제 2주제 : 유전자 교정 기술을 이용한 고추 신육종 연구 동향과 전망

Genome Editing Meditated Pepper Improvement

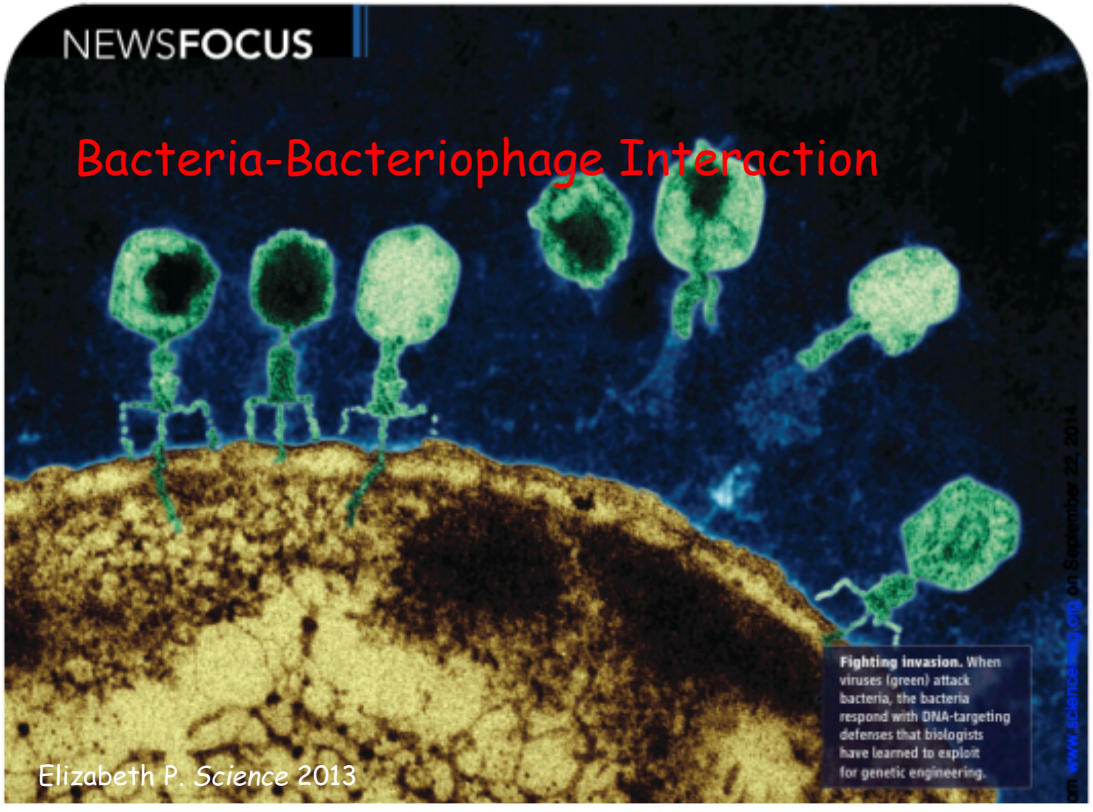
김혜란 강원대학교

Contents

- Understanding CRISPRs & recent updates
- CRISPRs-based editing in crops
- CRISPR-mediated gene editing in peppers

Understanding of CRISPRs & Recent updates

What is CRISPR/Cas9?



- Evolved bacterial immune system, CRISPR!
- Developed as genome-editing tools

CRISPR/Cas9, Prokaryotic adaptive immune system

(2007, Function)

Clustered CRISPR
Regularly associated
Interspaced proteins
Short
Palindromic
Repeats

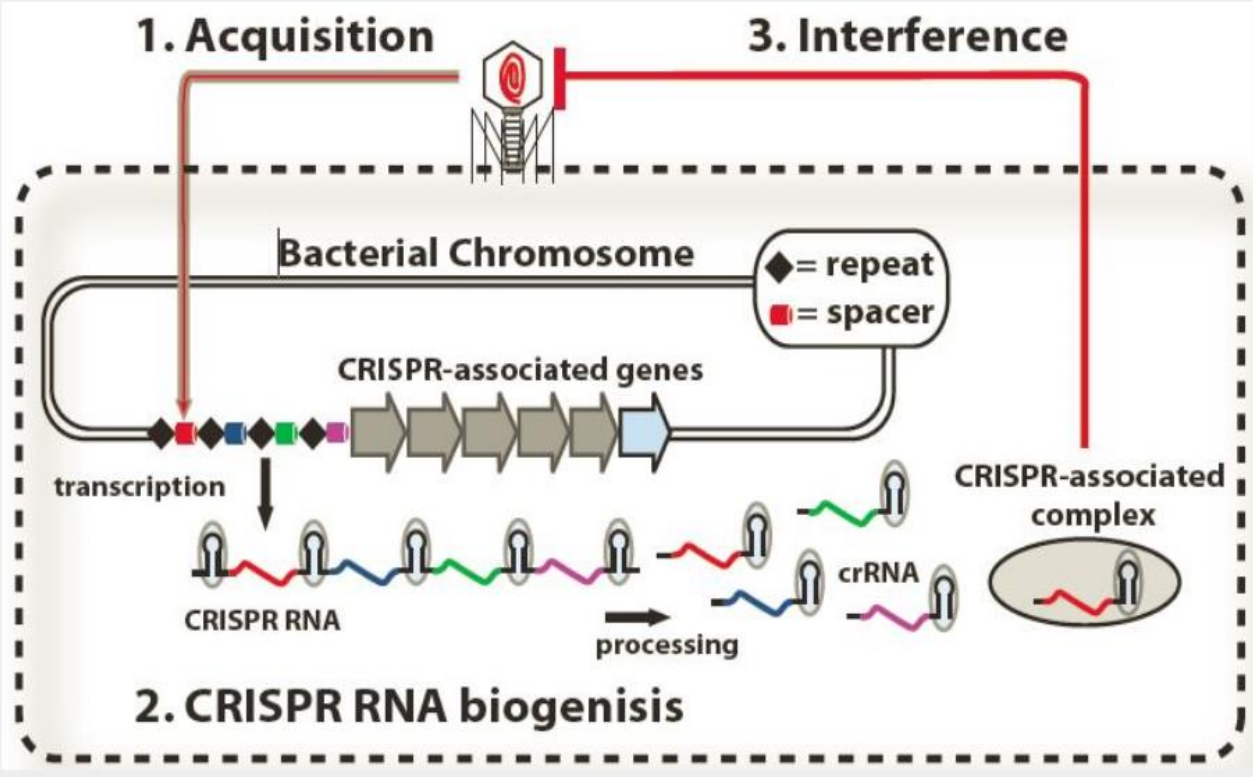


Figure 1: The three main steps in adaptive bacterial immunity via CRISPR systems.

CRISPR: Programmable DNA Scissors

(2012/ Engineering)

RESEARCH ARTICLE

A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek,[1,2]* Krzysztof Chylinski,[3,4]* Ines Fonfara,[3] Michael Hauer,[2]† Jennifer A. Doudna,[1,2,5,6]‡ Emmanuelle Charpentier[4]‡

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.

Bacteria and archaea have evolved RNA-mediated adaptive defense systems called clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) that protect organisms from invading viruses and plasmids (*1*–*3*). These defense systems rely on small RNAs for sequence-specific detection and silencing of foreign nucleic acids. CRISPR/Cas systems are composed of *cas* genes organized in operon(s) and CRISPR array(s) consisting of genome-targeting sequences (called spacers) interspersed with identical repeats (*1*–*3*). CRISPR/Cas-mediated immunity occurs in three steps. In the adaptive phase, bacteria and archaea harboring one or more CRISPR loci respond to viral or plasmid challenge by integrating short fragments of foreign sequence (protospacers) into the host chromosome at the proximal end of the CRISPR array (*1*–*3*). In the expression and interference phases, transcription of the repeat-spacer element into precursor CRISPR RNA (pre-crRNA) molecules followed by enzymatic cleavage yields the short crRNAs that can pair with complementary protospacer sequences of invading viral or plasmid targets (*4*–*11*). Target recognition by crRNAs directs the silencing of the foreign sequences by means of Cas proteins that function in complex with the crRNAs (*10*, *12*–*20*).

There are three types of CRISPR/Cas systems (*21*–*23*). The type I and III systems share some overarching features: specialized Cas endonucleases process the pre-crRNAs, and once mature, each crRNA assembles into a large multi-Cas protein complex capable of recognizing and cleaving nucleic acids complementary to the crRNA. In contrast, type II systems process pre-crRNAs by a different mechanism in which a trans-activating crRNA (tracrRNA) complementary to the repeat sequences in pre-crRNA triggers processing by the double-stranded (ds) RNA-specific ribonuclease RNase III in the presence of the Cas9 (formerly Csn1) protein (fig. S1) (*4*, *24*). Cas9 is thought to be the sole protein responsible for crRNA-guided silencing of foreign DNA (*25*–*27*).

We show here that in type II systems, Cas9 proteins constitute a family of enzymes that require a base-paired structure formed between the activating tracrRNA and the targeting crRNA to cleave target dsDNA. Site-specific cleavage occurs at locations determined by both base-pairing complementarity between the crRNA and the target protospacer DNA and a short motif [referred to as the protospacer adjacent motif (PAM)] juxtaposed to the complementary region in the target DNA. Our study further demonstrates that the Cas9 endonuclease family can be programmed with single RNA molecules to cleave specific DNA sites, thereby raising the exciting possibility of

[1]Howard Hughes Medical Institute (HHMI), University of California, Berkeley, CA 94720, USA. [2]Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA. [3]Max F. Perutz Laboratories (MFPL), University of Vienna, A-1030 Vienna, Austria. [4]The Laboratory for Molecular Infection Medicine Sweden, Umeå Centre for Microbial Research, Department of Molecular Biology, Umeå University, S-90187 Umeå, Sweden. [5]Department of Chemistry, University of California, Berkeley, CA 94720, USA. [6]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA.

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developing a simple and versatile RNA-directed system to generate dsDNA breaks for genome targeting and editing.

Cas9 is a DNA endonuclease guided by two RNAs. Cas9, the hallmark protein of type II systems, has been hypothesized to be involved in both crRNA maturation and crRNA-guided DNA interference (fig. S1) (*4*, *25*–*27*). Cas9 is involved in crRNA maturation (*4*), but its direct participation in target DNA destruction has not been investigated. To test whether and how Cas9 might be capable of target DNA cleavage, we used an overexpression system to purify Cas9

a fivefold molar excess of substrate DNA provided evidence that the dual-RNA–guided Cas9 is a multiple-turnover enzyme (fig. S6B). In

The History of CRISPR systems

Discovery

1987
First report of CRISPR clustered repeats
Ishino et al.

2000
Recognition that CRISPR families are present throughout prokaryotes
Mojica et al.

2002
Coined "CRISPR" name, defined signature Cas genes
Jansen et al.

2005
Identified foreign origin of spacers, proposed adaptive immunity function
Mojica et al.
Pourcel et al.

Identified PAM
Bolotin et al.

Function

2007
First experimental evidence for CRISPR adaptive immunity
Barrangou et al.

2008
CRISPR acts upon DNA targets
Marraffini et al.

Spacers are converted into mature crRNAs that act as small guide RNAs
Brouns et al.

2009
Type III-B Cmr CRISPR complexes cleave RNA
Hale et al.

2010
Cas9 is guided by spacer sequences and cleaves target DNA via DSBs
Garneau et al.

2011
tracrRNA forms a duplex structure with crRNA in association with Cas9
Deltcheva et al.

Type II CRISPR systems are modular and can be heterologously expressed in other organisms
Sapranauskas et al.

Engineering

2012
In vitro characterization of DNA targeting by Cas9
Jinek et al.
Gasiunas et al.

2014
Genome-wide functional screening with Cas9
Wang et al.
Shalem et al.

Crystal structure of apo-Cas9
Jinek et al.

Crystal structure of Cas9 in complex with guide RNA and target DNA
Nishimasu et al.

Applications

2013
Three papers of Cas9 edited plants Arabidopsis/Rice/ Nicotiana

First demonstration of Cas9 genome engineering in eukaryotic cells
Cong et al.
Mali et al.

2015
DNA-free, CRISPR/Cas9-based lettuce editing
Woo and Kim et al.

2016
Development of Base Editor
Kormor et al.

Development of anti-browning mushroom
Yang et al.

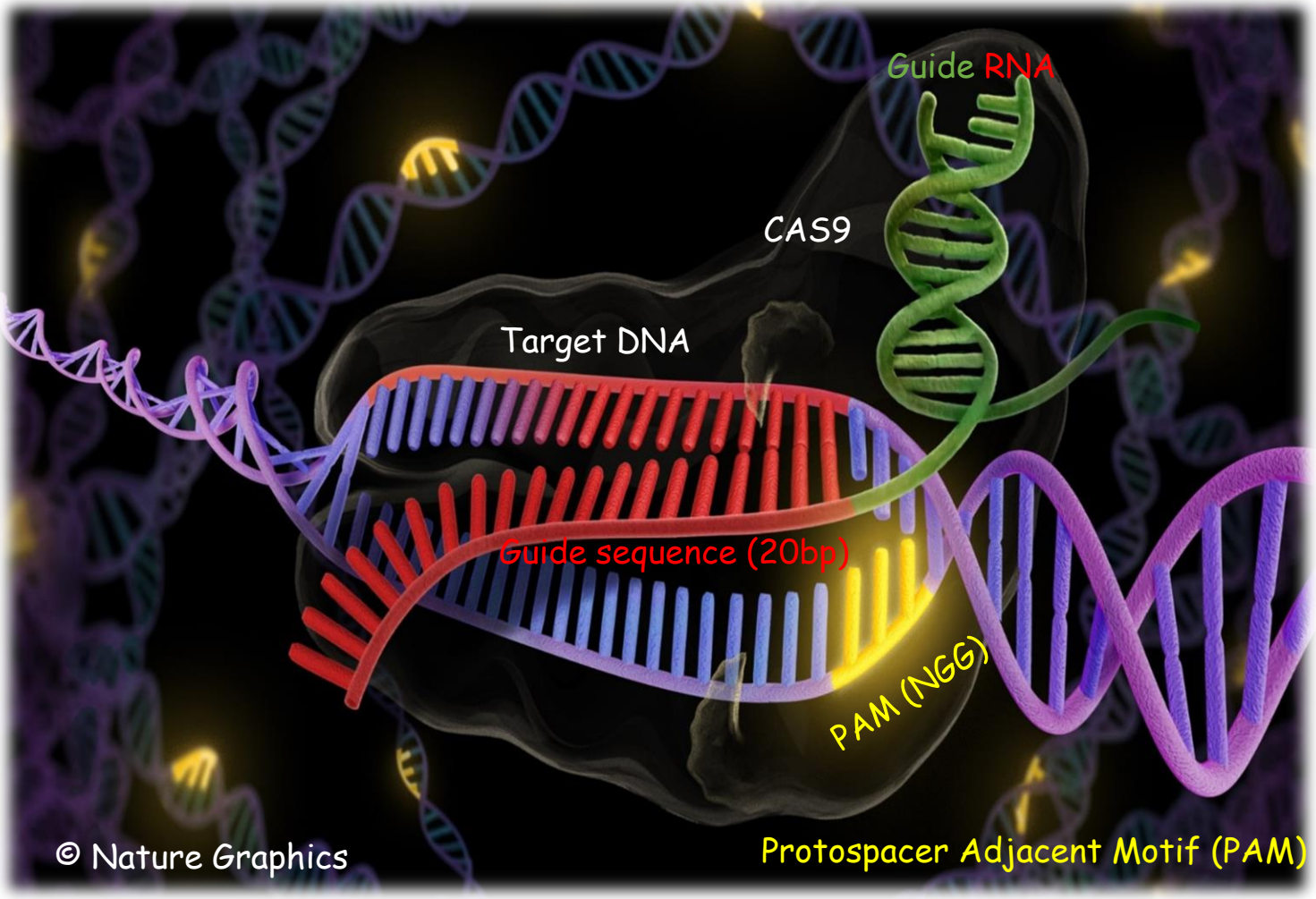
2017
Development of Adenine Base Editor
Gaudelli et al.

DNA-free, CRISPR/Cpf1-mediated soybean editing
Kim et al.

2018
***De novo* domestication of wild plants**
Zsögön et al.
Lemmon et al.

modified from Patrick D.H. et al., 2014 Cell 157, 1263-1278

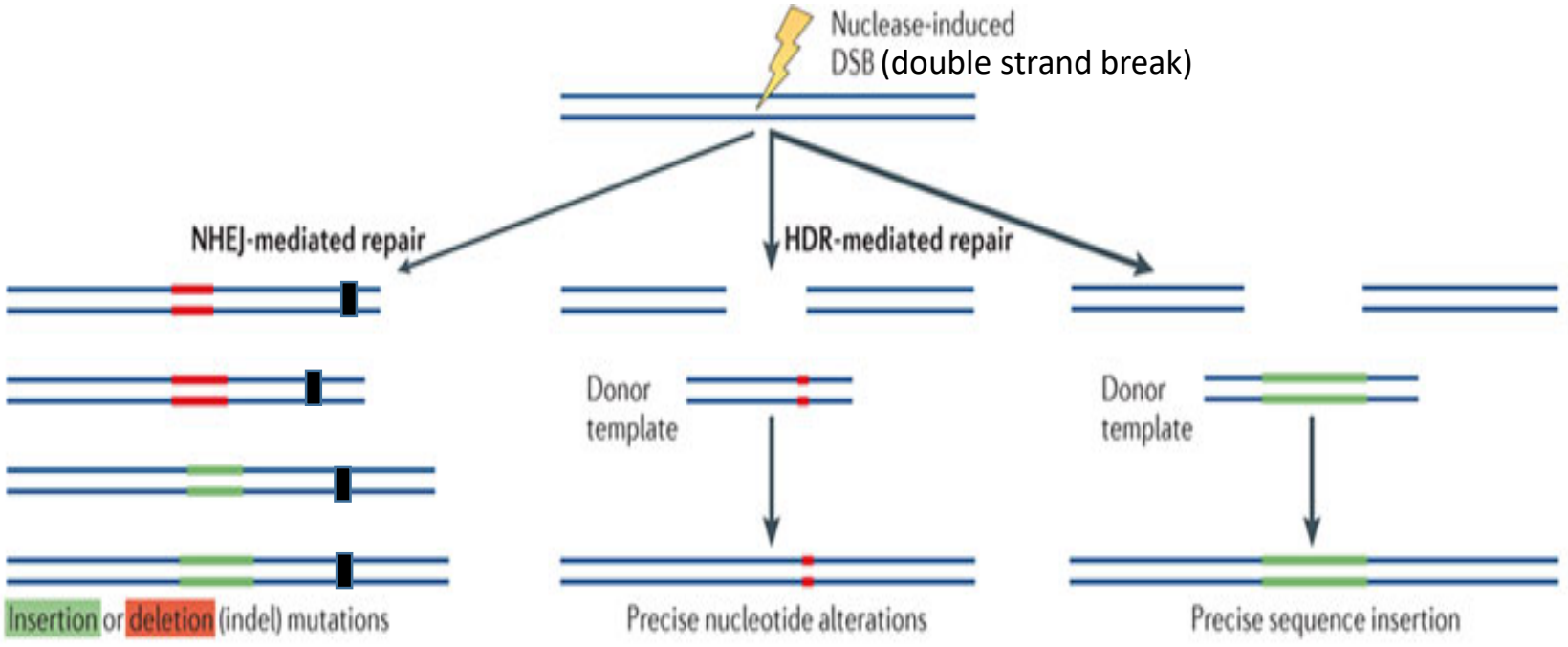
CRISPR/Cas9 at a target locus



Outcomes of CRISPR/Cas9

DNA repair : ① Non-Homologous End-Joining (NHEJ), error-prone

② Homology-Directed Repair (HDR)



▮ Premature stop codon

→ Generate gene knock in a target site

CRISPR/Cas9 applied three model plants

Rice,
Arabidopsis thaliana,
Nicotiana benthamiana
(2013, Aug)

Targeted genome modification of crop plants using a CRISPR-Cas system

To the Editor:

Although genome editi
using zinc finger nuclea
and transcription activa
nucleases (TALENs)[2] ca
modifications, new tech
robust, affordable and e
needed. Recent advance
the prokaryotic adaptiv
involving type II cluste
interspaced, short palin
(CRISPR), provide an a
editing strategy[3]. Type
are widespread in bacte
endonuclease, a CRISP
Cas9, to provide a defer
viral and plasmid DNA
complex with a synthet
(sgRNA), consisting of
RNA (crRNA) and *tran*
The sgRNA guides Cas9
and cleave target DNA.
nuclease domain and a
each cleaves one strand
stranded DNA. It can b
guided endonuclease to
specific genome editing
cells, zebrafish and mic

single customized sgRNA, encoded by a sequence of ~100 nt, is required to target a specific sequence, and Cas9 does not have to be reengineered for each new target site. The sgRNA:Cas9 system is therefore much more straightforward than ZFNs or TALENs.

To test whether sgRNA:Cas9 can induce

Note: Supplementary information is available in the online version of the paper (doi:10.1038/nbt.2650).

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (201263, 383601 and 31200273), the Ministry of Agriculture of China (2011ZX08002-004 and 2013ZX08010-002) and Chinese Academy of Sciences (KSCX2-EW-N-06, KSCX2-EW-J-6). We thank X. Wang for bioinformatics analysis.

AUTHOR CONTRIBUTIONS

Q.S., Y.W., J.L., Y.Z., K.C., Z.L., J.J.X designed the experiments; Q.S., Y.W and J.L. performed experiments; Q. J.-L.Q. and C.G. wrote the paper.

COMPETING FINANCIAL INTE
The authors declare no competing f

Qiwei Shan[1,4], Yanpeng Wang[1] Yi Zhang[1], Kunling Chen[1], Zhe Kang Zhang[1], Jinxing Liu[1], Jian Jin-Long Qiu[3] & Caixia Gao[1]

[1]State Key Laboratory of Plant C Chromosome Engineering, Instit and Developmental Biology, Chi Sciences, Beijing, China. [2]Institu Medicine, Peking University, Bei [3]State Key Laboratory of Plant C Institute of Microbiology, Chines of Sciences, Beijing, China. [4]The contributed equally to this work. e-mail: cxgao@genetics.ac.cn or

Multiplex and homologous recombination–mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9

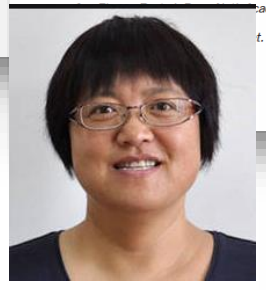
future implementation. We also introduced a facile method to manually design a shared sgRNA target site specific for multiple homologous target genes by aligning their coding sequences and carrying out a BLAST search to evaluate off-target possibilities (**Supplementary Fig. 3**). The sgRNA:pcoCas9 technology enables an easy reprogramming of DNA targeting specificity by changing the 20-nt guide sequence in the sgRNA without modifying the pcoCas9 protein. We have established a simple and rapid procedure to create a custom sgRNA through overlapping PCR (**Supplementary Fig. 5** and **Supplementary Table 1**). Thus, it is feasible to use single or tandemly expressed sgRNAs

Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease

To the Editor:

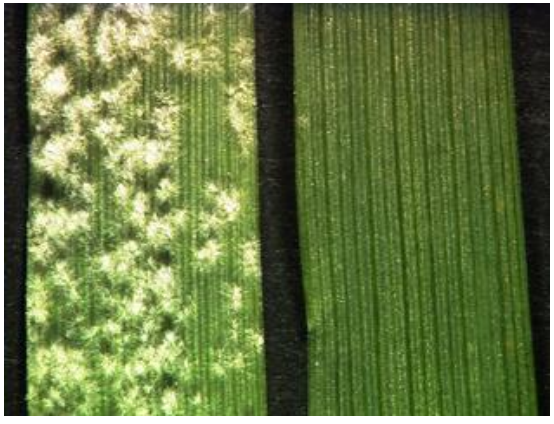
Sustainable intensification of crop production is essential to ensure food demand is matched by supply as the human population continues to increase[1]. This will require high-yielding crop varieties that can be grown sustainably with fewer inputs on less land. Both plant breeding and genetic modification (GM) methods make valuable contributions to varietal improvement, but targeted genome engineering promises to be critical to elevating future yields. Most such methods require targeting DNA breaks to defined locations followed by either nonhomologous end joining (NHEJ) or homologous recombination[2]. Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) can be engineered to create such breaks, but these systems require two different DNA binding proteins flanking a sequence of interest, each with a C-terminal FokI nuclease module. We report here that the bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR) system, comprising a CRISPR-associated (Cas)9 protein and an engineered single guide RNA (sgRNA) that specifies a targeted nucleic acid sequence[3], is applicable to plants to induce mutations at defined loci.

To test the potential of the Cas9 system to induce gene knockouts in plants, we took advantage of *Agrobacterium tumefaciens*–mediated transient expression assays (agroinfiltration) to co-express a Cas9 variant



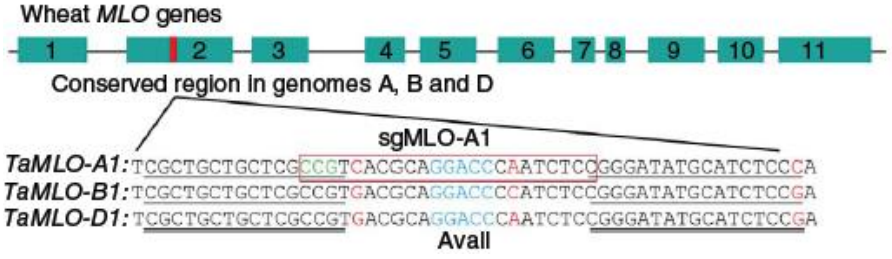
Powdery mildew resistant hexaploid bread wheat

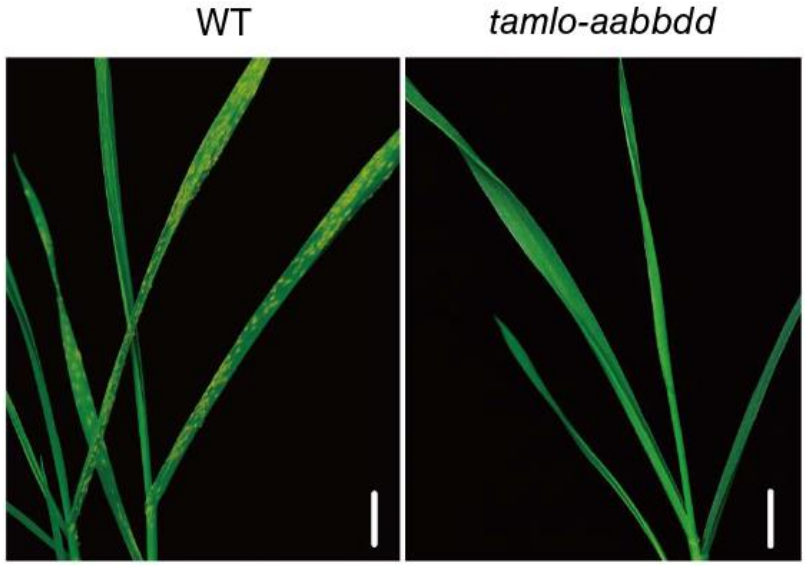
Barely-Powdery mildew (*Blumeria graminis*)



WT *mlo*

Wang *et al.*, NBT (2014 Jul)





Another CRISPR system: Cpf1 (Cas12a)

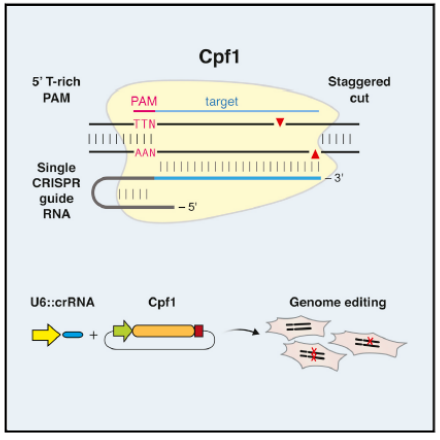
(2015, Oct)

Article

Cell

Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System

Graphical Abstract



Authors

Bernd Zetsche, Jonathan S. Gootenberg, Omar O. Abudayyeh, ..., Aviv Regev, Eugene V. Koonin, Feng Zhang

Correspondence

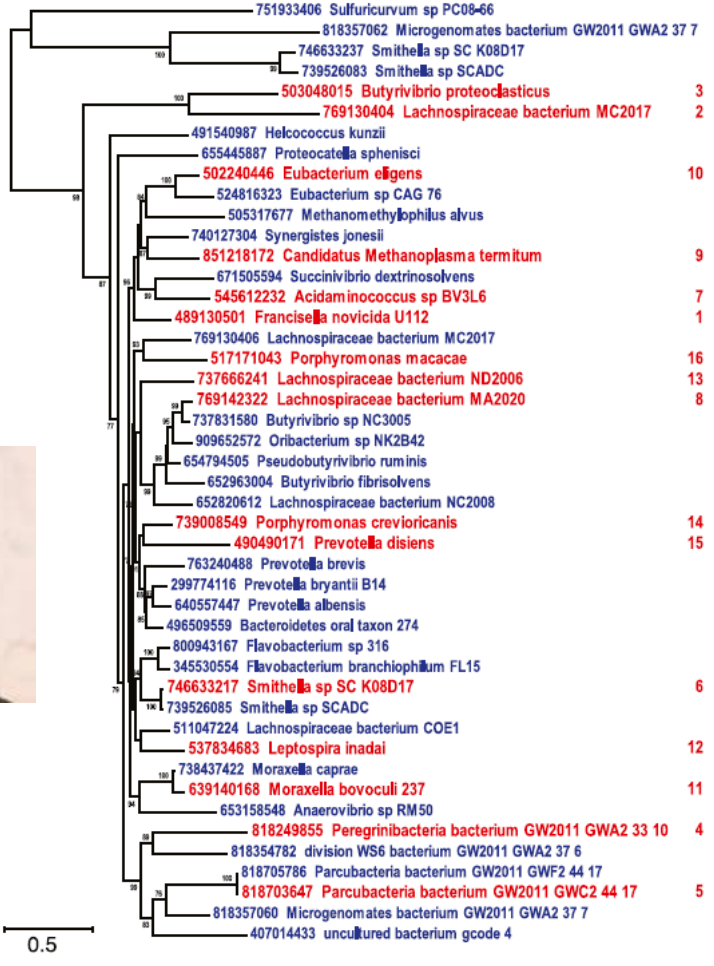
zhang@broadinstitute.org

In Brief

Cpf1 is a RNA-guided DNA nuclease that provides immunity in bacteria and can be adapted for genome editing in mammalian cells.

Highlights

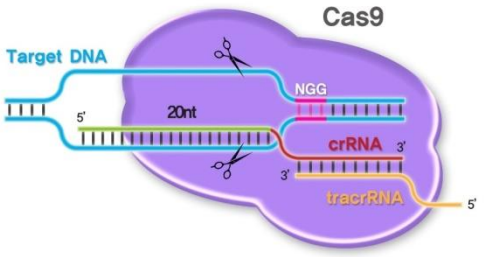
- CRISPR-Cpf1 is a class 2 CRISPR system
- Cpf1 is a CRISPR-associated two-component RNA-programmable DNA nuclease
- Targeted DNA is cleaved as a 5-nt staggered cut distal to a 5′ T-rich PAM
- Two Cpf1 orthologs exhibit robust nuclease activity in human cells

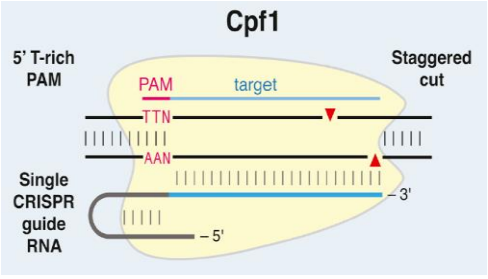


Zetsche et al., 2015, Cell *163*, 759–771
October 22, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.cell.2015.09.038

CellPress

Two CRISPR Tools





Cas9

- PAM:NGG
- gRNA: crRNA+tracrRNA
- DSB type: Blunt Cut

Cpf1

- PAM: TT(T)N
- gRNA: crRNA
- DSB type: Staggered Cut

Woo and Kim et al., 2015 Nat. Biotechnol.

Kim et al., 2017 Nat. Commun.

CRISPR-mediated Precise Plant Gene Editing

Precise Gene Editing in Plants

CRISPR offers an easy, exact way to alter genes to create traits such as disease resistance and drought tolerance.

Availability: 5-10 years

by David Talbot

Several genome-edited(GE) products with the support of USDA having "Am I regulate?" > The Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient (SECURE) rule

The common white button mushroom (*Agaricus bisporus*) has been modified to resist browning.

The US Department of Agriculture (USDA) will not regulate a mushroom genetically modified with the gene-editing tool CRISPR–Cas9.

Camelina or false flax is grown as an oilseed crop to produce vegetable oil and animal feed.

DuPont Pioneer's high amylopectin corn is the first CRISPR-edited plant likely to bypass USDA oversight. *Image: © Dinodia Photos / Alamy Stock Photo*

Table 1 CRISPR-edited plants in the pipeline that USDA will not oversee

Date of USDA response	Inquiring institution (location)	Plant trait engineered with CRISPR–Cas9
10/16/2017	USDA ARS, Plant Science Research Unit (St. Paul, Minnesota)	Soybean (*Glycine max*) with drought and salt tolerance; achieved by disrupting the *Drb2a* and *Drb2b* genes (double-stranded RNA-binding protein2 genes)
8/29/2017	Yield10 Bioscience (Woburn, Massachusetts)	Camelina with increased oil content; target genes not disclosed
4/07/2017	Donald Danforth Plant Science Center (St. Louis)	*Setaria viridis*, or green bristlegrass, with delayed flowering time; achieved by deactivating the *S. viridis* homolog of the *Zea mays* ID1 gene
4/18/2016	DuPont Pioneer (Johnston, Iowa)	Waxy corn with starch composed exclusively of amylopectin; achieved by inactivating the endogenous waxy gene *Wx1* that encodes a granule-bound start synthase catalyzing production of amylose
4/13/2016	The Pennsylvania State University (University Park, Pennsylvania)	White button mushroom (*Agaricus bisporus*) with anti-browning properties; achieved by knocking out a gene coding for polyphenol oxidase (*PPO*)

Source: USDA

Waltz E. *Nature Biotechnology* 2018

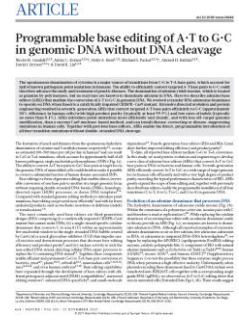
Base editors via engineered Cas9 proteins

Komor A. et al
Nature (2016. Apr)

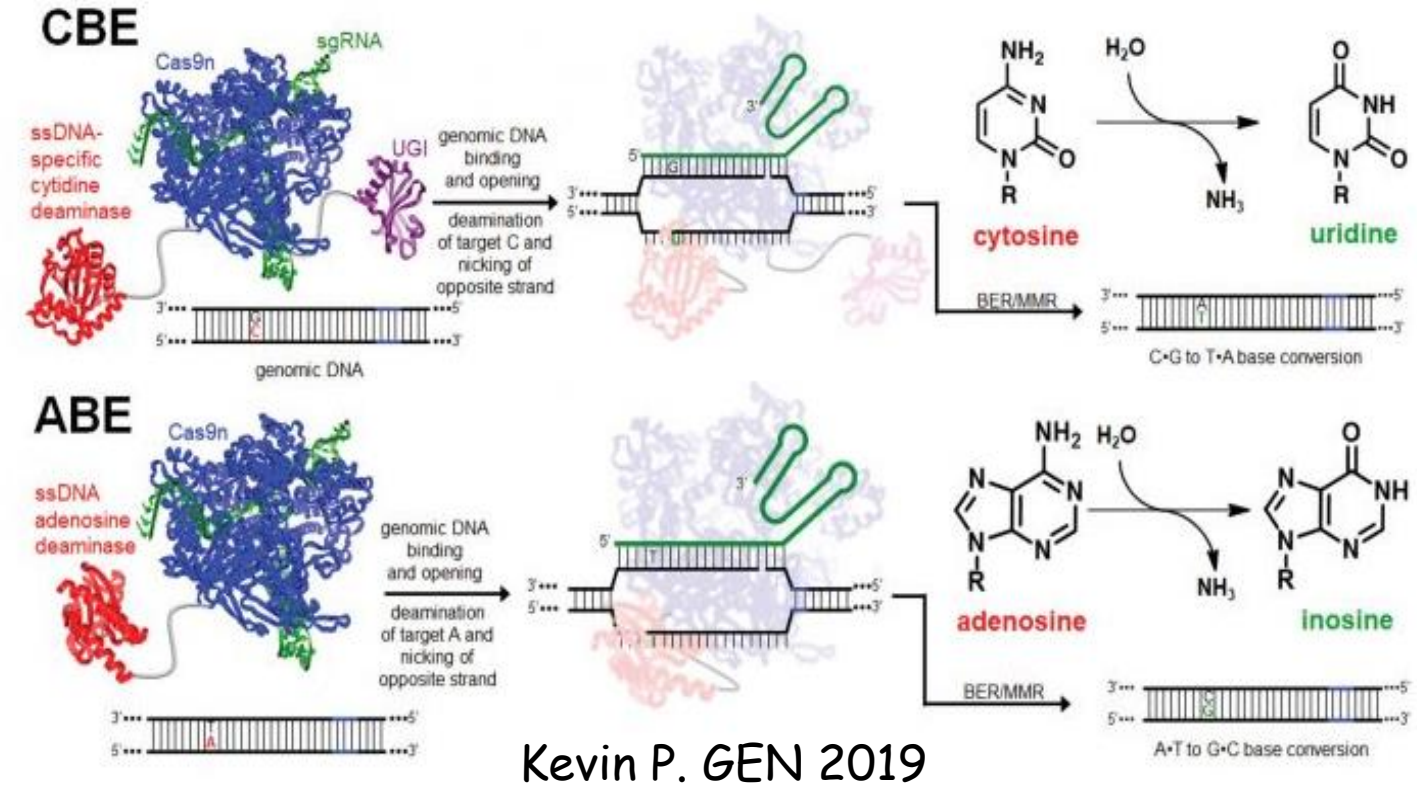

LETTER

Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage

Gaudelli N.M. et al
Nature (2017. Oct)


ARTICLE

Programmable base editing of A·T to G·C in genomic DNA without DNA cleavage



Kevin P. GEN 2019

Base editor applied three model plants

nature biotechnology

Published: 27 February 2017

Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion

Yuan Zong, Yanpeng Wang, Chao Li, Rui Zhang, Kunling Chen, Yidong Ran, Jin-Long Qiu, Daowen Wang & Caixia Gao ✉

Nature Biotechnology **35**, 438–440(2017) | Cite this article

5645 Accesses | **284** Citations | **49** Altmetric | Metrics

Abstract

Targeted base editing in plants without the need for a foreign DNA donor or double-stranded DNA cleavage would accelerate genome modification and breeding in a wide array of crops. We used a CRISPR–Cas9 nickase–cytidine deaminase fusion to achieve targeted conversion of cytosine to thymine from position 3 to 9 within the protospacer in both protoplasts and regenerated rice, wheat and maize plants at frequencies of up to 43.48%.

nature biotechnology

Published: 27 March 2017

Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion

Zenpei Shimatani, Sachiko Kashojiya, Mariko Takayama, Rie Terada, Takayuki Arazoe, Hisaki Ishii, Hiroshi Teramura, Tsuyoshi Yamamoto, Hiroki Komatsu, Kenji Miura, Hiroshi Ezura ✉, Keiji Nishida ✉, Tohru Ariizumi ✉ & Akihiko Kondo

Nature Biotechnology **35**, 441–443(2017) | Cite this article

5261 Accesses | **220** Citations | **51** Altmetric | Metrics

This article has been updated

Abstract

We applied a fusion of CRISPR-Cas9 and activation-induced cytidine deaminase (Target-AI
specified by single gui
induced multiple herb
editing using herbicid
marker-free plants wi
demonstrating the fea

CellPress PARTNER JOURNAL

Molecular Plant

Letter to the Editor

Generation of Targeted Point Mutations in Rice by a Modified CRISPR/Cas9 System

Dear Editor,

CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated Cas9 endonuclease)-mediated genome editing has revolutionized biological research and crop improvement because of its specificity, simplicity, and versatility (reviewed in Komor et al., 2016a). Editing a gene by CRISPR/Cas9 only has three requirements: (1) expression of the nuclear localized Cas9 protein; (2) production of a guide RNA (gRNA) molecule, whose first 20 nucleotides are complementary to the target gene; (3) the NGG PAM site that is located immediately adjacent to the 3′ end of the target sequence (Figure 1A). In the presence of a gRNA, Cas9 generates a double-strand break (DSB) at the target sequence. DSB repair by the error-prone, non-homologous end-joining (NHEJ) mechanism often leads to small deletions or insertions at the target site, providing a valuable venue to generate knockout and loss-of-function mutants. The majority of the reported CRISPR/Cas9-mediated gene editing in plants belongs to this category (Miao et al., 2013; Xie and Yang, 2013; Ma et al., 2015; Wang et al., 2015; Char et al., 2016; Gao et al., 2016). While such mutants are very valuable in defining gene functions, their applications in crop improvement are somewhat limited because many agriculturally important traits are conferred by single-nucleotide polymorphisms or by dominant gain-of-function point mutations. Homology-directed repair (HDR) of the DSB at specific locations can provide a feasible approach to achieve gene replacement. However, it has been very challenging to make use of HDR in plants to introduce point mutations because of the very low frequency of targeted integration. So far only a few special cases of CRISPR/Cas9-mediated HDR have been reported in plants (Sun et al., 2016). NHEJ-mediated repair may also be used to introduce point mutations through gene replacement if the target sequences of CRISPR/Cas9 are located in introns (Li et al., 2016). Nevertheless, it is still a major challenge to generate targeted point mutations in plants.

Cas9 has been engineered as a DNA-binding protein without the nuclease activities (dCas9), which has D10A and H840A mutations. A gRNA molecule brings dCas9 protein to a specific DNA target, thus offering unprecedented opportunities to modify structures or properties of DNA/chromatin near the target site (Komor et al., 2016a). Fusions of dCas9 with transcription activator or repressors have led to activation or repression of specific gene expression. A methyltransferase fusion with dCas9 caused modifications of histones in a gRNA-specific manner (Komor et al., 2016a). Recently, dCas9 fused with a cytidine deaminase enzyme was shown to have the ability to convert C to T (or G to A) within a window of approximately five nucleotides, providing a very useful tool to introduce point mutations at specific locus defined by a gRNA molecule (Komor et al., 2016b). Moreover, the system was further improved by combining a nicked Cas9 with only the D10A mutation (nCas9), a cytidine deaminase enzyme, and the uracil glycosylase inhibitor (UGI) that inhibits base-excision repair (Komor et al., 2016b). The improved version was named as BE3 (APOBEC1-XTEN-nCas9-UGI, the third generation of base editor, BE3 (Supplemental Figure 1). The complete sequence of BE3 unit, which is also available at https://www.addgene.org/73021/sequences/#depositor-full, targets the non-complementary strand for cleavage and confers desired base-editing outcomes through manipulating the cellular DNA repair response (Figure 1A). BE3 had 15%–75% base-editing efficiency in animal cells with very minimal indel formation (Komor et al., 2016b). Therefore, base editing expands the scope, ability, and efficiency of CRISPR/Cas9-mediated genome editing for introducing point mutations. However, it is not clear whether similar strategies will work in plant species, especially in crops.

In this study, we report the use of *n*Cas9 fused with a cytidine deaminase enzyme and the UGI to generate targeted point mutations in rice. We cloned the BE3 cassette to our pCXUN vector to generate pCXUN-BE3, which has the ability to target a specific locus in genome when a gRNA molecule is simultaneously expressed (Supplemental Figure 2). We used the maize (*Zea mays*) ubiquitin promoter to express BE3. We then inserted the expression cassette of a gRNA, which was under the control of the rice U3 promoter, into the PmeI site of pCXUN-BE3 (Supplemental Figure 2). We tested our base-editing vectors in rice for their feasibility and efficacy. Three targets were chosen: one target (P2) in the *OsPDS*, which encodes a phytoene desaturase, and two targets (S3 and S5) in the *OsSBEIIb*, which encodes a starch branching enzyme IIb in rice (Supplemental Table 1). We delivered the vectors into rice calli through *Agrobacterium*-mediated transformation.

For target S5 (Figure 1B), we transformed 93 calli and obtained 52 independent lines after two rounds of hygromycin selection and regeneration. We performed PCR amplification using the primer sets designed to specifically amplify *n*Cas9, gRNAs, and *hpt*II sequences, respectively (Supplemental Table 2). Results showed that all of the candidate plants tested contained *n*Cas9, gRNAs, and *hpt*II (Supplemental Figure 3). PCR/restriction enzyme (PR) analyses (see primers in Supplemental Table 2) were then used to screen for plants with targeted mutations (Figure 1B). Once the expected substitution of G to A in the target region (marked red in Figure 1B) takes place in S5 in *OsSBEIIb* gene, the *Bsl*NI restriction site will be disrupted. From 52 independent plants, we identified 23 lines that had *Bsl*NI-resistant bands (Figure 1B), suggesting that the mutagenesis efficiency is more than 40%. When the PCR

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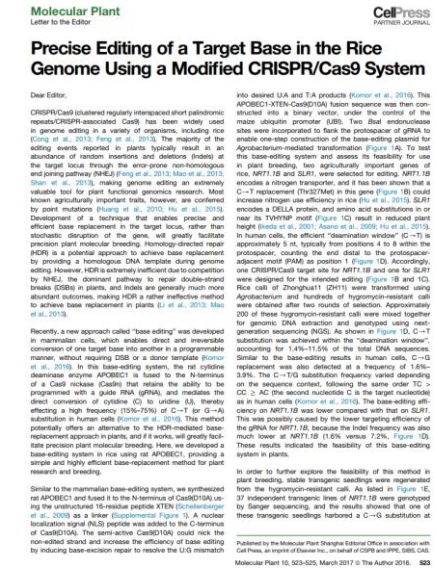

Molecular Plant

Letter to the Editor

CellPress PARTNER JOURNAL

Precise Editing of a Target Base in the Rice Genome Using a Modified CRISPR/Cas9 System

Dear Editor,

CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated Cas9) has been widely used in genome editing in a variety of organisms, including rice (Ding et al., 2013; Feng et al., 2013). The majority of the editing events reported in plants typically result in an abundance of random insertions and deletions (Indels) at the target locus through the error-prone non-homologous end joining pathway (NHEJ) (Feng et al., 2013; Mao et al., 2013; Shan et al., 2013), making genome editing an extremely valuable tool for plant functional genomics research. Most known agriculturally important traits, however, are conferred by point mutations (Huang et al., 2010; Hu et al., 2012). Development of a technique that enables precise and efficient base replacement in the target locus, rather than stochastic disruption of the gene, will greatly facilitate precision plant molecular breeding. Homology-directed repair (HDR) is a potential approach to achieve base replacement by providing a homologous DNA template during genome editing. However, HDR is extremely inefficient due to competition by NHEJ, the dominant pathway to repair double-strand breaks (DSBs) in plants, and Indels are generally much more abundant outcomes, making HDR a rather ineffective method to achieve base replacement in plants (Li et al., 2013; Mao et al., 2013).

Recently, a new approach called "base editing" was developed in mammalian cells, which enables direct and irreversible conversion of one target base into another in a programmable manner, without requiring DSB or a donor template (Komor et al., 2016). In this base-editing system, the rat cytidine deaminase enzyme APOBEC1 is fused to the N-terminus of a Cas9 nickase (Cas9n) that retains the ability to be programmed with a guide RNA (gRNA), and mediates the direct conversion of cytidine (C) to uridine (U), thereby effecting a high frequency (15%–75%) of C→T (or G→A) substitution in human cells (Komor et al., 2016). This method potentially offers an alternative to the HDR-mediated base-replacement approach in plants, and if it works, will greatly facilitate precision plant molecular breeding. Here, we developed a base-editing system in rice using rat APOBEC1, providing a simple and highly efficient base-replacement method for plant research and breeding.

Similar to the mammalian base-editing system, we synthesized rat APOBEC1 and fused it to the N-terminus of Cas9(D10A) using the unstructured 16-residue peptide XTEN (Schellenberger et al., 2009) as a linker (Supplemental Figure 1). A nuclear localization signal (NLS) peptide was added to the C-terminus of Cas9(D10A). The semi-active Cas9(D10A) could nick the non-edited strand and increase the efficiency of base editing by inducing base-excision repair to resolve the U:G mismatch into desired U:A and T:A products (Komor et al., 2016). This APOBEC1-XTEN-Cas9(D10A) fusion sequence was then constructed into a binary vector, under the control of the maize ubiquitin promoter (UBI). Two *Bsa*I endonuclease sites were incorporated to flank the protospacer of gRNA to enable one-step construction of the base-editing plasmid for *Agrobacterium*-mediated transformation (Figure 1A). To test this base-editing system and assess its feasibility for use in plant breeding, two agriculturally important genes of rice, *NRT1.1B* and *SLR1*, were selected for editing. *NRT1.1B* encodes a nitrogen transporter, and it has been shown that a C→T replacement (Thr327Met) in this gene (Figure 1B) could increase nitrogen use efficiency in rice (Hu et al., 2015). *SLR1* encodes a DELLA protein, and amino acid substitutions in or near its TVHYNP motif (Figure 1C) result in reduced plant height (Ikeda et al., 2001; Asano et al., 2009; Hu et al., 2015). In human cells, the efficient "deamination window" (C→T) is approximately 5 nt, typically from positions 4 to 8 within the protospacer, counting the end distal to the protospacer-adjacent motif (PAM) as position 1 (Figure 1D). Accordingly, one CRISPR/Cas9 target site for *NRT1.1B* and one for *SLR1* were designed for the intended editing (Figure 1B and 1C). Rice calli of Zhonghua11 (ZH11) were transformed using *Agrobacterium* and hundreds of hygromycin-resistant calli were obtained after two rounds of selection. Approximately 200 of these hygromycin-resistant calli were mixed together for genomic DNA extraction and genotyped using next-generation sequencing (NGS). As shown in Figure 1D, C→T substitution was achieved within the "deamination window", accounting for 1.4%–11.5% of the total DNA sequences. Similar to the base-editing results in human cells, C→G replacement was also detected at a frequency of 1.6%–3.9%. The C→T/G substitution frequency varied depending on the sequence context, following the same order TC > CC ≥ AC (the second nucleotide C is the target nucleotide) as in human cells (Komor et al., 2016). The base-editing efficiency on *NRT1.1B* was lower compared with that on *SLR1*. This was possibly caused by the lower targeting efficiency of the gRNA for *NRT1.1B*, because the Indel frequency was also much lower at *NRT1.1B* (1.6% versus 7.2%, Figure 1D). These results indicated the feasibility of this base-editing system in plants.

In order to further explore the feasibility of this method in plant breeding, stable transgenic seedlings were regenerated from the hygromycin-resistant calli. As listed in Figure 1E, 37 independent transgenic lines of *NRT1.1B* were genotyped by Sanger sequencing, and the results showed that one of these transgenic seedlings harbored a C→G substitution at

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Prime editor (2019, 10)

Article

Search-and-replace genome editing without double-strand breaks or donor DNA

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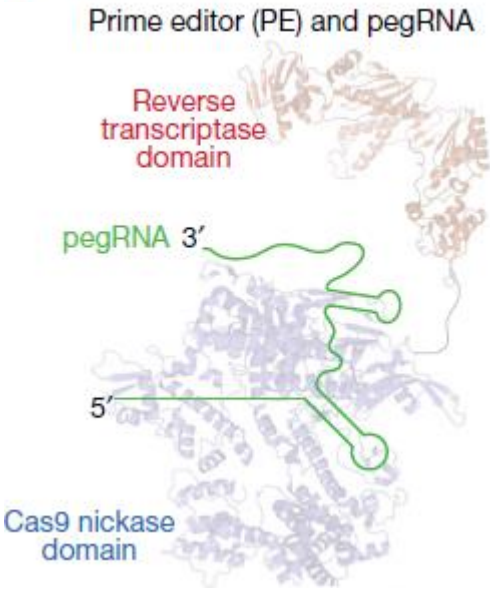
Most genetic variants that contribute to disease[1] are challenging to correct efficiently and without excess byproducts[2–5]. Here we describe prime editing, a versatile and precise genome editing method that directly writes new genetic information into a specified DNA site using a catalytically impaired Cas9 endonuclease fused to an engineered reverse transcriptase, programmed with a prime editing guide RNA (pegRNA) that both specifies the target site and encodes the desired edit. We performed more than 175 edits in human cells, including targeted insertions, deletions, and all 12 types of point mutation, without requiring double-strand breaks or donor DNA templates. We used prime editing in human cells to correct, efficiently and with few byproducts, the primary genetic causes of sickle cell disease (requiring a transversion in *HBB*) and Tay–Sachs disease (requiring a deletion in *HEXA*); to install a protective transversion in *PRNP*; and to insert various tags and epitopes precisely into target loci. Four human cell lines and primary post-mitotic mouse cortical neurons support prime editing with varying efficiencies. Prime editing shows higher or similar efficiency and fewer byproducts than homology-directed repair, has complementary strengths and weaknesses compared to base editing, and induces much lower off-target editing than Cas9 nuclease at known Cas9 off-target sites. Prime editing substantially expands the scope and capabilities of genome editing, and in principle could correct up to 89% of known genetic variants associated with human diseases.



Prime editor applied cereal plants

(2020 Mar. 16)

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BRIEF COMMUNICATION
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Prime genome editing in rice and wheat

Qiupeng Lin[1,2,6], Yuan Zong[1,6], Chenxiao Xue[1,2,6], Shengxing Wang[1], Shuai Jin[1,2], Zixu Zhu[1,2], Yanpeng Wang[1,2], Andrew V. Anzalone[3,4,5], Aditya Raguram[3,4,5], Jordan L. Doman[3,4,5], David R. Liu[3,4,5] and Caixia Gao[1,2]

Prime editors, which are CRISPR-Cas9 nickase (H840A)–reverse transcriptase fusions programmed with prime editing guide RNAs (pegRNAs), can edit bases in mammalian cells without donor DNA or double-strand breaks. We adapted prime editors for use in plants through codon, promoter, and editing-condition optimization. The resulting suite of plant

(Supplementary Fig. 1). We designed pOsU3-BFP-peg01 with an RT template for changing ACCCAC (threonine-histidine) to ACGTAC (threonine-tyrosine), with the edited bases at positions +1 and +2, counting from the first base 3′ of the pegRNA-induced nick. We introduced PPE, pUbi-BFP, pOsU3-BFP-peg01, and a nicking sgRNA into rice protoplasts. Flow cytometric analysis revealed 4.4%

Plant Prime Editing (PPE)
PPE2, PPE3, PPE3b
BFP → GFP

prime editor systems (PPEs): PPE2, PPE3, and PPE3b[11] (Fig. 1a). PPE2 consists of a nCas9(H840A) fused to an engineered M-MLV RT, and a pegRNA composed of a primer binding site (PBS) and an RT template[11]. PPE3 adds an additional nicking single guide RNA (sgRNA) to cleave the non-edited strand, which facilitates favorable DNA repair. In PPE3b, this nicking sgRNA targets the edited sequence, thereby preventing nicking of the non-edited strand until after editing occurs, resulting in fewer indels in mammalian cells[11].

We codon-optimized PPE genes for cereal plants and expressed them using the maize *Ubiquitin-1* (*Ubi-1*) promoter (Fig. 1b). We used the *OsU3* (or *TaU6*) and *TaU3* promoters to drive pegRNA and nicking sgRNA transcription, respectively. To test whether other RTs support prime editing, we replaced the engineered M-MLV RT with either the CaMV RT (RT-CaMV) from cauliflower mosaic virus[12] or a retron-derived RT (RT-retron) from *E. coli* B1.21 (ref. [13]) (Fig. 1b).

We first used our previously described[14] rice protoplast reporter system to test the PPE system for blue fluorescent protein (BFP) to green fluorescent protein (GFP) conversion, which requires changing codon 66 from CAC (histidine) to TAC (tyrosine) frequencies of up to 5.7% at the tested target sites in rice (Fig. 1e); in wheat, the frequencies of single nucleotide substitutions, including A-to-T, C-to-G, G-to-C, T-to-G, and C-to-A, reached 1.4% (Fig. 1f). PPE3 and PPE3b had a similar editing efficiency to PPE2 in the protoplast systems (Fig. 1e,f), indicating that the nicking sgRNA does not necessarily enhance prime editing efficiencies in plants, in contrast with observations in mammalian cells[11]. We also found that the PPE systems were less effective at the *OsCDC48-T3* and *OsEPSPS-T2* target sites despite the fact that indel frequencies generated by Cas9 nuclease at those sites were high (Fig. 1e and Supplementary Fig. 2), indicating that prime editing activity may not parallel Cas9 nuclease cleavage activity at some targets.

We observed PPE editing byproducts at 6 out of 21 tested targets at frequencies ranging from 0.5% to 4.9% (Fig. 1e,f). The main byproducts were pegRNA scaffold insertions or replacements (Supplementary Fig. 3), consistent with previous observations in mammalian cells[11].

When we examined prime editing of endogenous genes by the PPE-CaMV system, we found that PPE-CaMV generated the desired 6-bp deletion with 5.8% efficiency at the *OsCDC48-T1* site,

(2020 Mar. 25)

Molecular Plant
Correspondence
CellPress PARTNER JOURNAL

Plant Prime Editors Enable Precise Gene Editing in Rice Cells

Dear Editor,

Genome editing is revolutionizing plant research and crop breeding. Sequence-specific nucleases (SSNs) such as zinc finger nuclease (ZFN) and TAL effector nuclease (TALEN) have been used to create site-specific DNA double-strand breaks and to achieve precise DNA modifications by promoting homology-directed repair (HDR) (Steinert et al., 2016; Voytas, 2013). Later, RNA-guided SSNs such as CRISPR-Cas9, Cas12a, Cas12b, and their variants were applied for genome editing in plants (Li et al., 2013; Nekrasov et al., 2013; Tang et al., 2017; Zhong et al., 2019; Ming et al., 2020; Tang et al., 2019). However, HDR relies on simultaneous delivery of SSNs and DNA donors, which has been challenging in plants (Steinert et al., 2016; Zhang et al., 2019). Another challenge for realizing efficient HDR in plants is that DNA repair favors non-homologous end joining (NHEJ) pathways over HDR in most cell types (Puchta, 2005; Qi et al., 2013). Unlike SSN-induced HDR, which is limited by the choice of the donor and DNA repair mechanism, cytidine or adenine base editors that were developed in recent years can convert C to T or A to G within a 3–8 nucleotide targeting window in the protospacer, respectively (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017). Base editors, albeit highly effici
mutations, but ca
mutations or insert
contexts, the recent
called prime editor
(Anzalone et al., 201
fused with a reverse
editing DNA strand t
the nicked DNA and
prime editing-guide
modified single-guid
transcription (RT) te
primer in sequence a
as in HDR. PEs also
as base editors at ce

We were interested in
in plants and did our
seminal article by L
Prime Editor 3 syste
generated a Plant Pri
Cas9(H840A and the
(M-MLV) RT were b
(Figure 1A). The peg
non-edited strand we
respectively (Figure 1
the nsgRNA would ni
the editing site. We c

OsKO2, *OsDEP1*, and *OsPDS*) and decided to primarily use the 13-nt length for both the PBS and the RT templates. Rice protoplasts were transfected with the resulting T-DNA expression vectors of PPE3-V01 and the editing was analyzed by next-generation sequencing (NGS) of PCR amplicons. Anticipated prime editing outcomes were confirmed at all five sites, although the editing efficiencies at these sites were quite low (0.05%–0.15%) (Figure 1B). The positive reads of the NGS data were validated for precise incorporation of designed edits, and some reads revealed large deletions presumably due to paired nicking when pairing the Cas9H840A nickase with two guide RNAs (a pegRNA and an nsgRNA) in the PB3 system (Figure 1C). To minimize these deletion byproducts, we applied the PB3b strategy where the nsgRNA was designed to match the edited strand, but not the *wild*-type sequence. We also wanted to try out different target sites, hoping to see improved editing frequency at any of them. Hence, we targeted five additional sites in four genes (*OsALS*, *OsEPSPS*, *OsGRF4*, and *OsSPL14*) with our PPE3b-V01 system. This time, we also tried variable lengths for RT templates, ranging from 13 nt to 23 nt. Prime editing outcomes were again observed at these five sites, with the highest editing frequencies up to 0.4% (by *OsEPSPS*-pegR01) (Figure 1D). Analysis of the NGS data suggested most of positive reads represented precise prime

(2020 Mar. 28)

Molecular Plant
Correspondence
CellPress PARTNER JOURNAL

Versatile Nucleotides Substitution in Plant Using an Improved Prime Editing System

Dear Editor,

Base editors (BEs) based on the CRISPR/Cas9 system, including cytosine base editors and adenine base editors, which can efficiently perform four transition mutations (C-G-to-T-A and A-T-to-G-C), have been well studied and widely used to produce base mutations in a variety of organisms, including in plants such as rice. However, manipulation of many agronomic traits in crops may require the other eight nucleotide substitutions (A-T-to-C-G, C-G-to-A-T, T-A-to-A-T and G-C-to-C-G). In addition, for BEs, the target bases are limited to an editing

site (OsALS-2), with the edited base at position +9 (Figure 1I and 1G). The primer-binding site (PBS) lengths were 8 bp and 12 bp in pegRNA-02 and pegRNA-03 (Supplemental Table 2). The above three vectors were introduced into rice calli by *Agrobacterium*-mediated transformation and 87, 98, and 88 transgenic events were regenerated, respectively. Based on the Sanger sequencing results, the expected G-to-T and specific C-to-T substitutions were obtained at the OsALS-1 and OsALS-2 targets using pegRNA-01 and pegRNA-03, achieving editing efficiencies of 1.1% (1/87) and 1.1% (1/88)

Prime Editor-Plant version (PE-P1, PE-P2)
PE3
OsALS, *OsACC*, *OsDEP1*

the multiple Cs near the target C. Therefore, we first designed pegRNA-01 with a reverse transcriptase (RT) template 14 nt in

tween the 5′ flap that contains the unedited DNA sequence and the 3′ flap that contains the edited sequence copied from the

Precise Modifications of Both Exogenous and Endogenous Genes in Rice by Prime Editing

Dear Editor,

Harnessing genetic diversity and the introduction of elite alleles from wild relatives or landraces into commercial cultivars has been a major goal in crop breeding programs. Precise modification of the plant genome through clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein

a similar strategy will work efficiently in plant species, especially in crops.

In this study, to evaluate the feasibility and efficacy of PE3 prime editor in precision genome editing in rice, we first mutated Cas9 into nCas9(H840A) in our pCXUN-Ubi-NLS-Cas9-NLS-PolyA-E9 vector (Supplemental Figure 1A), in which the expression of the rice

PE3 + polycistronic tRNA
Inactive Hyg → active

(NHEJ) is the predominant DNA repair pathway in plants (Baltes
J et al., 2019), it remains challenging, especially in
Thus, it is essential to further exploit more efficient
nome-editing technology in order to accelerate
ment.

rime editing system, which enables targeted inser-
tions, deletions, and all 12 classes of point mutations, without
and breaks or a DNA donor repair template,
ion efficiently in mammalian cells (Anzalone
report, a third generation of prime editor
ered by fusing a mutated M-MLV-RT
kemia virus reverse transcriptase) to the C
talytically impaired Cas9 (H840A) (Cas9
d programmed with a prime editing guide
nposed of a single chimeric guide RNA
the specific site, a primer-binding site
e transcription (RT) template encoding the
3 complex binds the target DNA and nicks
strand, and uses a nicked genomic DNA
for the synthesis of an edited DNA flap by
RT template on the pegRNA. Subsequent
orates the edited DNA flap on the non-
ther copies the edit into the complementary
ing in stably edited DNA. At the same time,
RNA at various distances from the nicks
was used to direct a second cut on the
rease the chances of repairing this strand
sequence (Figure 1A). The prime editing
antially expand the scope and capabilities
precise modification of plant genomes
nt transversion and targeted gene/allele
improvement. It remains unclear whether

NLS-nCas9(H840A)-Linker1(33aa)-M-MLV-RT-Linker2(14aa)-NLS-PolyA-E9-Actin-Nos (hereafter referred to as the prime editor-basic) (Supplemental Figure 1C). In order to test the feasibility of the prime editor-basic vector in precision editing of exogenous gene, we further mutated the *hptII* gene in our prime editor-basic vector at position Gly 45 (GGA) to TGA and Tyr 46 (TAT) to TAG to generate a prime editor-basic-*hptII*-mutant vector (Supplemental Figure 1D). The introduction of these two point mutations will disable the ability of the *hptII* gene to confer hygromycin resistance on rice calli during selection. We then designed a pegRNA composed of an sgRNA, a 28-bp RT (including two synonymous mutations and two mutations to restore the two stop codons into original GGA and TAT) and a 13-bp PBS, which are reversely complementary to the non-target strand, and another nicking sgRNA for a second cut, which is located at a distance of 50-bp upstream from the nick induced by pegRNA on the non-target strand (Figure 1B). Then, taking advantage of the automatic tRNA self-processing activity *in vivo* (Xie et al., 2015), we used the polycistronic tRNA strategy to simultaneously produce pegRNA and nicking sgRNA. We cloned the tRNA-pegRNA-tRNA-sgRNA-tRNA-PolyA complex into this vector to generate prime editor-*hptII* mutant vector, in which the pegRNA and nicking sgRNA were separated by two tRNAs and driven by a single constitutive rice *Actin* promoter and terminated by a *PolyA* sequence to increase the stability of pegRNA and nicking sgRNA transcripts and a *Nos* terminator (Supplemental Figure 1E). We delivered this vector into rice (*Japonica cv. Zhonghua 11*) calli by particle bombardment. Subsequently, the calli were treated at 30°C for 4 h and then

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Prime editor applied cereal plants

(2020 Apr. 8)

(2020 Apr. 27)

(2020 May. 10)

PE2 > PE3

Inactive Hyg → active

Rice

PE2/ PE3

Inactive GFP → active

OsALS

PE2/ PE3

OsALS, OsTB1, OsIPA

Prime editing is achievable in the tetraploid potato, but needs improvement

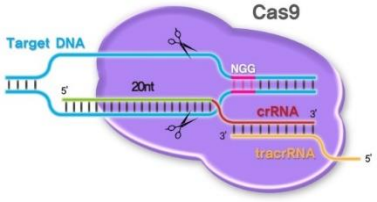
Prime editing efficiently generates … in two ALS genes of maize

CRISPR-based gene editing in crops

Gene Editing Plants without DNA

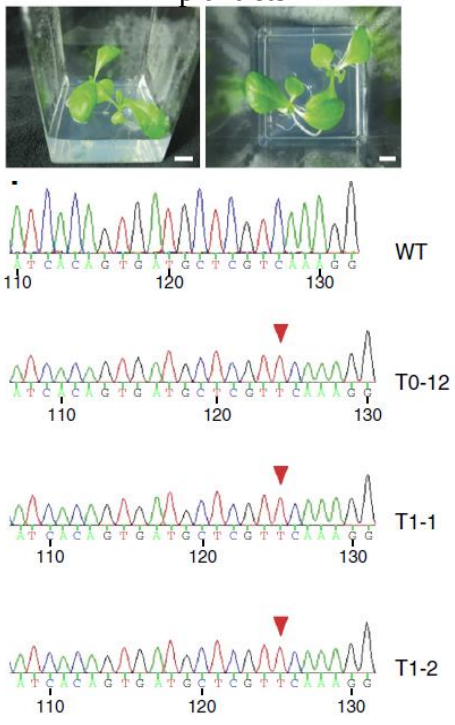
Woo J. and Kim E. et al., *Nat. Biotechnol.* 2015

Cas9 RNP



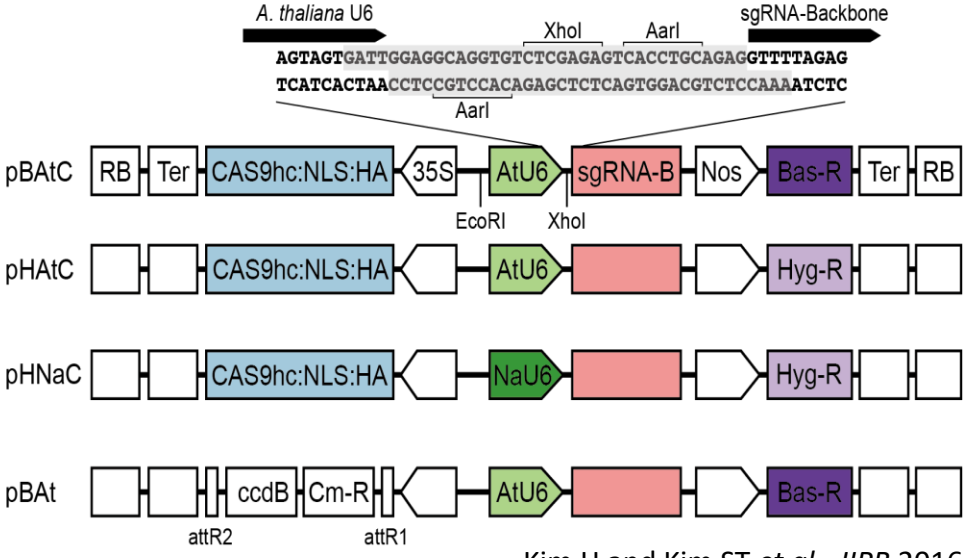
	N. attenuata *AOC*		*A. thaliana* *PHYB*		*O. sativa* *P450*	
RGEN RNP	–	+	–	+	–	+
Indels (%)						
T7E1	17		19		21	
NGS	44		16		19	

AOC		*PHYB*		*P450*	
CAAAAGACTGTCAATTC-CCTTGG	WT	CACTAGGAGCAACACCC-AACGGG	WT	CATATAGTTGGGTCATG-GCATGG	WT
CAAAAGACTGTCAATTCACCTTGG	+1	CACTAGGAGCAACACCCAAACGGG	+1	CATATAGTTGGGTCAT--GCATGG	-1
CAAAAGACTGTCAATTCTCCTTGG	+1	CACTAGGAGCAACACC--AACGGG	-1	CATATAGTTGGGTC----GCATGG	-3
CAAAAGACTGTCAATTCCCCTTGG	+1	CACTAGGAGCAACACCCCAACGGG	+1	CATATAGTTGGGc-----GCATGG	-4
CAAAAGACTGTCAATT--CCTTGG	-1	CACTAGGAGCAACAC---AACGGG	-2	CATATAGTTGGGT------CATGG	-5
		CACTAGGAGCAAC-----AACGGG	-4		



- Developed a method to edit the genome of plants without the introduction of foreign DNA (A DNA free, Cas9 RNP method)

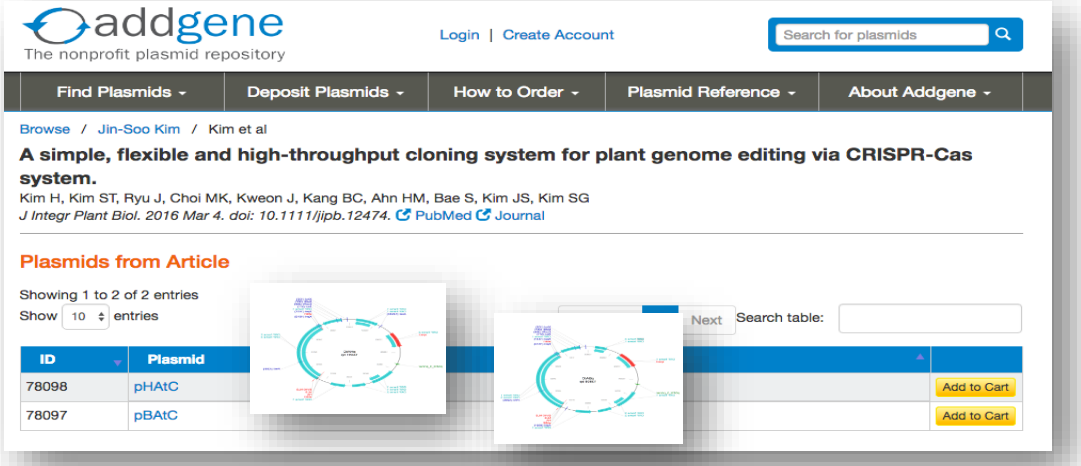
A Simple and Versatile Binary Vector, pH(B)AtC



Kim H and Kim ST *et al.*, *JIPB* 2016

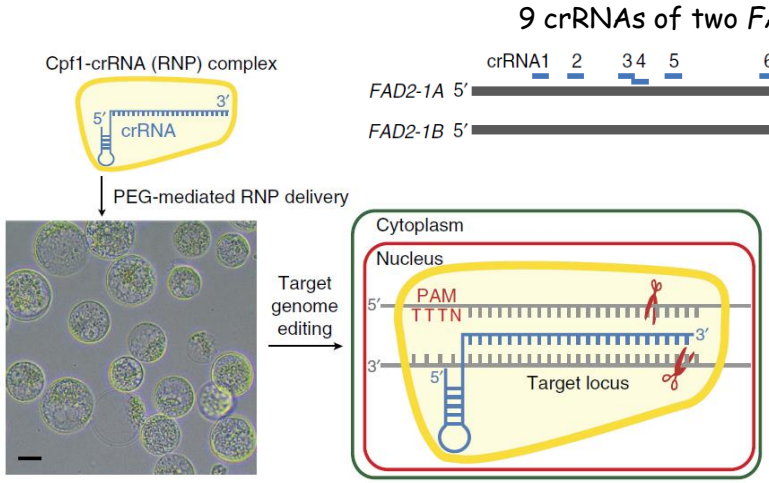
www.addgene.org /78097 or 78098

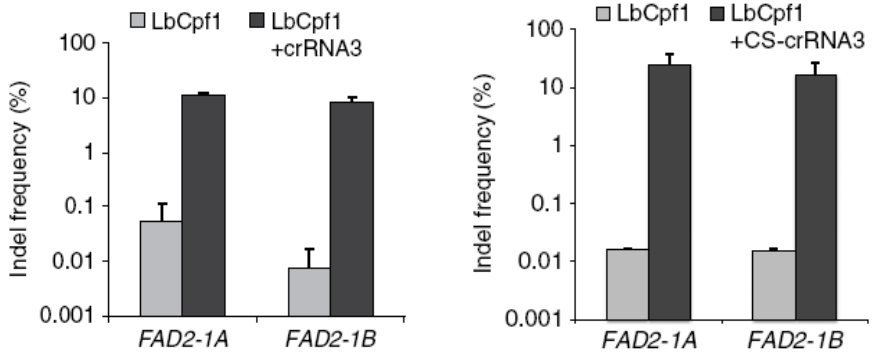
Institute for Basic Science



CRISPR-Cpf1 mediated DNA-free Soybean editing

Kim H. et al., *Nat. Commun.* 2017





LbCpf1 + CS-crRNA3

FAD2-1A locus

		Total reads#
CTTTTAGTCCCTTATTTCTCATGGAAAATAAGCCATCGCCGCCATCACTCCAACACAGGTTC	WT	55284
CTTTTAGTCCCTTATTTCTC-------ATAAGCCATCGCCGCCATCACTCCAACACAGGTTC	−7	3003
CTTTTAGTCCCTTATTTCTC----------AGCCATCGCCGCCATCACTCCAACACAGGTTC	−10	1052
CTTTTAGTCCCTTATTTCTC---------AAGCCATCGCCGCCATCACTCCAACACAGGTTC	−9	1008
CTTTTAGTCCCTTATTTCTCAT---------GCCATCGCCGCCATCACTCCAACACAGGTTC	−9	866
CTTTTAGTCCCTTATTTCTCAT----------CCATCGCCGCCATCACTCCAACACAGGTTC	−10	647
CTTTTAGTCCCTTATTTCTCATG--------GCCATCGCCGCCATCACTCCAACACAGGTTC	−8	593
CTTTTAGTCCCTTATTTCTCAT-GAAAATAAGCCATCGCCGCCATCACTCCAACACAGGTTC	−1	576
CTTTTAGTCCCTTATTTCT----------AAGCCATCGCCGCCATCACTCCAACACAGGTTC	−10	501
CTTTTAGTCCCTTATTTCTCAT--------AGCCATCGCCGCCATCACTCCAACACAGGTTC	−8	488
CTTTTAGTCCCTTATTTCTCATGG-AAATAAGCCATCGCCGCCATCACTCCAACACAGGTTC	−1	385

FAD2-1B locus

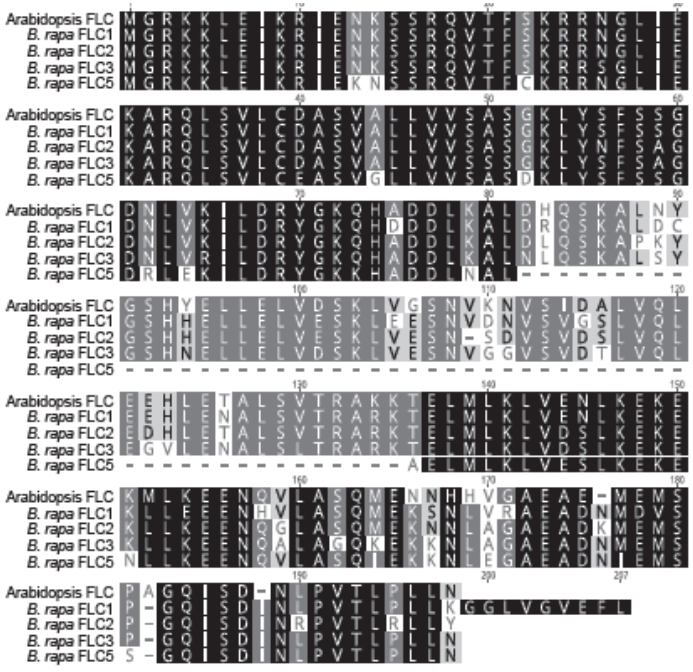
		Total reads#
CTTTTAGTCCCTTATTTCTCATGGAAAATAAGCCATCGCCGCCATCACTCCAACACAGGTTC	WT	15692
CTTTTAGTCCCTTATTTCTC-------ATAAGCCATCGCCGCCATCACTCCAACACAGGTTC	−7	2188
CTTTTAGTCCCTTATTTCTCAT---------GCCATCGCCGCCATCACTCCAACACAGGTTC	−9	1091
CTTTTAGTCCCTTATTTCTC---------AAGCCATCGCCGCCATCACTCCAACACAGGTTC	−9	696
CTTTTAGTCCCTTATTTCTCAT-GAAAATAAGCCATCGCCGCCATCACTCCAACACAGGTTC	−1	479
CTTTTAGTCCCTTATTTCTCAT----------CCATCGCCGCCATCACTCCAACACAGGTTC	−10	469
CTTTTAGTCCCTTATTTCTC----------AGCCATCGCCGCCATCACTCCAACACAGGTTC	−10	430
CTTTTAGTCCCTTATTTCTCAT--------AGCCATCGCCGCCATCACTCCAACACAGGTTC	−8	363
CTTTTAGTCCCTTATTT-----------AAGCCATCGCCGCCATCACTCCAACACAGGTTC	−12	352
CTTTTAGTCCCTTATTTCT----------AAGCCATCGCCGCCATCACTCCAACACAGGTTC	−10	305
CTTTTAGTCCCTTATTT-------------GCCATCGCCGCCATCACTCCAACACAGGTTC	−14	294

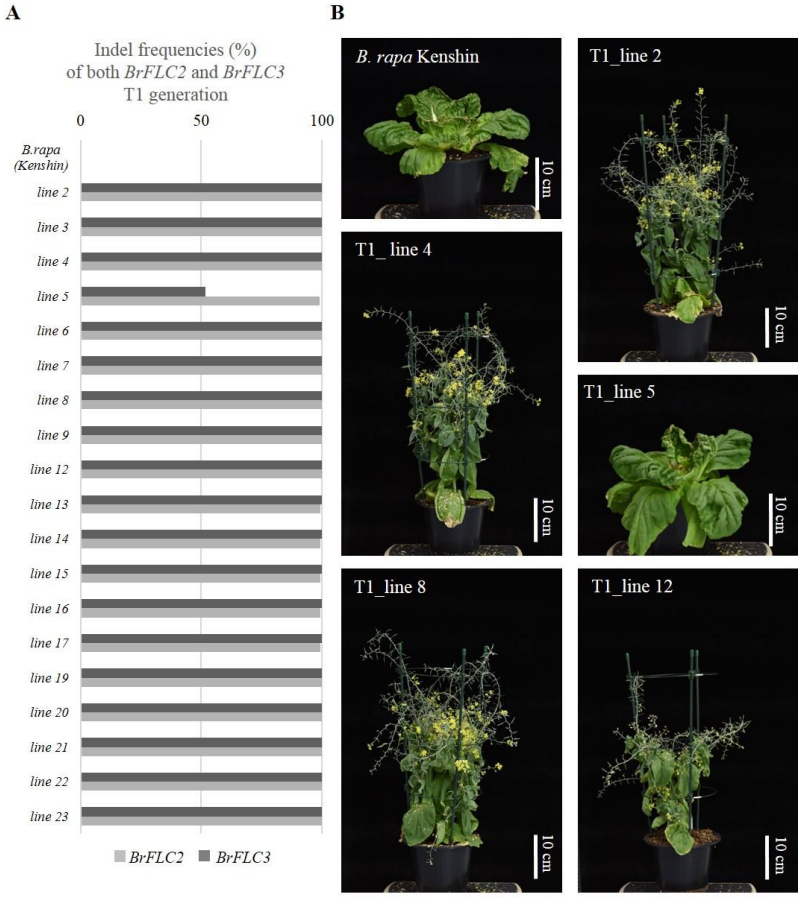
- Improved the DNA-free method to edit the genome of soybean with chemically synthesized crRNAs

Early-flowering Chinese cabbage via CRIRSP-Cas9 mediated editing

Jung S.Y. et al., *Plant Biotechnol Rep 2019*

Arabidopsis FLC orthologs in B. rapa

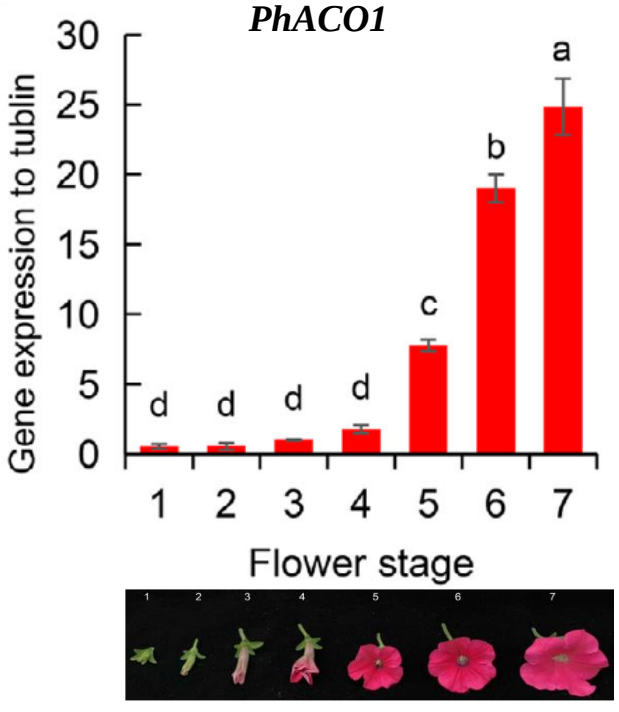




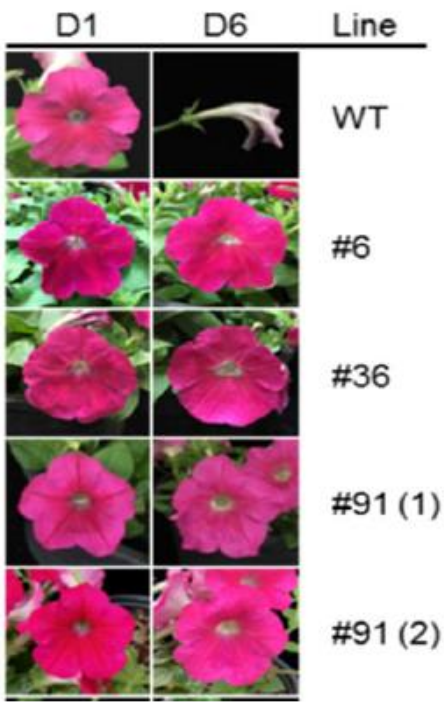
- Understood the role of BraFLC2 and BraFLC3 in flowering without vernalization

CRISPR-Cas9 mediated Petunia editing for enhanced flower longevity

Xu J. et al., *Plant Biotechnol. J 2020*



sg1	ATCAGCTTGGACAAAGT-GAATGG	WT	Reads (%)
#6	ATCAGCTTGGACAAAGT-GAATGG	WT	49.74
	ATCAGCTTGGACAAAGTtGAATGG	+1 T	44.04
#36	ATCAGCTTGGA-----T-GAATGG	-5 Del	49.24
	ATCAGCTTGGACAAAGT-GAATGG	WT	45.07
#91(1)	ATCAGCT----------GAATGG	-10 Del	100.0
#91(2)	ATCAGCTTGGAC----T-GAATGG	-4 Del	52.13
	ATCAGCTTGGACAAAGT-GAATGG	WT	40.17
	ATCAGCTTGGACAAAGTtGAATGG	+1 T	0.99
	ATCAGCTTGGAC------GAATGG	-5 Del	0.51



- Understood the role of PhACO1 in ethylene production and applied the CRISPR-Cas9 tool for the improvement of floricultural quality

CRISPR based precise gene editing in pepper

Capsicum (bell and hot peppers)

- The most economically important vegetable crop in the world, especially Asian countries
- A member of the *Solanaceae* family that include tobacco, tomato, and petunia
- Whole Genome sequence were released since 2014

  Genome Size: about 3.5 Gb,

  Capsicum annuum 'CM334' (Nat. Genet., 2014), 'Zunla-1' (PNAS, 2014)
- Strong research background in Korea

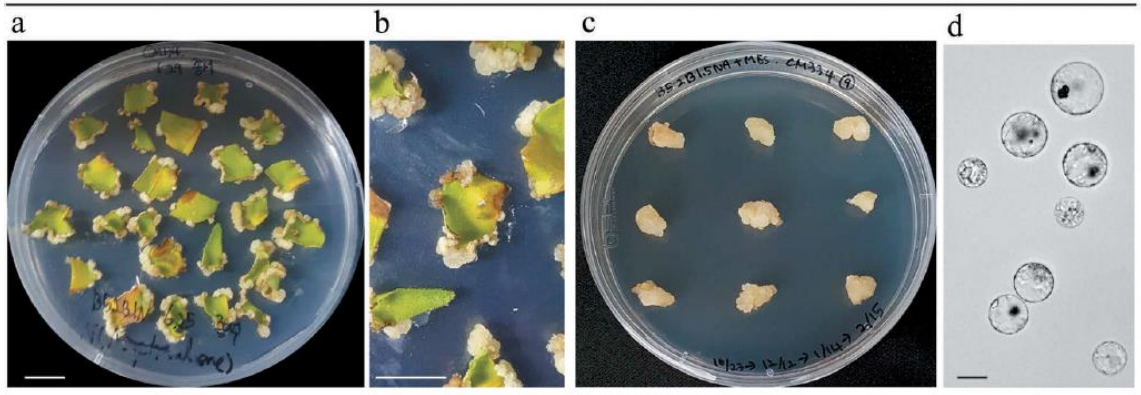
  Reference Genome Sequence

  Molecular markers for major traits,

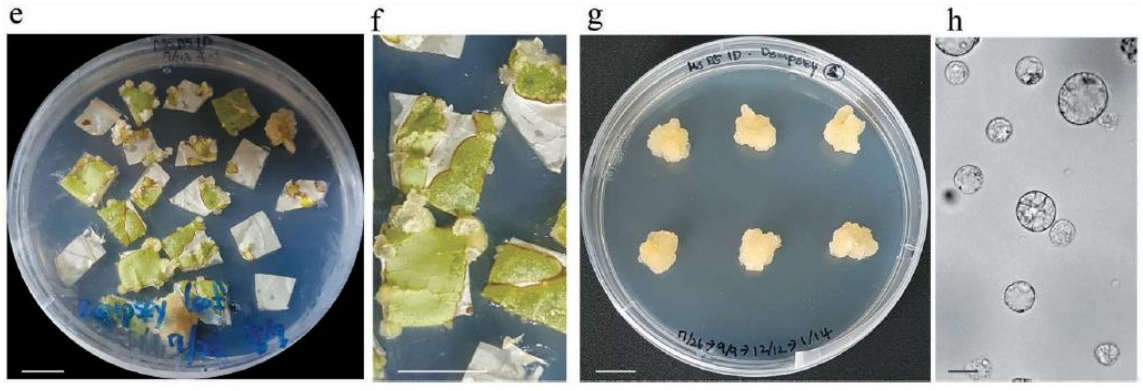
  High density genetic maps

Pepper leaf-induced callus formation in hot pepper CM334 and bell pepper Dempsey

CM334

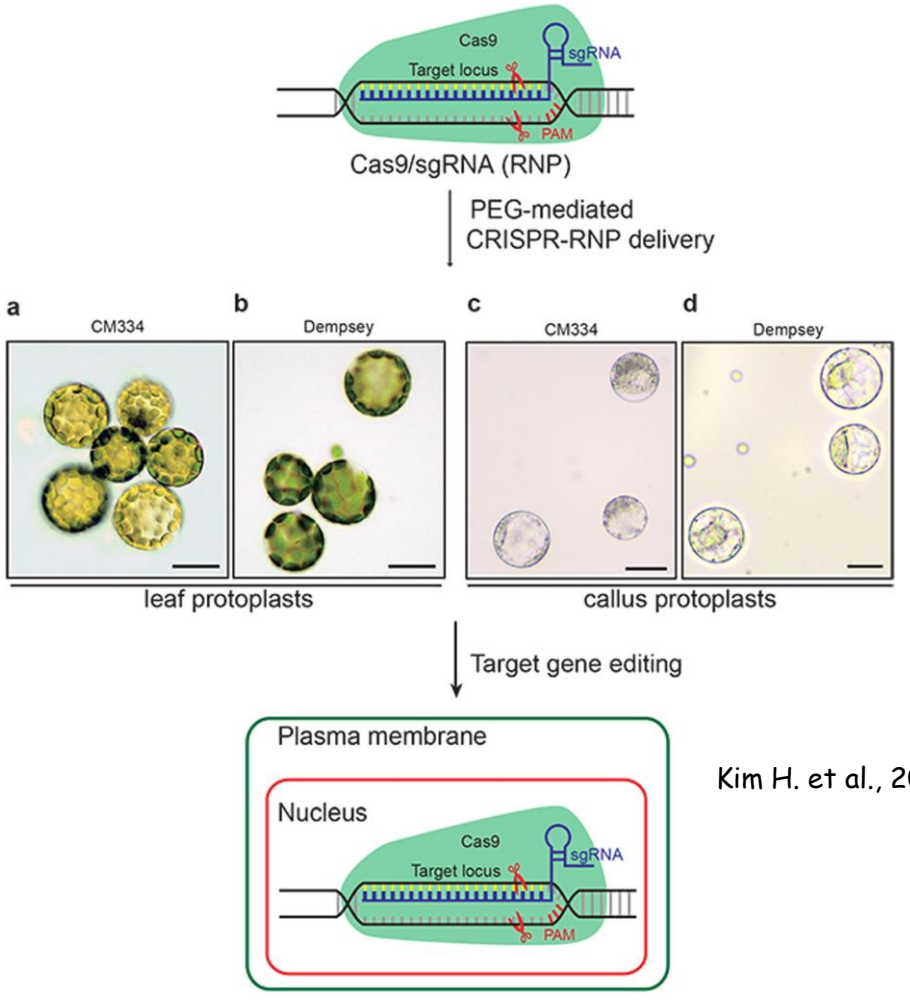


Dempsey



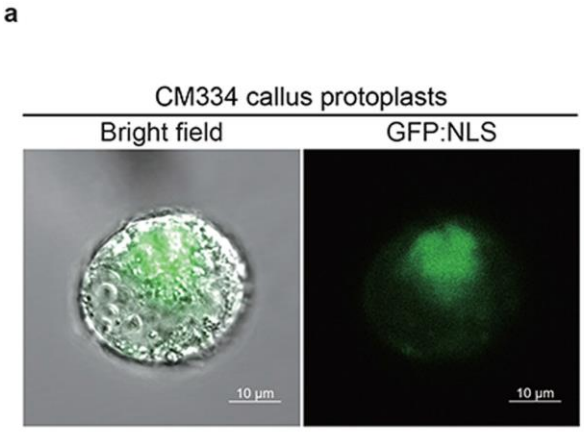
Kim H. et al., 2019 *Plant Signal. Behav.*

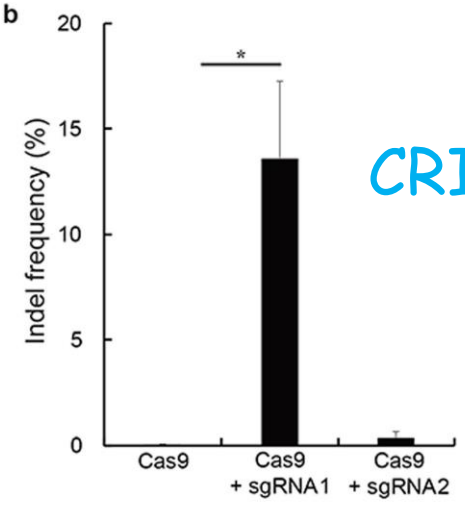
Schematic overview of CRISPR-mediated genome editing in pepper protoplasts.



Kim H. et al., 2020 *BMC Plant Biology*

CM334-callus-protoplasts as a screening system for CRISPR RNPs





CRISPR/Cas9

c

CaMLO2 sgRNA1 locus

			Indel %	Reads #	total #
CM334	TGGCATCCTTGT-AAGGCAGATGAAGATGTCAAGTCTGAGT	WT		14451	17535
			17.6	3084	
	TGGCATCCTTGT--AGGCAGATGAAGATGTCAAGTCTGAGT	-1	3.9	676	
	TGGCATCCTTGT----GCAGATGAAGATGTCAAGTCTGAGT	-3	3.7	644	
	TGGCATCCTTGT--AGGCAGATGAAGATGTCAAGTCTGAGT	-2	3.2	566	
	TGGCATCCTTGTaAAGGCAGATGAAGATGTCAAGTCTGAGT	+1	2.3	400	
	TGGCATCCTTGT------AGATGAAGATGTCAAGTCTGAGT	-5	1.8	312	

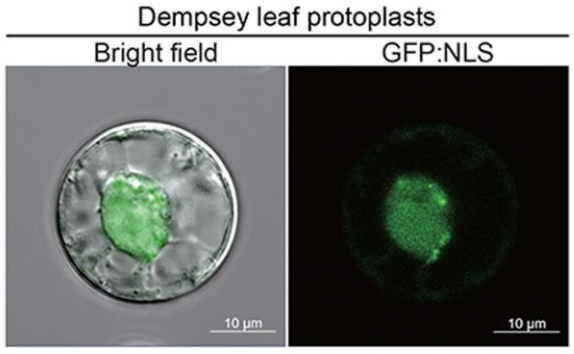
CaMLO2 sgRNA2 locus

		Indel %	Reads #	total #
TGAGTATGATGACCCTTGTTTAC-AAAAGGTACAATGGTTA	WT		24185	24226
		0.2	41	
TGAGTATGATGACCCTTG----C-AAAAGGTACAATGGTTA	-4	0.1	19	
TGAGTATGATGACCCTTGTTTACcAAAAGGTACAATGGTTA	+1	0.0	9	
TGAGTATGATGACCCTTGTTTAC--AAAGGTACAATGGTTA	-1	0.0	7	

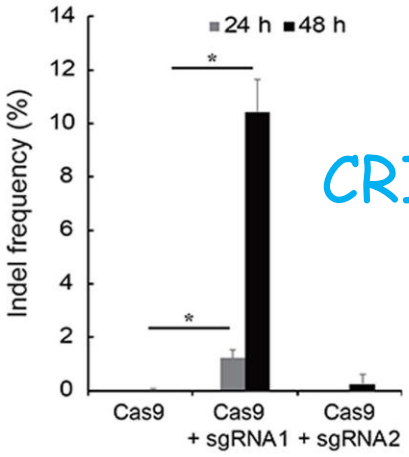
Kim H. et al., 2020 *BMC Plant Biology*

Dempsey-leaf-protoplasts as a screening system for CRISPR RNPs

a



b



CRISPR/Cas9

c

CaMLO2 sgRNA1 locus

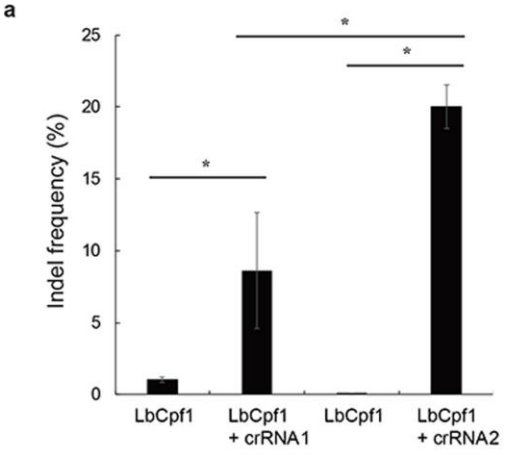
		Indel %	Reads #	total #
Dempsey	TGGCATCCTTGTAAGGCAGATGAAGATGTCAAGTCTGAGT WT		9587	10807
		11.3	1220	
	TGGCATCCTTGT-AGGCAGATGAAGATGTCAAGTCTGAGT -1	3.9	420	
	TGGCATCCTTGT---GCAGATGAAGATGTCAAGTCTGAGT -3	2.7	291	
	TGGCATCCTTGT--GGCAGATGAAGATGTCAAGTCTGAGT -2	1.8	191	
	TGGCATCCTTGT------GATGAAGATGTCAAGTCTGAGT -6	0.9	95	
	TGGCATCCTTG-------GATGAAGATGTCAAGTCTGAGT -7	0.6	65	

CaMLO2 sgRNA2 locus

		Indel %	Reads #	total #
	TGAGTATGATGACCCTTGTTTAC-AAAAGGTACAATGGTTA WT		8720	8764
		0.5	44	
	TGAGTATGATGACCCTTGTTTACtAAAAGGTACAATGGTTA +1	0.3	24	
	TGAGTATGAT----------AAAAGGTACAATGGTTA -13	0.2	20	

Kim H. et al., 2020 *BMC Plant Biology*

Development of callus-protoplasts screening systems for CRISPR-RNPs in peppers



CRISPR/Cpf1

b

CaMLO2 crRNA1 locus

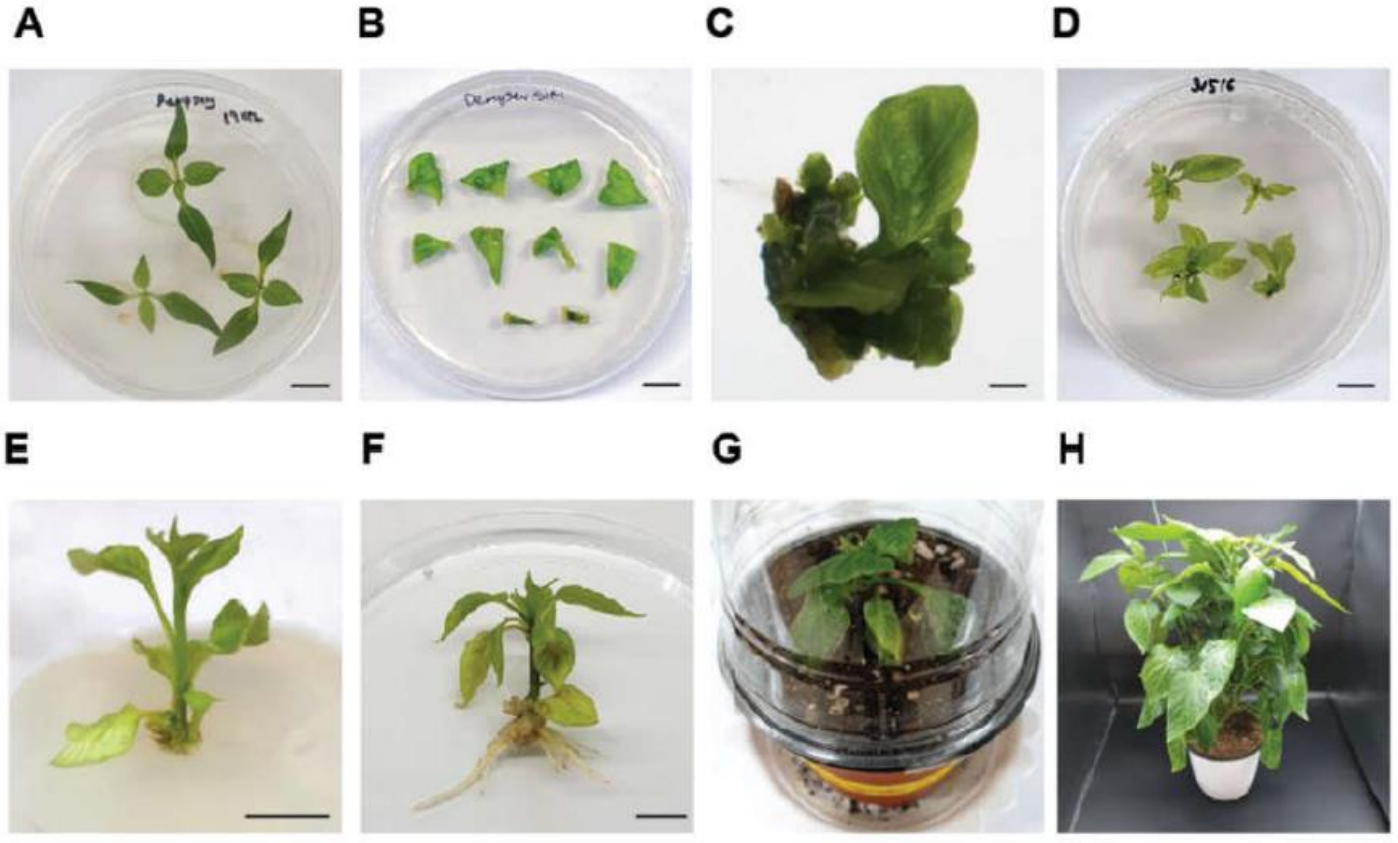
			Indel %	Reads #	total #
	CCATTTTTATTGAACAAATTATGCATCACCTTGGAGAGGT	WT		18045	20020
			9.9	1975	
CM334	CCATTTTTATTGAACAAATTATG------CTTGGAGAGGT	-6	1.6	318	
	CCATTTTTATTGAACAAATTATGCA------TGGAGAGGT	-6	0.6	119	
	CCATTTTTATTGAACAAATTATGCA-----TTGGAGAGGT	-5	0.5	106	
	CCATTTTTATTGAACAAATTA----------TGGAGAGGT	-10	0.4	87	
	CCATTTTTATTGAACAAATTATG-----CCTTGGAGAGGT	-5	0.4	86	

CaMLO2 crRNA2 locus

			Indel %	Reads #	total #
	ATACAAGACCCAGTTTCTAACTTATGTGTCCCCAAAAGTG	WT		21015	26032
			19.3	5017	
	ATACAAGACCC-------AACTTATGTGTCCCCAAAAGTG	-7	2.4	927	
CM334	ATACAAGACC------CTAACTTATGTGTCCCCAAAAGTG	-6	2.3	600	
	ATACAAGACC----TTCTAACTTATGTGTCCCCAAAAGTG	-4	2.2	551	
	ATACAAGACCC------------ATGTGTCCCCAAAAGTG	-12	1.7	446	
	ATACAAGACCCAG--TCTAACTTATGTGTCCCCAAAAGTG	-2	1.4	363	

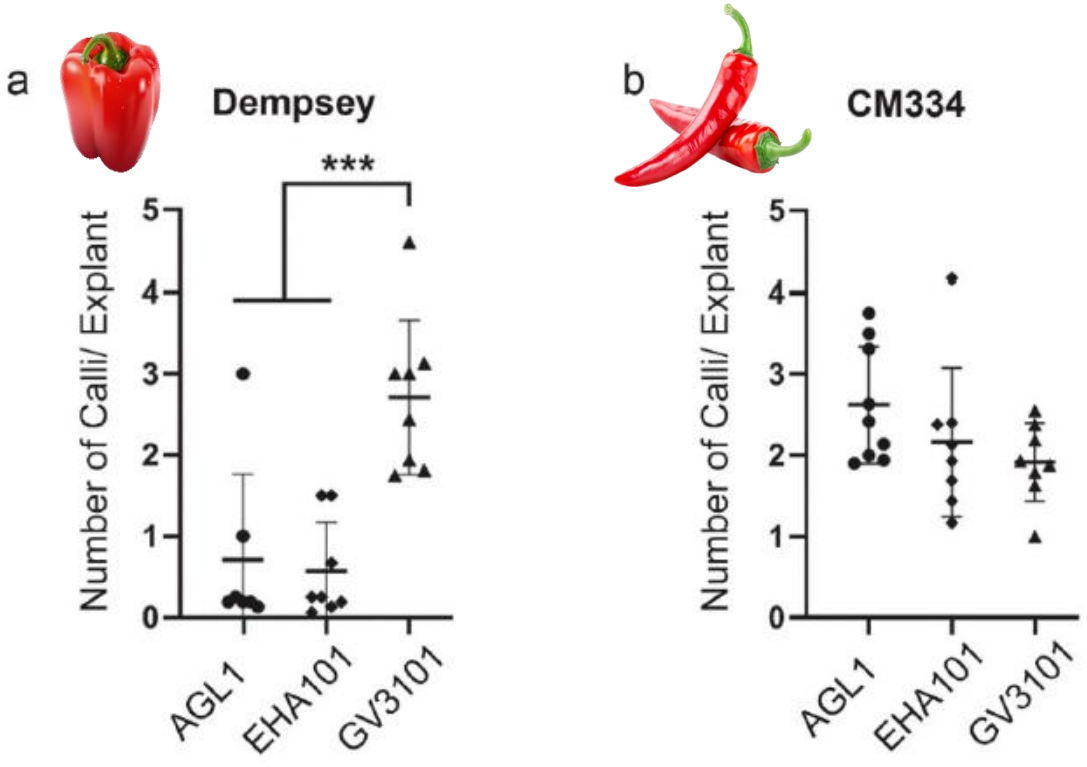
Kim H. et al., 2020 *BMC Plant Biology*

A Reliable Regeneration Method in Genome-Editable Bell Pepper "Dempsey"



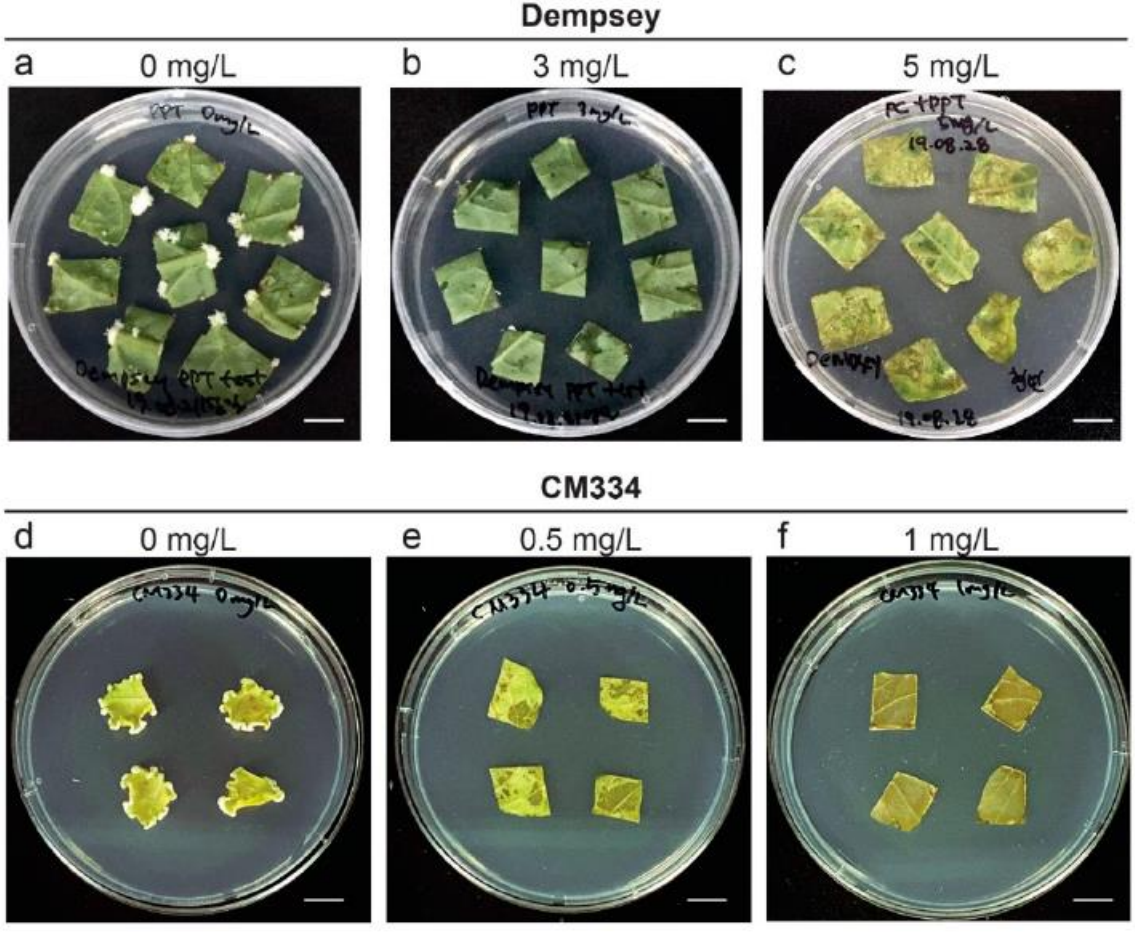
Won and Park et al., 2021 Horticulturae

Comparison of callus induction ratios among the tested Agrobacteria-mediated transformation in two peppers



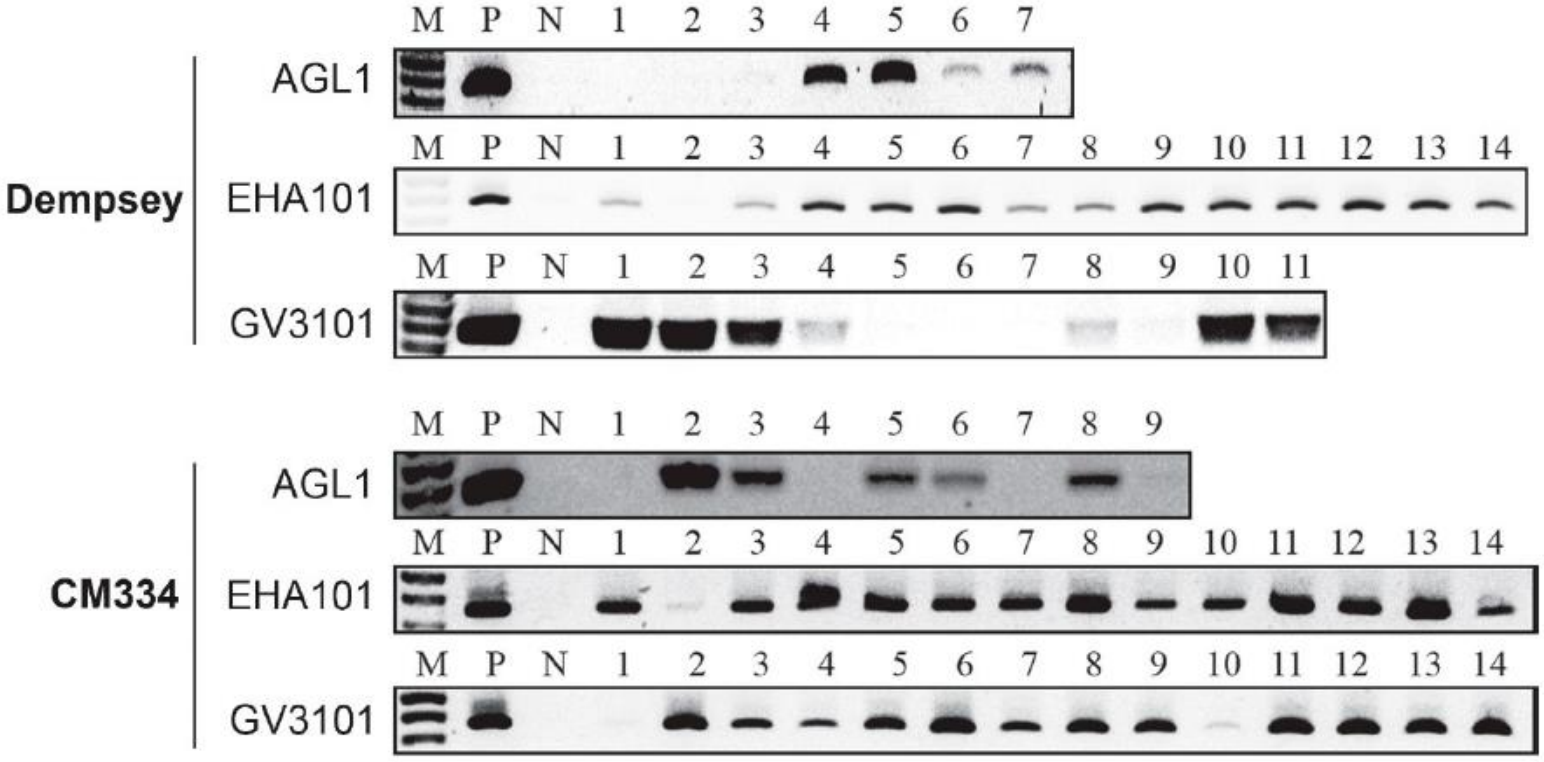
Park et al., 2021 Int. J. Mol. Sci.

Suitable Phosphinothricin (PPT) concentration for screening transformants in two peppers



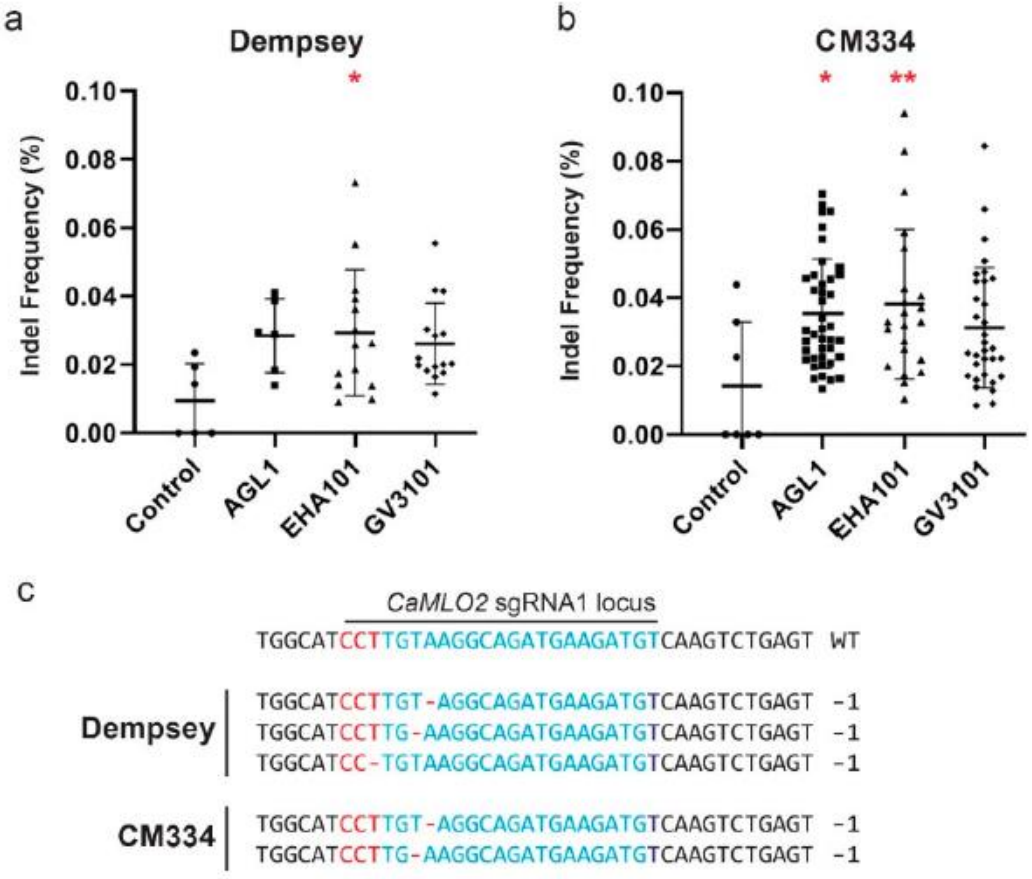
Park et al., 2021 Int. J. Mol. Sci.

PCR analyses of PPT-selected transformed calli of two pepper cultivars



Park et al., 2021 Int. J. Mol. Sci.

Comparison of indel frequencies and patterns of selected pepper calli



Park et al., 2021 Int. J. Mol. Sci.

SUMMARY

CRISPR systems

> Prokaryotic adaptive immunity system

> various genome engineering tools

> Improvement of soybean oil quality

> Prolonged Petunia flower

> Early-flowering Cabbage

> Development of callus-protoplasts screening systems for CRISPR-RNPs in peppers

> A Reliable Regeneration Method in Genome-Editable Bell Pepper "Dempsey"

> Agrobacterium-mediated *Capsicum annuum* gene editing in two cultivars

Acknowledgement

❖ PCGE lab members

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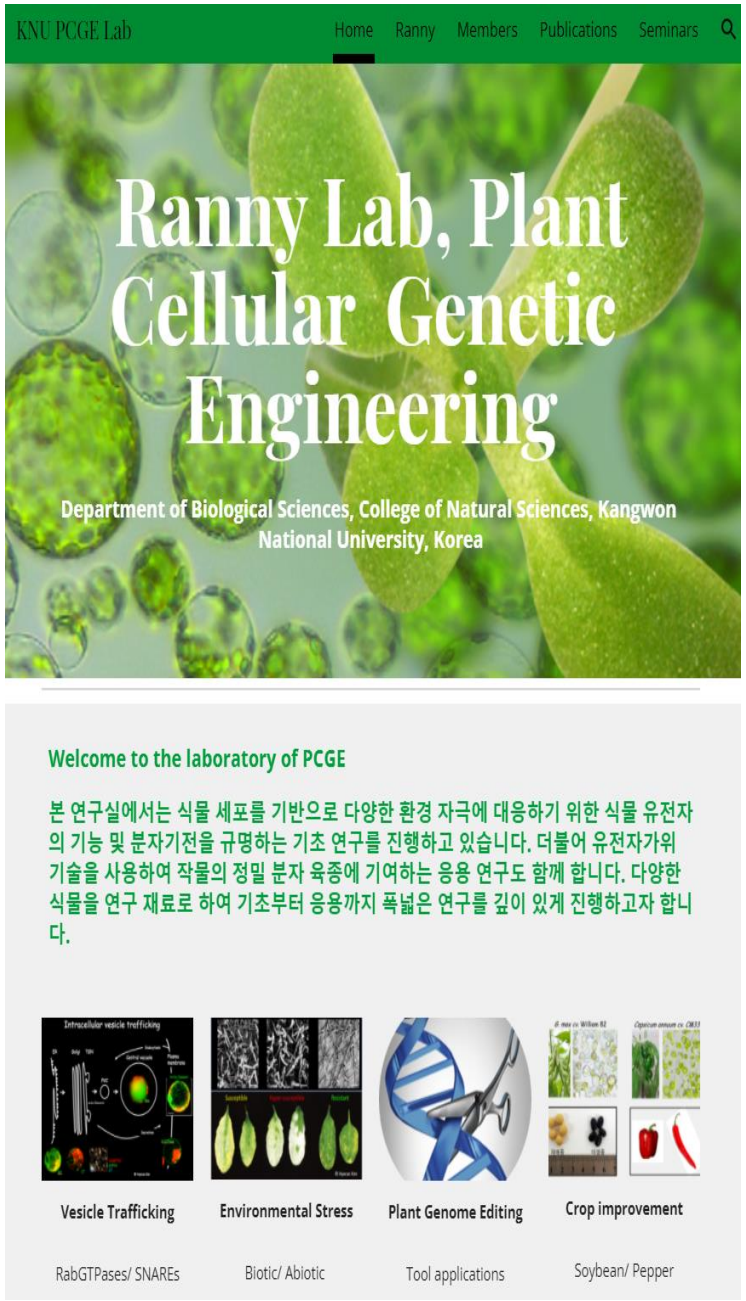
- KangHee Won
- Sung-il Park

C. annuum cultivars were provided by the Vegetable Breeding Research Center (VBRC), **Prof. Byoung-Cheorl Kang (SNU)**



Thank you for your attention!

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