed PCR products are then digested with

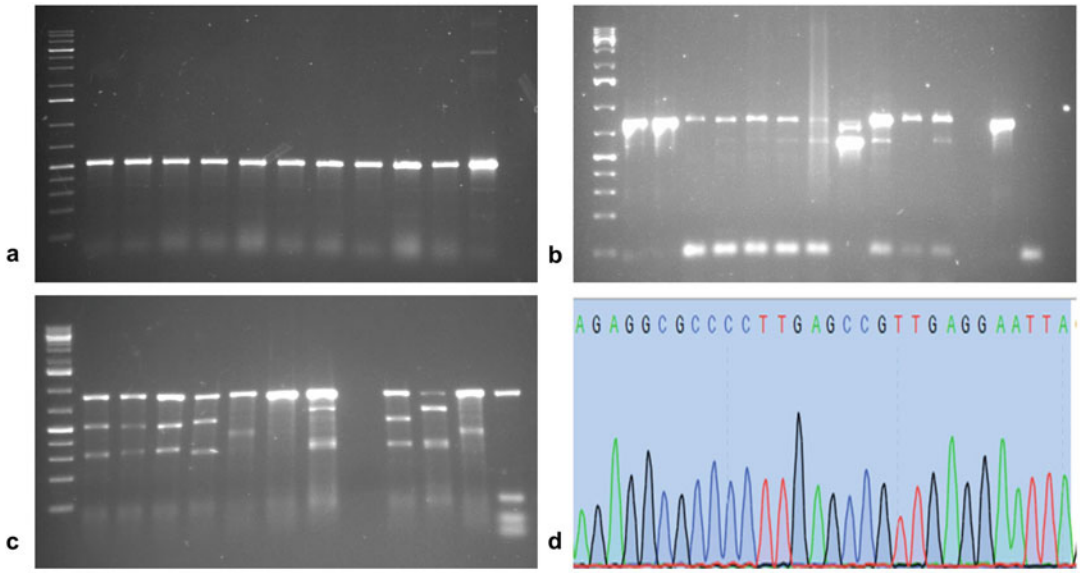

Fig. 4 Molecular characterization of genome-edited events of banana. (**a**) PCR analysis to confirm the presence of *Cas9* gene. (**b**) Detection of band shift in edited events by PCR. This is only possible if Cas9 cleaves the two gRNAs simultaneously in case of multiplexing. (**c**) T7 endonuclease I assay to detect mutations in edited events. (**d**) Sanger sequencing of edited events to detect the mutations

5 U of T7E1 for 2 h at 37 °C. The T7E1-digested products are separated on1% agarose gel stained with gel red.

3.6.5 Sequencing to Detect Targeted Mutations

The targeted mutations in the edited events can be further confirmed by sequencing [18] (Fig. 4d). The PCR-amplified fragment of the target site is purified with QIAquick PCR Purification Kit (Qiagen) according to the instruction manual. The purified products are cloned into *pCR™8/GW/TOPO®* according to the manufacturer's instruction, transformed into DH5α chemical competent *E. coli* cells, and selected on LB plates containing spectinomycin. About ten clones for each event are prepared for Sanger sequencing. The purified PCR products can be directly (without cloning) sequenced by next-generation sequencing. The sequencing reactions are prepared in a 96-well plate and sent for sequencing. The sequencing data are analyzed using software such as SnapGene (WWW.snapgene.com) or vector NTI [46]. The sequences of edited events and wild-type non-edited control are aligned to detect indels and type of mutations (deletions, additions, or/and replacements) at the target site(s).

3.6.6 Evaluation of Off-Target Mutations

There are several tools available for the detection of potential off-target mutations. We use the Cas9/gRNA analysis software Breaking-Cas (http://bioinfogp.cnb.csic.es/tools/breakingcas/) [47] to check for potential off-target sequences [18]. Further,

each gRNA including the PAM sequence is blasted against banana genomes A and B using the BLASTN program in the Banana Genome Hub (http://banana-genome-hub.southgreen.fr/organism/Musa/acuminata-ssp.-burmannica) to identify the genes with potential off-target sites. Once potential off-target sites are identified, if any, the fragment having these sites are amplified using the primers flanking the potential off-target sites. The PCR products are purified and sequenced to confirm the off-target mutations. The sequencing data is then analyzed using SnapGene software, Vector NTI, or any sequence analysis and design tool. From our experience, although the software usually picks potential off-targets, in most cases, such off-targets are not detected by sequencing. This is because we usually select gRNAs with minimal or no potential off-target effects.

3.7 Phenotyping of Genome-Edited Events

Phenotyping of edited events depends upon the target traits. This section describes the methods for phenotyping of genome-edited events developed for resistance to banana streak virus (BSV) and banana Xanthomonas wilt (BXW).

3.7.1 Phenotyping for BSV

BSV is a badnavirus which is integrated into the banana genome and known as endogenous BSV (eBSV). The plants with integrated eBSV do not show any disease symptoms. These proviruses can be activated into the infectious episomal BSV by various stress conditions. When the banana plants are stressed, the eBSV produces a functional episomal viral genome and infectious viral particles, and as a result, the plant develops disease symptoms. Environmental conditions such as water stress triggers its activation resulting to infection. The genome-edited banana plants were developed by knocking out the viral genome of eBSV integrated into the host genome [48]. To phenotype for this disease, edited plants confirmed by sequencing are acclimatized and grown in pots in the greenhouse. About 4-month-old potted plants are subjected to water stress for 2 weeks to induce BSV symptoms [48]. At the end of the stress period, the plants are assessed by visual observation for the presence or absence of BSV symptoms, which appeared in the form of broken or continuous streaks on the leaf lamina and vary from yellow to chlorotic, black, or brown color. The wild-type non-edited control plants are included in all the experiments. The BSV symptoms are further confirmed by molecular diagnostics to confirm the presence of a virus [48].

3.7.2 Phenotyping for BXW

The edited events developed with the aim to develop resistance to BXW caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) can be phenotyped using in vitro plantlets under laboratory conditions or potted plants in glasshouse [49, 50]. Bacterial culture is grown for 48 h in YTS media composition (1% yeast extract, 1% tryptone, 1% sucrose). The culture is then centrifuged for 5 min

at 3,560 × *g*. The supernatant is discarded, and the pellets are dissolved in sterile water to $OD_{600} = 1.0$. For rapid assay using in vitro plantlets, 100 μL of the fresh bacterial culture is injected in the pseudo stem of 4-week-old well-rooted plantlets using an insulin syringe. Inoculated plantlets are incubated at 26 ± 2 °C in the growth room and checked for the presence of symptoms as yellowing, necrosis, and complete wilting of plants up to 6 weeks [49].

For glasshouse screening, regenerated plants are weaned in a disposable cup containing sterile soil and incubated in the humid chamber for 6 weeks and transferred to pots. About 100 μL of the fresh bacterial culture is injected in the first fully opened leaf of 3-month-old plants. The plants are assessed for 8 weeks for the development of disease symptoms of chlorosis or necrosis of the leaves and final symptoms of complete wilting of the plants [50] with preliminary symptoms. The relative resistance of transgenic plants to BXW is evaluated 8 weeks after inoculation based on the reduction in wilting in comparison with control non-edited plants.

The wild-type control plants are included in all the experiments.

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Chapter 15

Homology-Directed Transgene-Free Gene Editing in *Chlamydomonas reinhardtii*

Aron Ferenczi and Attila Molnar

Abstract

Chlamydomonas reinhardtii is a microalgal model organism with a suite of molecular and genetic techniques, but routine editing of its nuclear genome is yet to be realized. DNA-based transformation techniques are prohibitively inefficient and lead to predominantly nonhomologous (i.e. off-target) integration. Standard CRISPR-based gene editing protocols have proved too ineffective to enable routine application. We have found that the use of CRISPR-Cpf1 in conjunction with single-stranded DNA (ssODN) repair templates achieves nuclear gene editing efficiencies as high as 20% as a proportion of total cells (Ferenczi et al. Proc Natl Acad Sci U S A 114:13567–13572, 2017). This produces edits with predictable outcomes in a transgene- and selection marker-free manner. The possibility to purchase all necessary reagents commercially with no preparation time (besides design) facilitates rapid and routine genetic engineering in this organism. Here we describe the use of this technique to knockout locus *FKB12*, which leads to rapamycin resistance and lends itself to an easy assay when adopting this gene-editing protocol.

Key words CRISPR, Cpf1, *Chlamydomonas reinhardtii*, ssODN, Homology-directed repair (HDR)

1 Introduction

Chlamydomonas reinhardtii is the most thoroughly studied microalga [1]. It has been quintessential to the fields of photosynthesis, cilia biology, lipid metabolism, carotenoid biosynthesis, and more [1]. It's acutely tractable owing to a suite of well-developed molecular and genetic techniques. Remarkably, however, gene editing of its nuclear DNA is yet to become routine practice.

Nuclear gene targeting in *C. reinhardtii* has historically been precluded by inefficient DNA-mediated transformation with predominantly nonhomologous (off-target) outcomes [2–4]. The emergence of gene editing enzymes like Cas9 now enables circumventing these attributes of *C. reinhardtii*. Critically, however, transgenic expression of gene editing enzymes is still subject to low transformation efficiency, pervasive transgene fragmentation [4] and transgene silencing [5, 6], which necessitates the use of positive

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selection markers [7–9]. This approach hinders successive rounds of edits being performed in a single strain and involves cloning for targeting each new locus. An alternative strategy, which bypasses transgenesis, is to deliver Cas9 protein as ribonucleoproteins (RNPs). Prior endeavours delivering Cas9 RNPs have achieved imprecise edits with poor gene editing efficiencies even when coupled with selection marker-assisted gene disruption (<1%), unconducive to routine editing applications [9–12].

Our gene editing technique for *C. reinhardtii* combines three key advantages: (1) it uses a homologous repair template to achieve precise predictable edits, (2) it is transgene- and selection marker-free, and (3) it is potentially efficient enough to enable mutant detection simply by colony PCR. The technique relies on inducing targeted, double-stranded breaks in the nuclear DNA using a CRISPR endonuclease called Cpf1 (or Cas12a) [13] and the use of single-stranded DNAs (ssDNAs or ssODNs) as repair templates to guide DNA repair and achieve precise, predictable gene edits. The increased gene editing efficiencies conferred through the use of the ssODNs enables a selection marker-free approach, whereby mutants can be identified by colony PCR [14]. Cpf1 is delivered as a protein, bypassing transgenic expression and cloning, making the technique non-transgenic.

Here we describe knocking out of gene *FKB12*, which leads to rapamycin resistance [15]. Editing of *FKB12* can, therefore, be readily assayed by plating cells on media supplemented with rapamycin [14]. We believe that this simple positive selection system will facilitate adoption of the technique both in CC-1883 and other strains. An overview of the method is provided in Fig. 1a.

2 Materials

Use deionized H_2O where H_2O is required. All solutions are autoclaved unless indicated otherwise.

2.1 TAP Medium

The following recipe has been accessed through the *Chlamydomonas* Resource Center website (https://www.chlamycollection.org/) in January of 2015, where it was referenced to the following publications [16, 17]. The current recipe on this website is for a slightly more dilute version of TAP, but we believe this to be interchangeable with the recipe described here.

1. Salts solution: 15 g NH_4Cl, 4 g $MgSO_4.7H_2O$, 2 g $CaCl_2{\cdot}2H_2O$, and water up to 1 L.
2. Phosphate solution: 5.76 g K_2HPO_4, 2.88 g KH_2PO_4, and water to 20 mL.

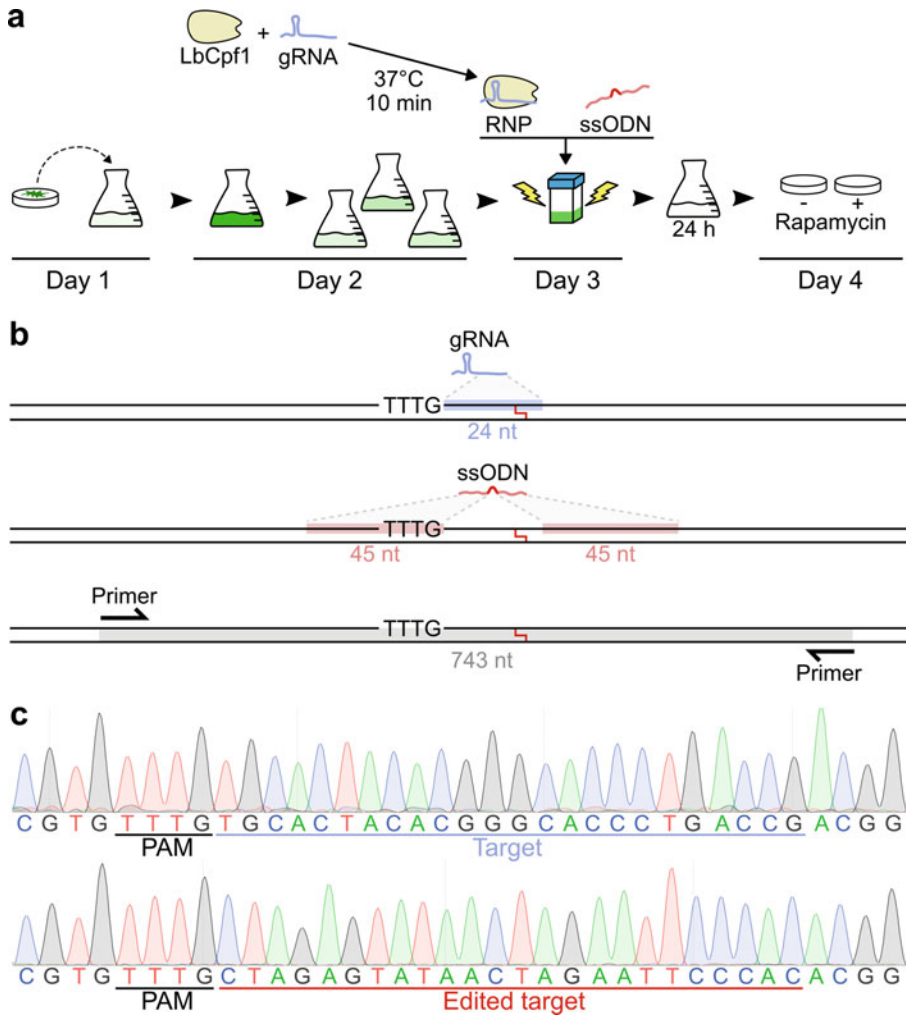

Fig. 1 (**a**) Schematic overview of gene editing protocol up to the point of cell plating. (**b**) Schematic overview of the design of the gRNA (top panel), ssODN (middle panel), and primers (bottom panel) for editing and analysing *FKB12*. The central bulge of the ssODN (the 'replacement sequence', dark red) represents non-homology with the target site (black). Cpf1-mediated, double-stranded DNA cleavage is indicated with a dark red line inside the 24 nt gRNA target. (**c**) Sanger chromatograms of the wild-type (top) and edited (bottom) *FKB12* target site. The diagram is adapted from SnapGene Viewer

3. Hutner's trace elements: Prepare the following solutions using H_2O (final volumes in brackets): 5.5 g $ZnSO_4 \cdot 7H_2O$ (25 mL), 2.85 g H_3BO_3 (50 mL), 1.265 g $MnCl_2 \cdot 4H_2O$ (12.5 mL), 0.403 g $CoCl_2 \cdot 6H_2O$ (12.5 mL), 0.393 g $CuSO_4 \cdot 5H_2O$ (12.5 mL), 0.275 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (12.5 mL), 1.25 g $FeSO_4 \cdot 7H_2O$ (12.5 mL), and 12.5 g disodium EDTA (62.5 mL). Mix all solutions except EDTA, bring to boil, and then add EDTA. The mixture should turn from an orange-brown colour to blue-green. Cool to 70 °C, and then add

21.25 mL KOH (20% w/v). Cool to room temperature and bring volume to 250 mL with H_2O. Oxidize for 1–2 weeks by bubbling with air or orbital shaking. During this process, the solution will turn dark purple and form a brown precipitate. Filter through Whatman #1 filter paper until the solution is clear of this precipitate. Do not autoclave. Aliquot and store at −20 °C. Keep a working stock at 4 °C (*see* **Note 1**).

4. Tris (tris(hydroxymethyl)aminomethane).
5. Glacial acetic acid.
6. Deionized H_2O.
7. TAP medium (per L): 2.42 g Tris, 25 mL salts solution, 0.375 mL phosphate solution, 1 mL Hutner's trace elements, 1 mL glacial acetic acid; the pH should be around 7.0–7.5 (*see* **Note 2**).

2.2 Chlamydomonas reinhardtii *Cultures*

1. *C. reinhardtii* strain CC-1883 (*Chlamydomonas* Resource Center) (*see* **Note 3**).
2. Incubation facilities with 100 μmol m^{-2} s^{-1} light and 28–30 °C (these are temperatures measured under the light where the cells will be incubated).
3. Orbital shaker.
4. Laminar flow hood.
5. TAP media: 5 × 10 mL aliquots (this includes 1 spare) in 100 mL Erlenmeyer flasks sealed with foam bungs, covered with aluminium foil, and then autoclaved (*see* **Notes 4** and **5**).
6. TAP plates: TAP with 1.5% agar (*see* **Note 6**), autoclaved and poured into 90 mm Petri dishes sealed with parafilm. Prepare in batches of 10 or 20.
7. Cell stain: 0.25 g iodine in 100 mL absolute ethanol. Do not autoclave. Alternative stains are described in [18].
8. Filtered pipette tips (1 mL).
9. Haematocytometer.
10. Light microscope with 200× total magnification.
11. Handheld tally counter.
12. Optional: inoculation loops.

2.3 C. reinhardtii *Electroporation*

1. Cpf1 (Cas12a) from *Lachnospiraceae* bacterium ND2006 (LbCpf1). LbCpf1 can be purchased from NEB [EnGen® Lba Cas12a (Cpf1)] or purified [14, 19, 20] (*see* **Note 7**).
2. gRNA: UAAUUUCUACUAAGUGUAGAUUGCACUACACGGGCACCCUGACCG (the target-specific sequence is underlined). Dissolve the gRNA using DEPC-treated, nuclease-free H_2O (Table 1) and aliquot in batches enough for

Table 1
Reagent stock concentrations, quantities, and volumes required for an electroporation reaction

	Molecular weight (kDa)	Concentration (mg/mL)	Concentration (μM)	Amount (nmol)	Volume (μL)
LbCpf1	145	20	138	0.262	1.90
gRNA	14.3	10	698	0.786	1.13
ssODN	35	35	1000	2.62	2.62
Cells	–	–	–	–	125
Sucrose (2 M)	–	–	2000	–	2.68
Total volume	–	–	–	–	133

Note that the final sucrose concentration should be 40 mM; sucrose volumes will need to be adjusted if the reagents concentrations deviate from those specified in the table

10–20 electroporation reactions; store at −80 °C (*see* **Notes 8** and **9**).

3. Single-stranded oligodeoxynucleotide (ssODN): GGTATACAGGCGTGAGCTTCCCCAAGACTGGCCAGACCGTG**TTTGCTAG**AGTA**TAACTAG**AATTCCCACACGGCAAGAAGTTCGACAGCTCCCGCGACCGTGGCGAGCCCTTCT (the PAM is bold, the 24 nt sequence replacing the target site is underlined and within it, the stop codons are bold) (*see* **Note 10**) (Fig. 1b). This can be ordered from commercial suppliers like Sigma-Aldrich or IDT. Note that the concentration of the ssODN should be 1 mM (Table 1).
4. 2 M sterile sucrose solution (*see* **Note 11**).
5. TAP media with 40 mM sucrose: 5 mL TAP, 0.1 mL sucrose (2 M), prepare in a 25 mL Erlenmeyer flask sealed with a foam bung, covered with aluminium foil, and then autoclaved.
6. Bio-Rad Gene Pulser Xcell with PC module.
7. 4 mm electroporation cuvettes (we use CELL PROJECTS Ltd., BR-204).
8. Filtered pipette tips (1 mL, 200 μL, 10 μL).
9. Parafilm.

2.4 Cell Plating

1. One plate of TAP agar and one of TAP agar supplemented with 10 μM rapamycin (*see* **Notes 12** and **13**).
2. Optional: sterile Duran bottle (*see* **Note 13**).
3. 50 mL TAP.
4. Starch (corn/potato).

5. Sterile Eppendorf tubes (*see* **Note 14**).
6. Absolute ethanol.
7. 100 mL sterile H_2O.

2.5 Colony PCR

1. Thermal Cycler.
2. Phire Plant Direct PCR Kit (Thermo Fisher).
3. PCR primers to amplify the *FKB12* target; forward: ATGGGTGTCGACGTCGCGACTA; reverse: GAAGTCGTGGCTGATGGTCAG.
4. QIAGEN MinElute PCR Purification Kit (or any alternative PCR purification kit).
5. BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher).
6. Agarose gel running equipment.

3 Methods

All steps are performed at room temperature. Work with cells is performed aseptically under a laminar flow hood (*see* **Note 15**). *C. reinhardtii* is cultured under 70–100 μmol m^{-2} s^{-1} constant white light at 28–30 °C (*see* **Note 16**). Liquid cultures are shaken at 100–130 rpm (Stuart SSL1 Orbital Shaker).

3.1 Chlamydomonas reinhardtii *Culture*

1. Streak out CC-1883 onto a TAP plate to obtain a freshly growing culture.
2. After growth is observed (typically 3 days), inoculate a 10 mL TAP aliquot using a 1 mL pipette tip or inoculation loop (*see* **Note 17**).
3. The next day, measure the cell density using a haematocytometer. To do this, aliquot 3 × 20 μL cell stain into Eppendorf or PCR tubes. Add 200 μL of culture to each stain aliquot, ensuring the culture is well mixed by swirling prior to taking each sample. A standard haematocytometer has a central 5 × 5 square with a volume of 0.1 mm^3, with each square subdivided into smaller 4 × 4 squares. Count the cells in the 5 × 5 portion for each sample, average the counts (removing outliers), multiply by 1.1 to account for the dilution caused by the stain (*see* **Note 18**), and multiply by 10^4 to obtain the density in cells/mL (e.g. a cell count of 100 equates to a 1.1×10^6 cells/mL).
4. Calculate the dilution required to obtain a density of 2×10^6 cells/mL by the next day. Assume a generation time of 8 h and the division of each cell into two daughter cells (*see* **Note 19**). Proceed to make this dilution by subculturing into a

new 10 mL TAP aliquot. Make two further dilutions, one less and one more dilute to ensure that at least one culture will be near target density the next day (*see* **Note 20**). It is optional to check for contamination prior to making the dilutions (*see* **Note 21**).

3.2 Electroporation

This procedure takes typically ~2 h.

1. Set up the laminar flow hood.
2. Start defrosting the Cpf1, gRNA, and ssODN on ice.
3. Count the cell densities of the three overnight cultures using a haematocytometer as described above (*see* **Note 22**)**.** Pick the culture that is closest to 2×10^6 cells/mL (*see* **Note 23**). It is optional to check for contamination prior to proceeding with this culture (*see* **Note 21**).
4. Prepare 2.5×10^5 cells per electroporation reaction in a volume of 125 μL (this equates to a density of 2×10^6 cells/mL). This may involve diluting or concentrating the culture. To dilute, add 2.5×10^5 cells into the electroporation cuvette and supplement with fresh TAP (*see* **Note 24**). To concentrate, aliquot 2.5×10^5 cells into a sterile Eppendorf tube (*see* **Note 14**), centrifuge (2 g, 15 min), remove and discard the surplus volume from the top, and then gently resuspend the cells in the remaining 125 μL by pipetting.
5. In the laminar flow hood, add 2.68 μL of sucrose (2 M) to the bottom of an electroporation cuvette (Table 1). Note that the volume of sucrose will need to be adjusted if the reagent concentrations deviate from those specified in Table 1 (*see* **Note 25**).
6. Add the cells to the cuvette and mix by flicking the cuvette.
7. In an Eppendorf or PCR tube, mix the 1.9 μL Cpf1 (138 μM) and 1.13 μL gRNA (698 μM) (Table 1) (*see* **Note 26**). To catalyse the formation of ribonucleoproteins (RNPs), incubate for 10 min at 37 °C (*see* **Note 27**).
8. Add 2.62 μL ssODN (1 mM) to the RNPs, and then add this to the electroporation cuvette and mix by flicking (*see* **Note 28**).
9. Electroporate using the following exponential decay pulse ('Exponential protocol') settings: 600 V, 50 μF, and 200 Ω, 4 mm. Flick the cuvette prior to electroporation to ensure mixture homogeneity (*see* **Note 29**).
10. Using the contents of a fresh 5 mL aliquot of TAP-sucrose, add 800 μL TAP-sucrose into the cuvette immediately after electroporation (*see* **Note 30**).

11. Transfer the cuvette's contents into the TAP-sucrose aliquot that was used in the previous step (*see* **Note 31**). Recover the culture for 24 h by shaking on an orbital shaker under light.

3.3 Plating

Depending on the number of cultures that require plating, this step typically takes 2–4 h.

1. Prepare fresh, sterile 30% (w/v) starch solution for plating (*see* **Note 32**). This step takes 15–20 min. Each plate requires 0.5 mL to be spread; prepare volumes accordingly (*see* **Note 33**). Weigh the starch in Eppendorf or 15 mL Falcon tubes. Wash the starch twice with absolute ethanol, three times with sterile H_2O, and once with TAP and finally resuspend in TAP (use the 50 mL TAP aliquot for this purpose). Washing consists of vortexing, centrifuging (maximum speed, 1 min), and then decanting (*see* **Note 34**). After decanting the first ethanol wash, only open the starch solution in a laminar flow hood.
2. To spread one electroporated culture onto two plates, aliquot 1 mL starch solution into a sterile Eppendorf tube (*see* **Note 14**). To this, add 50 μL of overnight-recovered culture (*see* **Note 35**). This will result in approximately 200–800 colonies on the TAP plate (*see* **Note 36**).
3. Mix the starch-cell solution by pipetting up and down several times using a 1 mL pipette (*see* **Note 37**). Spread the 1 mL volume as 2 × 450 μL onto a TAP and TAP-rapamycin plate (*see* **Notes 38** and **39**).
4. Dry the plates with the lids partially removed for 15–30 min, and then seal with parafilm (*see* **Note 40**).
5. Grow the plates under low light (5–15 μmol m^{-2} s^{-1}) to prevent the photodegradation of rapamycin (*see* **Note 41**). Colonies typically appear in 4 days, but we always wait 7 days to analyse colony counts and perform colony PCR.

3.4 Calculating Rapamycin Resistance

Determine the efficiency of gene editing by dividing the number of cells on the TAP-rapamycin plate by the number of cells on the TAP plate (*see* **Note 42**). We typically obtain 25–30% rapamycin-resistant cells.

3.5 Colony PCR

1. Aliquot 10 μL of the Phire Plant Direct PCR Kit's dilution buffer into PCR tubes.
2. Pick cells with a 10 μL pipette tip, touch onto the surface of a new TAP plate, and place into the dilution buffer aliquot (*see* **Notes 43** and **44**). Before removing the tip from the dilution buffer, mix well to ensure the cells have transferred into it.
3. Centrifuge the PCR tubes for a few seconds and add 0.5–1 μL of the supernatant into a 10 μL Phire PCR reaction as per

manufacturer's instructions. Cycling conditions: 98 °C, 5 min > (98 °C, 5 s > 67 °C, 5 °C > 72 °C, 10s) × 35 > 72 °C, 2 min (primers under Materials) (*see* **Note 45**).

4. Separate 5 μL of the PCR product on a 1.5% agarose gel to confirm specific PCR amplification.
5. Clean the remaining 5 μL PCR product using the QIAGEN MinElute PCR Purification Kit as per manufacturer's instructions.
6. Sequence the cleaned PCR product using the BigDye Terminator v3.1 Cycle Sequencing Kit as per manufacturer's instructions using the forward primer.
7. Send sequenced reactions for capillary analysis at your local sequencing facility (Fig. 1c).

4 Notes

1. Aliquot stocks in batches of 50 mL and store 5 mL refrigerated as the working stock.
2. We do not routinely check for or correct the pH.
3. This is a cell wall-deficient strain with the *cw15* genotype. As such, it requires starch plating as described in the Methods.
4. The aluminium foil serves to keep the neck of the flask sterile; therefore, ensure it sufficiently covers it.
5. The purpose is to have single-use sterile TAP aliquots to minimize the potential for contamination. We have previously autoclaved several hundred millilitres of liquid TAP and aliquoted it into sterile Erlenmeyer flasks in a laminar flow hood as it was required but found it difficult to determine contamination prior to starting experiments. We prepare TAP aliquots in batches of 20 and store them at 4 °C.
6. Anecdotal evidence suggests that *C. reinhardtii* can be sensitive to the quality of the agar. We use Duchefa Phyto agar P1003.1000 sold by Melford.
7. We purify LbCpf1 from *E. coli* as described in [14]. We have not described its purification here as we felt it is a separate technique covered in numerous other publications [14, 19, 20].
8. Notes on gRNA design: First, identify a protospacer-adjacent motif (PAM) of TTTV [13, 21] within the gene's coding sequence (CDS) and as close as possible to the translation start site. The 24-nucleotide sequence downstream of the PAM is the gRNA target site (Fig. 1b). The LbCpf1-induced double-stranded DNA cut occurs 18–22 nt downstream of the

PAM [13]. Ensure this cut is within the CDS. Check for potential (homologous) off-targets in the *C. reinhardtii* genome using BLAST [22]. It is best to avoid targets with any hits, but if one has no alternative target site, consider what is known about Cpf1's gRNA mismatch tolerances to evaluate the risk of these off-targets being targeted [13, 21, 23]. Finally, to turn the target sequence into a full-length gRNA, replace all occurrences of T with U, and then concatenate it downstream of the LbCpf1 gRNA repeat sequence (UAAUUUCUACUAAGUGUAGAU). The end result is a 45-nt gRNA. If disruption of a gene's coding sequence (CDS) is desired and there is a lack of PAMs in the CDS, consider intronic PAM sites with the cut site falling within the exon. In addition, consider the design flexibility offered through the ssODN design, whereby cut sites just outside of exons can still be useful to achieve disruption of the CDS. In such cases where ssODNs will overlap intron-exon boundaries, take care to preserve the splice junction motif (*see* **Note 10**).

9. We order gRNAs from Sigma-Aldrich and Synthego, but they can also be generated through in vitro transcription (IVT) [14]. In our experience, as a function of the time input requirements and yields, IVT costs considerably more than purchasing gRNAs. In vitro transcription using our method [14] takes 3 days, could be performed in a maximum of 200 μL volumes (bottlenecked by the step of polyacrylamide gel purification), and yielded 50–100 μg (~3–7 nmol) per 100 μL reaction. We recommend purchasing *FKB12*-targetting gRNA in order to hone the editing assay described here.

10. Notes on ssODN design. The ssODNs consist of 45 nt homology arms that are directly upstream and downstream of the target site (the upstream arm includes the PAM) and a central 24 nt sequence replacing the target site ('replacement sequence') (Fig. 1b). For the replacement sequence, we modified a 24 nt eGFP sequence to include stop codons in every reading frame as well as an *Eco*RI site. We add stop codons in every frame to capture editing events that have additional insertions or deletions [14]. It is not necessary for the replacement sequence to match the target site in size, though we design all ssODNs this way. What is key is that the replacement sequence contains stop codons. For universal applications, we have designed a 12 nt sequence that contains stop codons in each reading frame in both directions: **CTAATTAACTAA** (sense and antisense stop codons are underlined and bold, respectively). ssODN design flexibility also allows the use of gRNAs where the cut sites are in intronic sequences. In this case, position the replacement sequence appropriately to place

the stop codons within the gene's CDS, taking care to preserve the splice junction motif (usually a few nucleotides long).

11. During filter sterilization, we aliquot 2 M sucrose solution in batches of a few hundred microlitres. These are subsequently opened only under sterile conditions in a laminar flow hood.
12. We typically prepare TAP-rapamycin plates on the day that they are required (the day of cell plating). Ahead of time, we prepare and autoclave the volume of TAP agar that will be required for the TAP-rapamycin plates (20 mL/plate) and use this to prepare the TAP-rapamycin on the day of cell plating. Alternatively, we prepare TAP-rapamycin plates no more than 1 week before use in order to prevent the degradation of rapamycin. In this case, store TAP-rapamycin at 4 °C and in the dark to prevent rapamycin's photodegradation.
13. With certain batches of rapamycin, we experience precipitation when adding the rapamycin stock (10 mM, in DMSO) directly into the sterile, molten TAP agar. This can be mitigated by adding rapamycin into a sterile Duran bottle and diluting it by adding lukewarm, sterile TAP agar (30–50 °C) in increments with swirling to mix in between each addition. Even this way, very fine grains of rapamycin precipitate can form, which may lead to a speckled pattern of condensation on the media surface as it solidifies. In our experience, this does not affect rapamycin-mediated counter-selection of wild-type cells. Note that this requires a separate sterile Duran bottle.
14. We autoclave sealed containers of Eppendorf tubes that are only opened under the flow hood for sterile applications. This minimizes the potential for contamination.
15. Contamination while working with *C. reinhardtii* can ruin experiments. To minimize the chances of contamination, observe sterile technique, including wiping all surfaces of the laminar flow hood with 70% ethanol, letting the hood run for 30 min prior to starting work inside it, spraying all items that enter the hood with 70% ethanol (and each time with items that enter it multiple times), using filtered pipette tips, and spraying gloves and lab coat sleeves with 70% ethanol. In addition, it is recommended to keep a waste container for pipette tips inside the flow hood rather than outside of it.
16. Stocks may be maintained under lower light (e.g. 10–30 μmol m^{-2} s^{-1}) to limit growth and thereby minimize the re-streaking frequency.
17. The culture should be pale green after inoculation for densities to be 1×10^5 to 1×10^6 cells/mL by the next day, which is desirable for the purposes of cell counting.

18. Settling of stained cells occurs in minutes. Adequate mixing of the stained cells is, therefore, paramount for accurate cell counts. Before loading each sample onto a haematocytometer, pipette up and down 5–10 times using a volume of 100–200 μL to mix.
19. These values work well in our lab under the culture conditions described here. Lab-to-lab variation may require these values to be adjusted and can be easily determined from one overnight culture where densities 24 h or 48 h apart are known.
20. For the additional subcultures, we subculture volumes that are half and double the calculated volume; e.g. if the calculated dilution requires adding 2 mL culture into 10 mL fresh TAP, make the two subcultures using 1 mL and 4 mL of culture.
21. Check for contamination by centrifuging 1 mL of culture (17 k g, 1 min). A white surface layer on top of the green algal pellet indicates bacterial contamination.
22. Set up triplicate stains for all three cultures (a total of nine samples) and count one sample of each culture. Of the culture that is closest to the target density, proceed to count the other two samples. Future experience with culture colours will help guide this process to minimize cell counting.
23. We typically use cultures between 1×10^6 and 3×10^6 cells/mL.
24. Use a spare 10 mL aliquot of TAP for this purpose. It is recommended to discard this aliquot of TAP after use, though an experienced experimenter may choose to do otherwise.
25. At this point, loosening but not removing the cuvette lid allows for easy handling during subsequent steps.
26. We do not perform this step in a laminar flow hood, nor do we use dedicated sterile Eppendorf tubes as both the Cpf1and gRNA are non-sterile solutions, though we very rarely experience contamination of the subsequently plated cells.
27. When using desalt-purified gRNAs from Sigma-Aldrich, we sometimes observe precipitation during incubation. We have anecdotal evidence that precipitation is eliminated by PAGE purification of the gRNAs. However, we have not noticed this precipitation to be detrimental to the efficacy of editing but is nevertheless not desirable.
28. On occasion, the ssODN suspension is sticky and difficult to pipette. This is the result of the ssODN being insufficiently dissolved. To remedy this, heat the ssODN at 65 °C for 2 min, vortex, and cool on ice prior to adding to the RNPs. If this does not completely dissolve the ssODN, try incubating for longer or at a higher temperature.

29. After the electroporation is completed, the electroporator will display two values: the time constant and delivered voltage. These are typically around 6.5 ms and 585 V, respectively.
30. We believe the immediate addition of TAP-sucrose after electroporation increases cell survival rate post-electroporation but have not designed experiments to test this.
31. If using a 1 mL pipette tip, a good technique is to pipette from the side of the electroporation cuvette and to simultaneously tilt the cuvette as the pipette's plunger is being depressed. Surface tension will draw out all of the liquid from the cuvette.
32. Using starch is vital for improved and consistent plating efficiencies of cell wall-deficient (*cw*) strains of *C. reinhardtii* such as CC-1883 [24]. Without it, cells struggle to form colonies and colony counts can be confounded by conditions that affect colony formation (e.g. agar moisture content).
33. It is better to prepare 1 mL of starch solution per plate since this step is inexpensive, yet time-consuming. Occasional mistakes in later steps will benefit from a surplus of the starch solution having been prepared.
34. Some loss of starch and hence a white hue of the decanted supernatants is normal.
35. The cell culture is colourless at this point.
36. Initially, it is recommended to spread additional plates with more and/or fewer cells (e.g. quarter, half, double, quadruple volumes of cells) to account for lab-to-lab variation in colony numbers. For example, we have noticed the amount and composition of the Cpf1's storage buffer to affect cell survival during electroporation (perhaps exerting some osmoprotective effect), which in turn affects colony numbers.
37. This pipetting is vital to ensure the cells are evenly distributed on the two plates.
38. When spreading the cells, leave a 0.5 cm margin along the edge of the plate. This serves two functions. First, in case of condensation inside the plate during the subsequent incubation period, it prevents cells around the edge from being washed together as the condensate typically swirls around the edges of the plate. Second, if counting cells using a software like Open CFU [25] or ImageJ, labelling around the edge of the plate will not overlap with the colonies.
39. The starch solution spreads easier if the agar medium is moist. Starch will stick to overdry plates, making it difficult to spread evenly. It is, therefore, beneficial to dry plates that will be used for spreading cells for a minimum amount of time after pouring.

40. Dryness of the starch solution is seen by the glossy (wet) surface turning matt.
41. We place plates (upside down) under 70–100 μmol m^{-2} s^{-1} and overlay them with 3–6 layers of white tissue roll as described by Shimogawara et al. [24]. A superior method would be to use neutral density filters.
42. We photograph the plates (without opening them, plates being upside down) and count cells using the free Open CFU software [25]. The software has a function to draw a mask to exclude false positives. This is under 'ROIs and Mask' > 'Draw Mask'. If processing many plate images, it helps to take plate images in a fixed position using a stand. This way, the mask does not have to be redrawn for each image.
43. Using 0.5% agar TAP speeds up colony growth of CC-1883, presumably due to its cell wall-deficient phenotype (*cw15*).
44. Our dilution buffer tends to be colourless to pale green after the addition of cells. We occasionally notice inconsistencies in PCR amplification and have suspected cell concentration to be an issue. Despite this, we have not noticed a correlation between the green colouration of the dilution buffer and PCR success.
45. Where the mutant colony phenotype is unknown, we recommend pooling colonies and performing PCR with a mutation-specific primer. To do this, after placing picked cells into 10 μL dilution buffer, centrifuge and then use 1 μL of each to create pools of 8 samples. Add 1 μL of the pooled sample into a 10 μL PCR reaction. In pools where there is specific PCR amplification, screen the individual samples. After identifying mutants, repeat the PCR using primers that are suitable for sequencing.

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Correction to: A Procedure to Design Guide RNA, Assemble Fragments, and Detect Mutation for Genome Editing in Flax

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Glossary

Adult stem cell—An undifferentiated cell found in a differentiated tissue in an adult organism that can renew itself and can differentiate to yield specialized cell types of the tissue in which it is found.

Allele—One of several possible versions of a gene. Each one contains a distinct variation in its DNA sequence. For example, a "deleterious allele" is a form of a gene that leads to disease.

Aneuploidy—The presence of an abnormal number of chromosomes in a cell. The aneuploid set differs from the normal set by only one or a small number of chromosomes.

Assisted reproductive technology (ART)—A fertility treatment or procedure that involves laboratory handling of gametes (eggs and sperm) or embryos. Examples of ART include in vitro fertilization and intracytoplasmic sperm injection.

Archaea—Microbes that look similar to bacteria but are actually more closely related to eukaryotes, such as humans. Archaea are single-celled organisms that don't have a nucleus and can only be seen with a microscope. The archaea are found in diverse habitats including in extreme environments.

Autologous transplant—Transplanted tissue derived from the intended recipient of the transplant. Such a transplant helps to avoid complications of immune rejection.

Bacteria—Bacteria are single-celled organisms that are invisible to the naked eye, don't have a nucleus, and can have many shapes. They're found in all types of environments, from Arctic soil to inside the human body. Most bacteria are mostly beneficial to human health and other living hosts but certain pathogenic bacteria can cause illness.

Base—The four "letters" of the genetic code (A, C, T, and G) are chemical groups called bases or nucleobases. A = adenine, C = cytosine, T = thymine, and G = guanine. Instead of thymine, RNA contains a base called uracil (U).

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Base pair—Different chemicals known as bases or nucleobases are found on each strand of DNA. Each base has a chemical attraction for a particular partner base, known as its complement. C matches up with G, while A pairs with T or U. These bonded genetic letters are called base pairs. Two strands of DNA can zip together to form a double-helix shape when complementary bases match up to form base pairs.

Blastocyst—A preimplantation embryo in placental mammals (about 5 days after fertilization in humans) of 50–150 cells. The blastocyst consists of a sphere made up of an outer layer of cells (the trophectoderm), a fluid-filled cavity (the blastocoel or blastocyst cavity), and a cluster of cells in the interior (the inner cell mass). Cells from the inner cell mass, if grown in culture, can give rise to embryonic stem cell lines.

Biosafety—"Biosafety" means the need to protect human and animal health and environment from the possible adverse effects of the products of modern biotechnology.

Biosecurity—Biosecurity, as originally conceptualized, is a set of preventive measures designed to reduce the risk of transmission of infectious diseases in crops and livestock, quarantined pests, invasive alien species, and living modified organisms. The emerging nature of biosecurity threats means that small-scale risks blow up rapidly; thus an effective policy becomes a challenge for there are limitations on time and resources available for analyzing threats and estimating the likelihood of their occurrence. Biosecurity has multiple meanings and is defined differently according to various disciplines. The term was first used by the agricultural and environmental communities. Starting from the late 1990s in response to the threat of biological terrorism, biosecurity encompasses the prevention of the intentional removal (theft) of biological materials from research laboratories. These preventive measures are a combination of systems and practices put into its place at bioscience laboratories to prevent the use of dangerous pathogens and toxins for malicious use, as well as by customs agents and agricultural and natural resource managers to prevent the spread of these biological agents.

Cancer—A type of disease caused by uncontrolled growth of cells. Cancerous cells may form clumps or masses known as tumors and can spread to other parts of the body through a process known as metastasis.

Cas—Abbreviation used for CRISPR-associated proteins. It may refer to genes (*cas*) or proteins (Cas) that protect bacteria and archaea from viral infection.

Cas9 (CRISPR-associated protein 9)—A specialized enzyme known as a nuclease that has the ability to cut DNA sequences. Cas9 makes up part of the "toolkit" for the CRISPR-Cas9 method of genome editing. This enzyme is derived from the CRISPR-Cas bacterial immune system that has been co-opted for genome engineering. In CRISPR immunity, cutting viral DNA prevents it from destroying the host

cell. In genome engineering, cutting genomic DNA initiates a repair process that ends up making a change or "edit" to its sequence.

C2c2—A Class 2 type VI Cas protein which targets single-stranded RNA as opposed to DNA, recently renamed as Cas13. C2c2 is an RNA-guided, RNA-activated nonspecific RNAse. Target sequence recognition is mediated by complementary non-G protospacer flanking sites (PFS). Upon binding of the C2c2 complex, cleavage occurs through the C2c2's nonspecific RNAse activity at U residues in single-stranded RNA regions of the target RNA, without requiring complementarity to the crRNA guide sequence.

Cascade—CRISPR-associated complex for antiviral defense. Multi-subunit Cas protein complex required for crRNA processing and maturation and CRISPR-mediated interference. Homologs from the archaea-type cascade complex from *E. coli* are found in all Type I CRISPR-Cas subtypes. The other CRISPR (sub)types have distinct ribonucleoprotein complexes (Type II, a multi-domain protein Cas9; Type III, a multi-subunit RAMP complex).

Cell—The basic unit of life. The number of cells in a living organism ranges from one (e.g., yeast) to quadrillions (e.g., blue whale). A cell is composed of four key macromolecules that allow it to function (protein, lipids, carbohydrates, and nucleic acids). Among other things, cells can build and break down molecules, move, grow, divide, and die.

Chimera—An organism composed of cells derived from at least two genetically different individuals.

Choriocarcinoma—A type of tumor that originates from the trophoblast, the precursor of the placenta, and invades the uterine wall.

Chromatin—The complex of DNA and proteins that forms chromosomes. Some of the proteins are structural, helping to organize and protect the DNA, while others are regulatory, acting to control whether genes are active or not and to promote DNA replication or repair.

Chromosome—The compact structure into which a cell's DNA is organized, held together by proteins. The genomes of different organisms are arranged into varying numbers of chromosomes, and human cells have 23 pairs.

Cleave—The scientific term for cut or break apart. Typically refers to splitting apart a long polymeric molecule like DNA, RNA, or protein. For example, a nuclease like Cas9 can be directed to cleave DNA at a specific location.

Cleavage—The process of cell division in the very early embryo before it becomes a blastocyst [10], p. 261. Also used to describe breaking or cutting DNA.

Clinical application—The use of a biomedical reagent, procedure, or device to treat a clinical condition.

Clinical trial—A supervised and monitored experimental test in patients of a newly developed clinical application to ensure minimization of risk and optimization of efficacy. Clinical trials are required before a treatment is approved for general use.

Complementary—Describes any two DNA or RNA sequences that can form a series of base pairs with each other. Each base forms a bond with a complementary partner. T (DNA) and U (RNA) bond with A, and C complements G. For example, in CRISPR immunity, the spacer sequence in a guide RNA is complementary to a sequence found in a viral genome. When the RNA bases pair with complementary DNA bases from an invading virus, the Cas9 protein will cut the target to stop the viral infection.

Cpf1 (Cas12a)—A protein derived from *Prevotella* and *Francisella* that has been co-opted for genome engineering. It can make staggered cut in the target DNA, compared to blunt cut by Cas9. Uses an RNA molecule as a guide to find a complementary DNA sequence. Once the target DNA is identified, Cpf1 cuts both strands. In CRISPR immunity, cutting viral DNA prevents it from destroying the host cell. In genome engineering, cutting genomic DNA initiates a repair process that ends up making a change or "edit" to its sequence.

CRISPR (clustered regularly interspaced short palindromic repeats)—Pronounced "crisper." An adaptive immune system found in bacteria and archaea, co-opted as a genome engineering tool. Acronym of "clustered regularly interspaced short palindromic repeats," which refers to a section of the host genome containing alternating repetitive sequences and unique snippets of foreign DNA. CRISPR-associated surveillance proteins use these unique sequences as molecular mugshots as they seek out and destroy viral DNA to protect the cell.

CRISPRa—CRISPR activation, using a guide RNA and nuclease-deficient or nuclease-dead Cas9 (dCas9) linked to one or more activation domains to increase transcription of a target gene.

CRISPRr/CRISPRi—CRISPR repression, or CRISPR interference, using a dCas9 or dCas9-repressor with a guide RNA to decrease transcription of a target gene.

CRISPR RNA (crRNA)—During CRISPR immunity, the host cell generates crRNA molecules, each containing one spacer that is complementary to a portion of a viral genome. crRNAs guide CRISPR immune proteins to find and destroy matching invader viral sequences.

CRISPR screening—A technique that lets scientists see the effects of turning gene expression up or down with CRISPRa and CRISPRi. Instead of checking one gene at a time, a single CRISPR screen can provide information about thousands of different genes at a time.

Cultured cell—A cell maintained in a tissue culture allowing expansion of its numbers.

dCas9—Catalytically inactive, or "dead," Cas9. This mutated version of the Cas9 protein cannot cut but still binds tightly to a particular DNA sequence specified by the guide RNA. Can be used to physically block the process of transcription, turning off a specific gene, or to shuttle other proteins to a particular site in the genome.

Deontology ethics—A normative theory regarding which choices are morally required, forbidden, or permitted.

DNA—Abbreviation of deoxyribonucleic acid, a long molecule that encodes the information needed for a cell to function or a virus to replicate. Forms a double-helix shape that resembles a twisted ladder. Different chemicals called bases, abbreviated as A, C, T, and G, are found on each side of the ladder, or strand. The bases have an attraction for each other, making A stick to T while C sticks to G. These rungs of the ladder are called base pairs.

Differentiation—The process whereby an unspecialized early embryonic cell acquires the features of a specialized cell, such as a heart, liver, or muscle cell.

Diploid—Cells that contain a full set of DNA, half from each parent. In humans, diploid cells contain 46 chromosomes (in 23 pairs).

Divergence (evolutionary)—During evolution, variations occur in the sequences of genes; if these variations confer some advantage, natural selection increases their prevalence. Different selective pressures select for different variations so that the prevalence of different gene variants diverges in different populations.

Dominant—A pattern of inheritance of a gene or trait in which a single copy of a particular allele (gene variant) confers a function independent of the nature of the second copy of the gene in a diploid cell of an organism.

Double-strand break (DSB)—When both strands of DNA are broken, two free ends are created. May be made intentionally by a tool such as Cas9. Cells repair their DNA to prevent cell death, sometimes changing the DNA sequence at the site of the break. Initiating or controlling this process with the intent to alter a DNA sequence is known as genome engineering.

Ectoderm—The outermost of the three primitive germ layers of the embryo; it gives rise to skin, nerves, and brain.

Ectopic—Found in an unusual location, such as an ectopic pregnancy outside the uterus.

Electrophoresis—The meaning of electrophoresis is "to bear electrons" which is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field. Electrophoresis of positively charged particles (cations) is sometimes called cataphoresis, while electrophoresis of negatively charged particles (anions) is sometimes called anaphoresis. Electrophoresis is used in laboratories to separate macromolecules based on size. The technique applies a

negative charge so proteins move towards a positive charge. Electrophoresis is used extensively in DNA, RNA, and protein analysis.

Embryo—An animal in the early stages of growth and differentiation that are characterized by cleavage (cell division of the fertilized egg), differentiation of fundamental cell types and tissues, and the formation of primitive organs and organ systems; the developing human individual from the time of implantation to the end of the eighth week after conception, after which stage it becomes known as a fetus.

Embryonic germ (EG) cell—A pluripotent stem cell that migrates during early development to the future gonads to form the progenitors of egg or sperm cells. The properties of EG cells are similar to those of embryonic stem cells but may differ in the DNA methylation of some imprinted regions.

Embryonic stem (ES) cell—A primitive (undifferentiated) cell from the embryo that has the potential to become a wide variety of specialized cell types (i.e., is pluripotent). It is derived from the inner cell mass of the blastocyst. An embryonic stem cell is not an embryo; by itself, it cannot produce the necessary cell types, such as trophectoderm cells, so as to give rise to a complete organism. Embryonic stem cells can be maintained as pluripotent cells in culture and induced to differentiate into many different cell types.

Endoderm—Innermost of the three primitive germ layers of the embryo; it later gives rise to the lungs, liver, and digestive organs.

Endogenous—Originating from within a cell or an organism.

Endometrium—The inner epithelial lining of the uterus into which embryos implant.

Endonuclease—A protein that breaks down a polynucleotide chain of DNA by cutting the internal bonds linking the nucleotides.

Enhancement—Improving a condition or trait beyond a typical or normal level.

Enucleated cell—A cell whose nucleus has been removed.

Enucleation—A process whereby the nuclear material of a cell is removed, leaving only the cytoplasm. When applied to an egg, can involve the removal of the maternal chromosomes, when they are not surrounded by a nuclear membrane.

Enzyme—A molecule, typically a protein, that causes or catalyzes a chemical change. Usually an enzyme's name describes a molecule involved in the activity it performs and ends with the suffix -ase. For example, lactase is a well-known enzyme that breaks down lactose, a sugar found in milk. Cas9 is a nuclease, an enzyme that breaks apart the backbone of nucleic acids (RNA or DNA).

Epiblast—A specific layer of cells in an early vertebrate embryo that gives rise to the entire embryo other than the yolk sac and placenta. Epiblast cells are pluripotent and can give rise to embryonic stem cells.

Epigenetic—Refers to changes to a cell's gene expression that do not involve altering its DNA code. Instead, the DNA and proteins that hold onto DNA are "tagged" with removable chemical signals. Epigenetic marks tell other proteins how to read the DNA, which parts to ignore, and which parts to transcribe into RNA.Comparable to sticking a note that says "SKIP" onto a page of a book, a reader will ignore this page but the book itself has not been changed.

Epigenetic effects—Changes in gene expression that occur without changing the DNA sequence of a gene; for example, in the epigenetic effect called genomic imprinting, chemical molecules called methyl groups attach to DNA and alter the gene's expression.

Epigenome—A set of chemical modifications to the DNA of the genome and to proteins that bind to DNA in the chromosomes to affect whether and how genes are expressed.

Eukaryote—A domain of organisms whose cells contain a nucleus and other organelles. Eukaryotes are often large and multicellular (e.g., elephants) but can also exist as microscopic, single cells (e.g., yeast). This category of life includes humans. Compare to prokaryotes (bacteria and archaea).

Ex vivo—Latin: "out of the living"; outside an organism.

Exogenous—Introduced or originating from outside a cell or an organism.

Expression—A product being made from a gene; can refer to either RNA or protein. When a gene is turned on, cellular machines "express" this by transcribing the DNA into RNA and/or translating the RNA into a chain of amino acids. For example, a "highly expressed" gene will have many RNA copies produced, and its protein product is likely to be abundant in the cell. CRISPRi and CRISPRa are methods for turning gene expression down or up, respectively.

Fertilization—The process whereby male and female gametes (sperm and egg) unite.

FokI—The nuclease from which the cleavage domain has been abstracted and joined to zinc finger (ZF) or transcription activator-like effector (TALE) DNA-binding domains. The FokI cleavage domain cuts only one strand of the DNA (a nick), so a pair of ZFNs or TALENs is required to create double-strand breaks. The FokI cleavage domain has also been linked to nuclease-deficient Cas9 (dCas9), and this fusion must also dimerize to cut DNA.

Gain of function—A type of mutation that results in an altered gene product that possesses a new molecular function or a new pattern of gene expression [11], p. 1.

Gamete—A reproductive cell (egg or sperm). Gametes are haploid (having only half the number of chromosomes found in somatic cells—23 in humans), so that when two gametes unite at fertilization, the

resulting one-cell embryo (zygote) has the full number of chromosomes (46 in humans).

Gastrulation—The procedure by which an animal embryo at an early stage of development produces the three primary germ layers—ectoderm, mesoderm, and endoderm.

Gene—A segment of DNA that encodes the information used to make a protein. Each gene is a set of instructions for making a particular molecular machine that helps a cell, organism, or virus function.

Gene drive—A mechanism for preferential inheritance of a particular DNA sequence. Usually, offspring have a semi-random chance of inheriting a given stretch of DNA from either parent. In a scientist-designed gene drive, a gene is engineered to have a 100% chance of being passed on. Gene drives can force the inheritance of a desirable trait through a population of organisms. For example, this approach could potentially make all mosquitoes incapable of transmitting the malaria parasite.

Gene editing—"Precisely altering genetic sequences, in living cells including those of human, at much higher accuracy and efficiency than ever before possible" (David Baltimore, Nobel Prize winner in 1975). In other words, any technique using site-directed mutagenesis to make small changes to DNA sequences at a known location is called gene editing or genome editing.

Gene expression—The process by which RNA and proteins are made from the instructions encoded in genes. Gene expression is controlled by proteins and RNA molecules that bind to the genome or to the RNA copy and regulate their levels of production and those of their products. Alterations in gene expression change the functions of cells, tissues, organs, or whole organisms and sometimes result in observable characteristics associated with a particular gene.

Gene gun—In genetic engineering, a gene gun or biolistic particle delivery system is a device used to deliver exogenous DNA (transgenes), RNA, or protein to cells. By coating particles of a heavy metal with a gene of interest and firing these micro-projectiles into cells using mechanical force, an integration of desired genetic information can be induced into cells. The technique involved with such micro-projectile delivery of DNA is often referred to as biolistics. This device is able to transform almost any type of cell and is not limited to the transformation of the nucleus; it can also transform organelles, including plastids and mitochondria.

Gene targeting—A procedure used to produce an alteration in a specific gene.

Gene therapy—Delivering corrective DNA to human cells as a medical treatment. Certain diseases can be treated or even cured by adding a healthy DNA sequence into the genomes of particular cells. Scientists and doctors typically use a harmless virus to shuttle genes into targeted cells or tissues, where the DNA is incorporated somewhere within the

cells' existing DNA. CRISPR genome editing is sometimes referred to as a gene therapy technique.

Gene transfer—Any process often used to describe the transfer of genes into cells, as used in gene therapy.

Genetic element—A segment of the DNA in a genome that has some particular property conferred by its sequence, such as a gene encoding a protein or RNA, more often used to refer to sequences that are not such genes but may control gene expression or genome organization.

Genetically modified organism (GMO)—A genetically modified organism has had its DNA intentionally altered using scientific tools. Any organism can be engineered in this manner, including microbes, plants, and animals. The exact definition of a genetically modified organism and what constitutes genetic engineering varies, with the most common being an organism altered in a way that "does not occur naturally by mating and/or natural recombination." A wide variety of organisms have been genetically modified (GM), from animals to plants and microorganisms. Genes have been transferred within the same species, across species (creating transgenic organisms), and even across kingdoms. New genes can be introduced, or endogenous genes can be enhanced, altered, or knocked out.

Genome—The entire genetic material (DNA sequence) of an organism or virus. The genome is essentially a huge set of instructions for making individual parts of a cell and directing how everything should run. In humans, the genome is organized into 23 pairs of homologous chromosomes.

Genome editing—Intentionally altering the genetic code of a living organism. Can be done with ZFNs, TALENs, or CRISPR. These systems are used to create a double-strand break at a specific DNA site. When the cell repairs the break, the sequence is changed. Can be used to remove, change, or add DNA.

Genome sequencing—Genome sequencing or whole genome sequencing (also known as WGS, full genome sequencing, complete genome sequencing, or entire genome sequencing) is ostensibly the process of determining the complete DNA sequence of an organism's genome at a single time. This entails sequencing all of an organism's chromosomal DNA as well as DNA contained in the mitochondria and, for plants, in the chloroplast. In practice, genome sequences that are nearly complete are also called whole genome sequences.

Genome surgery—Repairing harmful DNA through a one-time genome-editing procedure. Unlike taking a drug that will temporarily reduce long-term symptoms, altering a patient's genetic code with the CRISPR-Cas9 "molecular scalpel" would permanently and directly reverse the cause of a genetic disease.

Genomics—The study of the genome, all the DNA from a given organism. It involves a genome's DNA sequence, organization and

control of genes, molecules that interact with DNA, and how these different components affect the growth and function of cells.

Genotype—Genetic constitution of an individual.

Germ cells (or germline cell)—The cells involved in sexual reproduction: eggs, sperm, and precursor cells that develop into eggs or sperm. The DNA in germ cells, including any mutations or intentional genetic edits, may be passed down to the next generation. In contrast, the genetic material in somatic cells (all the cells in the body except for germ cells) cannot be inherited by offspring. Note that genome editing in an early embryo is considered to be germline editing since any DNA changes will likely end up in all cells of the organism that is eventually born.

Germ layer—In early development, the embryo differentiates into three distinct germ layers (ectoderm, endoderm, and mesoderm), each of which gives rise to different parts of the developing organism.

Gestation—The period of development of an organism from fertilization of the egg until birth.

Governance—The process of exercising oversight through traditions (standards of practice) or regulations by which individuals and communities are held accountable. Governance often involves such policy tools as professional standards of practice and codes of conduct; formal guidelines, agreements, and treaties; and legislation or other governmental regulation.

Guide molecule—A protein or short section of RNA used to guide the genome-editing machinery to the desired location in the DNA sequence.

Guide RNA (gRNA)—A two-piece molecule that Cas9 binds and uses to identify a complementary DNA sequence. Composed of the CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). Cas9 uses the tracrRNA portion of the guide as a handle, while the crRNA spacer sequence directs the complex to a matching DNA sequence. Scientists have also formed a version of the guide RNA that consists of a single molecule, the single-guide RNA (sgRNA).

Haploid—Refers to a cell (usually a gamete or its immediate precursor) having only one set of chromosomes (23 in humans). In contrast, body cells (somatic cells) are diploid, having two sets of chromosomes (46 in humans).

Heritable genetic change—Modifications to genes that could be passed down through generations.

Heterozygous—Having two different variants (alleles) of a specific gene on the two homologous chromosomes of a cell or an organism.

Homologous recombination—Recombining of two like DNA molecules, including a process by which gene targeting produces an alteration in a specific gene.

Homology-directed repair (HDR)—A way for a cell to repair a break in its DNA by "patching" it with a piece of donor DNA. The donor DNA must contain similar sequences, or homology, to the broken DNA ends for it to be incorporated. HDR is a more precise repair pathway than nonhomologous end joining. In genome engineering, a researcher designs and adds in the donor DNA, potentially allowing scientists to replace a disease-causing gene with a healthy copy.

Homozygous—Having the same variant (allele) of a specific gene on both homologous chromosomes of a cell or an organism.

Implantation—The process by which an embryo becomes attached to the inside of the uterus (7–14 days in humans).

In vitro—Latin: "in glass"; in a laboratory dish or test tube; in an artificial environment.

In vitro fertilization (IVF)—An assisted reproduction technique in which fertilization is accomplished outside the body.

In vivo—Latin: "in the living"; in a natural environment, usually in the body of the subject. This term is often also used to refer to events in "living" cells in culture.

Indel—Abbreviation for insertion or deletion. Refers to the random removal or addition of nucleotides from a DNA sequence. This can be enough to stop a gene from functioning (imagine removing a page from the middle of an instruction manual). Indels occur when DNA is broken and "sloppily" repaired by the cell in a process called nonhomologous end joining (NHEJ).

Induced pluripotent stem (iPS) cell—A cell induced by the introduction or activation of genes conferring pluripotency and stem cell-like properties. Thus, cells already committed to a particular fate (e.g., skin) can be induced to become pluripotent. This is useful in regenerative medicine because the iPS cells can be introduced back into the donor of the original cells with much less risk of transplant rejection.

Insertional mutagenesis—The alteration of the sequence of a gene by the insertion of exogenous sequence such as by integration of viral sequences.

Institutional review board (IRB)—An administrative body in an institution (such as a hospital or a university) established to protect the rights and welfare of human research subjects recruited to participate in research activities conducted under the auspices of that institution. The IRB has the authority to approve, require modifications in, or disapprove research activities in its jurisdiction, as specified by both federal regulations and local institutional policy.

Interference—Process by which invasive DNA or RNA is targeted by crRNA-loaded Cas proteins. This process relies on sequence homology and complementarity between the crRNA and the target nucleic acid. In some cases, ancillary elements are necessary for target interference, such as tracrRNA and PAMs, and for preventing autoimmunity.

Leader—AT-rich sequence located upstream of the first CRISPR repeat. This sequence serves as a promoter for the transcription of the repeat-spacer array. Also, this sequence defines CRISPR locus orientation for transcription and polarized spacer acquisition.

Lentivirus—A subclass of retroviruses, viruses whose genome are made of RNA but during viral replication becomes copied into a DNA form that can integrate into the DNA genome of a cell. Often used as carriers of genes (vectors) to introduce genes into cells.

Ligase—An enzyme that catalyzes joining of two pieces of DNA.

Loss of function—A type of mutation in which the altered gene product lacks the molecular function of the wild-type gene.

Meganuclease—A special type of enzyme that binds to and cuts DNA at specific DNA sequences of a length that occurs at few sites in the genome. These are natural enzymes (and their synthetic derivatives) that catalyze DNA rearrangement events via DNA cleavage. They can be used in genome editing for both nonhomologous end joining and homology-directed repair—mediated alterations. It was the study of these that first revealed the basic mechanisms of DNA cleavage and the DNA repair processes on which genome editing depends.

Mesoderm—The middle layer of the embryo, which consists of a group of cells derived from the inner cell mass of the blastocyst; it is formed at gastrulation and is the precursor to blood, bone, muscle, and connective tissue.

Microbe—A microscopic organism. Can be single-celled or multicellular and is sometimes used to refer to viruses, although they are not considered to be alive. Examples include bacteria, yeast, and algae.

Microinjection—It is the use of a glass micropipette to inject a liquid substance at a microscopic or borderline macroscopic level. The target is often a living cell but may also include intercellular space. Microinjection is a simple mechanical process usually involving an inverted microscope with a magnification power of around 200× (though sometimes it is performed using a dissecting stereo microscope at 40–50× or a traditional compound upright microscope at similar power to an inverted model).

Mitochondrial transfer (or mitochondrial replacement)—Novel procedures designed to prevent the maternal transmission of mitochondrial DNA (mtDNA) diseases.

Mitochondrion (plural, mitochondria)—A cellular structure in the cytoplasm that provides energy to the cell. Each cell contains many mitochondria. In humans, a single mitochondrion contains 37 genes on a circular mitochondrial DNA, compared with about 35,000 genes contained in the nuclear DNA.

Mosaicism—Variation among cells, such that the cells are not all the same, for example, in an embryo when not all the cells are edited.

Multipotent stem cells—Stem cells from the embryo, fetus, or adult, whose progeny are of multiple differentiated cell types and usually, but not necessarily, all of a particular tissue, organ, or physiological system.

Murine—Derived from mice.

Mutation—A change in a DNA sequence. Mutations can occur spontaneously during cell division or can be triggered by environmental stresses, such as sunlight, radiation, and chemicals.

Nick—When only one strand of DNA is broken, there is a gap called a nick in the backbone, but the DNA does not separate. A tool like CRISPR-Cas9 may be used to generate a nick.

Nickase—A nuclease that cuts only one strand of the DNA double helix. nCas9 (D10A) cuts the target DNA strand which is complementary to guide RNA, whereas nCas9 (H840A) cuts the noncomplementary strand.

Non-homologous end joining (NHEJ)—A way for a cell to repair a break in its DNA by attaching the free DNA ends. This pathway is "sloppier" than homology-directed repair and often results in the random addition or removal of nucleotides around the site of the DNA break, causing insertions or deletions in the genetic code. In genome engineering, this allows scientists to stop a gene from working (similar to removing a page from the middle of an instruction manual).

Normative theory—A theory of how people should make decisions, as opposed to how they actually do or will make decisions.

Nuclease—An enzyme that breaks apart the backbone of RNA or DNA. Breaking one strand generates a nick and breaking both strands generates a double-strand break. An endonuclease cuts in the middle of RNA or DNA, while an exonuclease cuts from the end of the strand. Genome engineering tools like Cas9 are endonucleases.

Nucleic acid—A term for DNA and RNA. Refers to nucleotides, the basic chemical units that are strung together to make DNA or RNA. One of the four macromolecules that make up all living things (protein, lipids, carbohydrates, and nucleic acids).

Nucleotide—One of the basic chemical units strung together to make DNA or RNA. Consists of a base, a sugar, and a phosphate group. The phosphates can link with sugars to form a string called the DNA/RNA backbone, while the bases can bind to their complementary partners to form base pairs.

Off-target effect—A direct or indirect, unintended, short- or long-term consequence of an intervention on an organism other than the intended effect on that organism [8, 9], p. 184.

Off-target event (or off-target cleavage)—When a genome-editing nuclease cuts DNA at a location other than the one for which it was targeted. This can occur because the off-target sequence is similar to but not identical with the intended target sequence.

Oocyte—Developing egg; usually a large and immobile cell.

Pathogen—A microbe that causes illness. Most microorganisms are not pathogenic to humans, but some strains or species are harmful.

Phage—A type of virus that infects bacteria or archaea, formally called bacteriophage.

Phenotype—Observable properties of an organism that are influenced by both its genotype and its environment.

Plasmid—A self-replicating circular DNA molecule. A plasmid can be engineered to carry and express genes of interest in target cells.

Pluripotent stem cell (PSC)—A stem cell that includes in its progeny all cell types that can be found in a postimplantation embryo, fetus, or developed organism.

Population—All of the individuals of a given species within a defined ecological area.

Preclinical research—Research conducted to investigate potential clinical applications but not involving humans. For example, research on molecules, cells, tissues, or animals.

Precursor cell or progenitor cell—In fetal or adult tissues, it is a partially committed but not fully differentiated cell that divides and gives rise to differentiated cells.

Preimplantation genetic diagnosis (PGD)—Before an in vitro-fertilized embryo is implanted in a woman's uterus, it can be screened for specific genetic mutations that are known to cause particular genetic diseases or for chromosomal abnormalities. One or more cells are removed from the preimplantation embryo for testing and the surviving embryo that is implanted is one that is not carrying the genetic abnormality.

Prenatal diagnosis—Detection of abnormalities and disease conditions while a fetus is developing in the uterus. Many techniques for prenatal diagnosis, such as chorionic villus sampling and amniocentesis, require sampling placental tissue or fetal cells found in the amniotic fluid or fetomaternal circulation. Others, such as ultrasonography, can be performed without cell or tissue samples.

Primitive streak—An elongated band of cells that forms along the axis of an embryo early in gastrulation by the movement of lateral cells toward the axis and that develops a groove along its midline through which cells move to the interior of the embryo to form the mesoderm.

Prokaryote—A category of living organisms that encompasses all bacteria and archaea. Prokaryotes are microscopic, single-celled organisms that do not have a nucleus or other membrane-bound organelles. Compare to eukaryotes.

Pronucleus—The haploid nucleus of an oocyte or sperm, either prior to fertilization or immediately after fertilization, before the sperm and egg nuclei have fused into a single diploid nucleus.

Protein—A large complex molecule made up of one or more chains of amino acids. Proteins perform a wide variety of activities in the cell.

Protospacer adjacent motif (PAM)—A short sequence that must be present next to a DNA target sequence for Cas9 to bind and cut. Prevents cleavage of host CRISPR array, where PAM is not present.

RAMP—Repeat-associated mysterious proteins. Subset of Cas proteins with an RNA recognition motif (RRM fold). Some RAMPs have endoribonuclease activity involved in the maturation and processing of crRNA.

Recessive—A recessive allele of a gene is one whose effects are masked by the second allele present in a diploid cell or organism, which is referred to as dominant.

Recombinant DNA—A recombinant DNA molecule is made up of DNA sequences that have been artificially modified or joined together so that the new genetic sequence differs from naturally occurring genetic material.

Recombinant DNA Advisory Committee (RAC)—Oversees and reviews proposals for research funded by the National Institutes of Health (NIH) or similar projects conducted at institutions funded by NIH that involve recombinant or synthetic DNA, such as gene therapy.

Recombinant DNA Technology—Recombinant DNA (rDNA) molecules are DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome. Recombinant DNA is the general name for a piece of DNA that has been created by combining at least two strands. Recombinant DNA is possible because DNA molecules from all organisms share the same chemical structure and differ only in the nucleotide sequence within that identical overall structure. Recombinant DNA molecules are sometimes called chimeric DNA, because they can be made of material from two different species, like the mythical chimera. R-DNA technology uses palindromic sequences and leads to the production of sticky and blunt ends.

Recombination—The process, natural or engineered, in which two pieces of DNA undergo breakage and reunion to generate a new combination of DNA segments.

Regenerative medicine—Medical treatments that seek to replace defective, damaged, or missing tissue by engineered cells, tissues, or implants, often involving stem cells.

Repeats—Short sequence repeated within a CRISPR array, separated by spacers. Highly similar sequences that form direct repeats spaced by

short variable sequences of conserved length. The CRISPR repeat sequence is critical for crRNA maturation and processing and is functionally coupled with cas genes to form a functional CRISPR-Cas system. Only a subset of all repeat types are palindromic.

Restriction enzyme—An enzyme from bacteria that is used to cut DNA at defined sequences, used in DNA analysis and in joining DNA fragments through the cut ends.

Retrovirus—A virus whose genome is made of RNA but during viral replication becomes copied into a DNA form that can integrate into the DNA genome of a cell. Often used as carriers of genes (vectors) to introduce genes into cells. A subset of retroviruses is called lentiviruses.

Ribonucleoprotein complex (RNP)—An assembly of molecules containing both protein and RNA. Often used to describe Cas9 protein bound to guide RNA (gRNA), which together form an active enzyme. For genome editing in cells, Cas9 can be delivered as a pre-assembled RNP or as DNA or RNA encoding the genetic instructions for the protein and RNA components.

Risk—The probability of an effect on a specific endpoint or a set of endpoints due to a specific set of a stressor or stressors. An effect can be beneficial or harmful.

Risk assessment—The process by which all available evidence on the probability of effects is collected, evaluated, and interpreted to estimate the probability of the sum total of effects.

R-loop—Section of DNA associated with RNA forming a loop. Structure in which RNA hybridizes with double-stranded DNA. The RNA base-pairs with a complementary sequence in one of the strands of a DNA molecule, causing the displaced strand to form a loop.

RNA—Abbreviation of ribonucleic acid. Transcribed from a DNA template and typically used to direct the synthesis of proteins. CRISPR-associated proteins use RNAs as guides to find matching target sequences in DNA.

RNAi—RNA interference. Process in eukaryotes by which small noncoding RNA molecules guide enzymatic cleavage of complementary mRNAs through the RNA-induced silencing complex (RISC).

Seed sequence—Short sequence within the crRNA which requires perfect base-pairing with the target sequence. Short stretch of nucleotides (7–9 nt) which enthalpically drives hybridization between the interfering crRNA and the complementary target strand in the vicinity of the PAM site, which supports R-loop formation and generally results in interference.

Selective advantage—Some variants of genes provide a trait that confers a survival or a reproductive advantage that can be selected by natural selection and therefore increases in prevalence in a population.

Signature gene—Key cas gene which defines a CRISPR-cas type. Idiosyncratic cas gene(s) that define the type of a particular CRISPR-cas system, which are cas3, cas9, and cas10 for Type I, Type II, and Type III, respectively.

Single-guide RNA (sgRNA)—A version of the naturally occurring two-piece guide RNA complex engineered into a single, continuous sequence. The simplified single-guide RNA is used to direct the Cas9 protein to bind and cleave a particular DNA sequence for genome editing.

Somatic cell—Any cell of a plant or animal other than a reproductive cell or reproductive cell precursor. Latin: soma = body.

Somatic cell nuclear transfer (SCNT)—The transfer of a cell nucleus from a somatic cell into an egg (oocyte) whose nucleus has been removed.

Spacer—Small variable nucleotide sequence within a CRISPR array, flanked by repeats. A spacer is the sequence derived from invasive genetic elements (notably viruses and plasmids) which is integrated within the CRISPR locus.

Spermatogonial stem cells—The self-replicating precursors of sperm cells.

Spoligotyping—Spacer oligonucleotide typing. Strain typing method based on the detection of specific CRISPR spacers, typically through hybridization of labeled CRISPR PCR amplicons.

Stem cells—Cells with the potential to turn into a specialized type of cell or to divide to make more stem cells. Most cells in your body are differentiated—that is, their fate has already been decided and they cannot morph into a different kind of cell. For example, a cell in your brain cannot transform into a skin cell. Embryonic stem cells are found in developing embryos, while adult stem cells are found in tissues including the bone marrow, blood, and fat. Adult stem cells replenish the body as it becomes damaged over time.

Stem cell therapy—The use of stem cells in regenerative medicine to replace defective, damaged, or missing tissue.

Strand—A string of connected nucleotides; can be DNA or RNA. Two strands of DNA can zip together when complementary, bases match up to form base pairs. DNA typically exists in this double-stranded form, which takes the shape of a twisted ladder or double helix. RNA is typically composed of just a single strand, though it can fold up into complex shapes.

Syncytiotrophoblast cell—A cell derived from trophectodermal cells from the early mammalian embryo that fuse (into multinucleate syncytia) and contribute to the structure and function of the placentae.

Synthetic biology—The development of living cells from separate genetic components, using engineering principles to build desired functions into living organisms.

Synthetic DNA—DNA molecules that are chemically or by other means synthesized or amplified; they may be chemically or otherwise modified but can base-pair, or be recombined with, naturally occurring DNA molecules.

T cells—Types of white blood cells that are of crucial importance in the immune system. They cooperate with other immune cells in killing infected or cancerous cells but can also participate in inflammation or in autoimmunity when they become activated against an organism's own cells or tissues.

Target sequence—Specific sequence of DNA bases within the genome that is the target of genome-editing tools. For CRISPR/Cas9 methods, this will be a 20-nucleotide sequence that the gRNAs are designed to recognize (i.e., they will contain a complementary sequence of the same length).

Therapy (or therapeutic intervention)—The treatment or prevention of disease or disability.

Tissue culture—The growth of cells or tissue segments in vitro in an artificial medium for experimental research.

Totipotent cell—A stem cell that has unlimited developmental capability. The totipotent cells of the very early embryo (an embryo prior to the blastocyst stage) have the capacity to differentiate into extraembryonic tissues, membranes, the embryo, and all postembryonic tissues and organs.

Transcription—The process by which DNA information is copied into a strand of RNA; performed by an enzyme called RNA polymerase.

Transcription activator-like effector nuclease (TALEN)—A class of engineered restriction enzymes generated by the fusion of a transcription activator-like effector DNA-binding domain (which binds to a specific DNA sequence) to a DNA-cleavage domain (nuclease) to be used as a genome-editing tool (adapted from NASEM [8], p. 186). TALENs followed zinc finger nucleases and preceded CRISPR/Cas9 as genome-editing tools.

Trans-activating CRISPR RNA (tracrRNA)—Abbreviation for trans-activating CRISPR RNA, pronounced "tracer RNA." In the CRISPR-Cas9 system, the tracrRNA base-pairs with the crRNA to form a functional guide RNA (gRNA). Cas9 uses the tracrRNA portion of the guide as a handle, while the crRNA spacer sequence directs the complex to a matching viral sequence.

Transcription factor—A protein that binds to control regions (enhancers and promoters) of genes to activate or repress their transcription (or expression).

Transfection—A method by which experimental DNA may be introduced into a cell.

Transgene—A gene or genetic material that has been introduced into a cell or organism. Transgenes can be integrated at random or targeted to a specific site by homologous recombination or by genome editing using methods of homology-directed repair.

Transgenic organism—An organism into which one or more genes from another species (transgenes) have been transferred or otherwise artificially introduced.

Transhumanism—A class of philosophies of life that seek the continuation and acceleration of the evolution of intelligent life beyond its currently human form and human limitations by means of science and technology, guided by life-promoting principles and values [7].

Translation—The process of forming a protein molecule from information contained in a messenger RNA, a step in gene expression following transcription (copying of RNA from DNA).

Trophectoderm—The outer layer of the developing blastocyst that will ultimately form the embryonic side of the placenta.

Undifferentiated—Not having developed into a specialized cell or tissue type.

Unipotent stem cell—A stem cell that both divides and gives rise to a single mature cell type, such as a spermatogenic stem cell, which only gives rise to sperm. Alternatively called a progenitor.

Utilitarianism—The morally right action as the action that produces the most "good."

Variant—Genes have many variants in a population that can differ somewhat in function, some being advantageous and some being deleterious or nonfunctional.

Vector—A vehicle that transfers a gene into a new site (analogous to insect vectors that transfer a virus or parasite into a new animal host). Vectors used in molecular cell biology and genetic engineering include plasmids and modified viruses engineered to carry and express genes of interest in target cells. The most clinically relevant viral vectors for gene transfer include retroviral, lentiviral, adenoviral, and adeno-associated viral vectors.

Virtue ethics—A focus on moral character as opposed to duties (deontology) or consequences (consequentialism).

Virus—An infectious entity that can only persist by hijacking a host organism to replicate itself. Has its own genome but is technically not considered a living organism. Viruses infect all organisms, from humans to plants to microbes. Multicellular organisms have sophisticated immune systems that combat viruses, while CRISPR systems evolved to stop viral infection in bacteria and archaea.

Wild type (noun); wild-type (adjective)—The "normal" type of an organism or a gene.

X-inactivation—The process in which one X chromosome of the two present in a female mammalian cell is inactivated so that only the genes of one X chromosome are expressed.

Zinc finger—A small protein structure based on naturally occurring transcription factors that bind to defined DNA sequences to control the activity of nearby genes. Zinc fingers can be custom engineered to target a specific section of the DNA sequence for use in genome engineering.

Zinc finger nuclease (ZFN)—A genetic engineering tool wherein one portion of the protein recognizes a specific DNA sequence and another part cuts DNA. Made by attaching a series of smaller DNA-binding domains together to recognize a longer DNA sequence. This DNA-binding domain is fused to a nuclease that will cut nearby DNA. Like CRISPR-Cas9 and TALENs, it can be used to alter DNA sequences. One of the first and a reliable method of genome editing.

Zygote—The one-cell embryo formed by the union of sperm and egg at fertilization.

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