**CHALLENGES AND PERSPECTIVES IN HOMOLOGY-DIRECTED GENE TARGETING IN MONOCOT PLANTS**

Tien Van Vu1,2, Yeon Woo Sung1, Jihae Kim1, Duong Thi Hai Doan1, Mil Thi Tran1, Jae-Yean Kim1,3,\*

1Division of Applied Life Science (BK21 Plus program), Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea.

2National Key Laboratory for Plant Cell Biotechnology, Agricultural Genetics Institute, Km 02, Pham Van Dong road, Co Nhue 1, Bac Tu Liem, Hanoi 11917, Vietnam.

3Division of Life Science, Gyeongsang National University, 501 Jinju-daero, Jinju 52828, Republic of Korea.

***corresponding author, email: kimjaeyean@gmail.com***

# Abstract

Continuing crop domestication/re-domestication and modification is a key determinant for adaptation and fulfil of food requirements of a global explosive population under many increasingly challenging factors such as climate changes and reduction of arable lands. Monocotyledon crops are not only responsible for around 70 % of the total global crop production indicating their important roles for human life but also the first crops to be challenged with the above mentioned hurdles, and hence should be the first ones to be engineered and/or de novo domesticated/re-domesticated. It is quite a long period of time after the first green revolution; the world is again facing the challenges to feed a predictive 9.7 billion of people in 2050 since the world hunger decline trend was reverted in 2015. One of the major lessons we have learnt from the first green revolution is the importance of novel and advanced trait-carrying crop varieties that could perfectly adapt to new agricultural practices. New plant breeding techniques (NPBTs) such as genome editing could help us to cope with the mission to create the novel and advanced crops. Considering the importance of NPBTs in crop genetic improvement, we attempt to summarize and discuss about the latest progress of the major approaches like site directed mutagenesis using molecular scissors, , base editors and especially homology-directed gene targeting (HGT), the most challenging but highly potential precise genome modification approach in plants. We therefore suggest potential approaches for improvement of practical HGT focusing on monocots and discuss about a potential approach for regulation of genome edited products.

**Keywords:** Gene targeting (GT), Homology-directed repair (HDR), homology-directed gene targeting (HGT), CRISPR/Cas, Targeted mutagenesis, Precision breeding, Monocots.

Contents

[Abstract 1](#_Toc19116226)

[BACKGROUND 3](#_Toc19116227)

[Status of food production using monocots 3](#_Toc19116228)

[Food requirement in 2050 vs present production, a big challenge. 4](#_Toc19116229)

[Introduction of the NPBT and genome editing-based precision breeding 5](#_Toc19116230)

[REVIEW 6](#_Toc19116231)

[Genome editing technologies 6](#_Toc19116232)

[Targeted mutagenesis, using molecular scissors to form DSBs 6](#_Toc19116233)

[Precision editing 12](#_Toc19116234)

[HDR-based gene targeting in monocots 17](#_Toc19116235)

[HDR mechanism in plant 17](#_Toc19116236)

[Gene targeting via HDR in monocots 22](#_Toc19116237)

[Potential solutions and perspectives 30](#_Toc19116238)

[The role of homologous donor templates 30](#_Toc19116239)

[Positive-negative selection 31](#_Toc19116240)

[Overexpression of genes involving in HDR pathway 31](#_Toc19116241)

[Knockout of genes relating to HDR pathway 33](#_Toc19116242)

[Favorable tissue culture conditions for gene targeting 35](#_Toc19116243)

[Cell Cycle Synchronization 35](#_Toc19116244)

[In planta gene targeting 36](#_Toc19116245)

[HDR-based monocot events and regulation aspects 36](#_Toc19116246)

[Conclusions 40](#_Toc19116247)

[Funding 41](#_Toc19116248)

[Acknowledgements 41](#_Toc19116249)

[Authors’ Contributions 41](#_Toc19116250)

[Competing Interest 41](#_Toc19116251)

[References 45](#_Toc19116252)

# Key terminologies used in this publication

|  |  |
| --- | --- |
| **Abbreviation** | **Description** |
| NPBTs | New plant breeding techniques |
| FAO | Food and Agriculture Organization |
| GR | Green revolution |
| GT | Gene targeting |
| HDR | Homology-directed repair |
| DSBR | Double-stranded break repair |
| HR | Homologous recombination |
| SDSA | Synthesis-dependent strand annealing |
| HGT | Homology-directed gene targeting |
| ZFNs | Zinc-finger nucleases |
| TALENs | Transcription activator-like effector nucleases |
| CRISPR | Clustered regularly interspaced short palindromic repeat |
| Cas | CRISPR-associated protein |
| C-NHEJ | Canonical non-homologous end joining |
| A-NHEJ | Alternative non-homologous end joining |
| MMEJ | Microhomology-mediated end joining |
| SSA | Single stranded annealing |
| BER | Base excision repair |
| NER | Nucleotide excision repair |
| dHj | Double Holiday junction |
| CO | Cross-over |
| NCO | Non-crossover |
| HptII | Hygromycin phosphotransferase II |
| NptII | Neomycin phosphotransferase II |
| DT-A | Diphtheria toxin A |
| SDN | Site Directed Nucleases |

# BACKGROUND

## **Status of food production using monocots**

Most of the present important crop plants were domesticated from around 10,000-13,000 years ago by our ancestors. The domestication of food crops has forever changed human life from hunting-gathering to immobilized living communities (Hickey et al., 2019; Meyer and Purugganan, 2013). Of the food crops, cereals might be the first ones to be artificially selected and intentionally planted for foods (Asano et al., 2011; Meyer et al., 2012). The domestication process has still been conducted though modern breeding techniques that have totally changed the ways of selection and adaptation of crop traits (Meyer et al., 2012). The monocots majorly contributing to daily ingredients are rice, maize and wheat. In 2018-2019, production of corn, wheat and rice has accounted for approximately 70 % of the total world crop production (FAO, 2019a; USDA, 2019). In the first half of 20th century, the world population rapidly increased disproportionally to increment of food production leading to a deadly prediction in the second half (Khush, 2001). Nowadays we know that the large-scale famine did not happen thanked to the first green revolution (GR) that had doubled cereal grain production within just ten years since it started in the years 1950s. Wheat and rice played the major roles in the first GR indicating the pivotal role of monocot crop in human life.

## Food requirement in 2050 vs present production, a big challenge.

The first GR accelerated world food production, which first-time reached at 1 billion tons in 1950 but needed only ten years to double the number by the uses of high yield varieties, chemical fertilizers and pesticides, and by the adoption of new cultivation method using irrigation system (Khush, 2001). Seventy years after the start of the first GR, the world is again facing the same challenge to feed a much bigger population. Unfortunately, the miracles of the first GR is now reaching its limitations. The increment of yield and production of food crops are slowing down and will not be matched with the requirement of 9.7 billion of people in 2050 at the present scenario (UN, 2019), please find more details in Food and Agriculture Organization (FAO) report (2009) and its revised version published in 2012). A recent report from FAO detailed that the world hunger decline trend was reverted in 2015 and the number of hungers are slowly increasing at present. As of 2018, over 820 million people are still living under hunger line (FAO, 2019b). It is worthy noted that the conclusion was drawn considering the present situation of food production and agriculture even under the whole-world supports for the conventional as well as the modern molecular-assisted breeding and smart agriculture practices (Ray et al., 2013). Obviously, the world agriculture is now challenging with many novel negative factors such as more vigorous climate changes, soil nutrition deficiency, and global sea level rise etc., but the other hurdles are remain the same as before the first GR. This reality indicates that to cope with the challenges and to fulfil the food production demand, the world has to accept and apply new technologies, especially new plant breeding techniques (NPBTs) (Lusser et al., 2011) for crop improvement and agriculture practices (Zaidi et al., 2019). Moreover, the world demands a second GR which can sustain and secure food production for mankind.

## **Introduction of the NPBT and genome editing-based precision breeding**

NPBTs, especially the recently emerged genome editing technology, are able to offer various solutions to improve crop traits such as (a) crops that can well adapt to environmental changes resulting in sustainability of yield, (b) crops that efficiently use limited resources to produce more foods and (c) crops with improved nutritional values. Genome editing technology is a two-stage process that includes (1) the generation of single-stranded/double-stranded breaks (SSBs/DSBs) or deamination of nucleotides (in case of base editors) and (2) repair of the targeted sites by the host cells. The repair process could be error-prone via canonical non-homologous end joining (C-NHEJ) or alternative NHEJ (A-NHEJ or microhomology-mediated end joining (MMEJ))/ Single stranded annealing (SSA) /base excision repair (BER) or nucleotide excision repair (NER) pathway or error-free through HGT (or homologous recombination (HR)) or oligonucleotide-directed mutagenesis (ODM) (Figure 1 and 3). The repair pathways in animals as well as plants were extensively reviewed elsewhere (Belhaj et al., 2013; Bortesi and Fischer, 2015; Doudna and Charpentier, 2014; Hsu et al., 2014; Rees and Liu, 2018). NHEJ, SSA and BER/NER approaches are highly efficient in generating unpredictable error-prone products while ODM, HGT and base editor (BE) (?) have been considered as techniques for precision editing of genes in plants. However, in plants the main obstacle to HGT applications is its extremely low efficacy (Paszkowski et al., 1988; Puchta et al., 1996). Many attempts have been conducted to improve plant HGT for practical applications. Important enhancements have been shown with CRISPR/Cas complexes with or without homologous donor template delivery and amplification by ssDNA replicons (Baltes et al., 2014; Cermak et al., 2015; Gil-Humanes et al., 2017). In this review, we summarize recent data regarding genome editing approaches in monocot plants with special focusing on HGT and provide perspectives for monocot crop improvement and commercialization.

# REVIEW

## Genome editing technologies

### Targeted mutagenesis, using molecular scissors to form DSBs

Since the discovery of restriction enzymes, the field of biotechnology has entered a new era of molecular engineering facilitated by the recombinant DNA technology. Several generations of molecular scissors have been discovered, characterized and developed for DSB-based targeted genome mutagenesis. It has been improved from the long recognition sequence homing nucleases to protein-dependent DNA binding nucleases such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), and ultimately to the 3rd generation RNA-guided molecular scissors CRISPR /Cas (Figure 2). With the inventions of target-specific synthetic molecular scissors, the specific modification of a gene of interest in a living organism has become possible. Consequently, there are two key factors of targeted mutagenesis induced by the molecular scissors: 1) The precise recognition of targeted sequence, and 2) the DSB formation frequency.

The host amendment of the DSB errors would lead to error-free or error-prone outcome depending on many factors including cell cycle state and availability of homologous DNA templates at the damaged sites. In plant somatic cells, DSB repair by either of the two major pathways homology-directed repair (HDR) or non-homologous end joining (NHEJ) usually lead to either error-free or error-prone products. Majority of the error-prone products appears as insertion or deletion (indel) DNA mutation resulted from C-NHEJ, or A-NHEJ (Figure 1). A possibly lower portion of error prone products may be resulted from SSA repair in the absence of homologous donor template and from Holiday junction resolution in the last steps of double-stranded break repair (DSBR) sub-pathway if the DSB flanking sequences of sister chromatids are not perfectly matched (Figure 1). In this part we summarize brief information of the above mentioned molecular scissors. Extensive reviews for the same could be found elsewhere (Carroll, 2011; Gaj et al., 2013).

#### Generation 0: Homing nucleases

Homing nucleases are endonucleases (Mw<40kDa) that recognize long DNA sequences (14-40nt) for their cutting activity (Figure 2A). Homing nucleases can work alone as monomers or in pair as homodimers (Chevalier and Stoddard, 2001). Since it recognizes longer sites than type II restriction enzymes, more specific targeting is allowed on host cells (approximately once every 7×109 [bp](https://en.wikipedia.org/wiki/Base_pair)) (Chevalier and Stoddard, 2001; Jasin, 1996). However, this feature also introduces limitation in the scarce of targetable sites on the genome of host cells. To compensate this, there have been studies to engineer these nucleases for a wider range of binding and cutting sites or combination of different homing nucleases for recognizing multiple sites (Chevalier et al., 2002). Engineered homing nucleases often cleave correct sites as efficiently as wild-type (Chevalier and Stoddard, 2001). However, the engineering of homing nucleases for wider applications is still inefficient, laborious and time-consuming.

#### Generation 1, 2: protein-guided DSB formation, ZFN and TALENs

ZFNs are derived from discovery of zinc finger, a finger-like DNA binding motif found in TFIIIA, a transcription factor of Xenopus laevis’s egg (Miller et al., 1985). Its structure is comprised of 30 repetitive amino acid sequences and stabilized by zinc ion (Berg, 1988; Miller et al., 1985). Berg (1988) suggested that the zinc finger protein structure might play a key role in the recognition of DNA sequences. ZFN was first developed in 1996 by fusing a non-specific DNA cleavage domain of FokI, a type II-S restriction enzyme, to the C-terminal of the zinc finger motifs (Kim et al., 1996). Typically, three consecutive nucleotides can be specifically recognized by one zinc finger motif and therefore several connected zinc finger motifs fused to FokI can bind the target DNA of interest (Kim et al., 1996). ZFN is the first artificial restriction enzyme that recognizes desirable sites on the genome. Due to binding specificity and dimerization-dependent FokI activity requirement, ZFNs were typically designed in pairs to recognize 9-18 bp by using connected 3-6 zinc finger motifs on both sense and anti-sense strand of targeted sequences spacing by 5-7 bp in between the ZFNs (Bitinaite et al., 1998; Kim et al., 1996; Laity et al., 2001; Urnov et al., 2010) (Figure 2B). Post cleavages, the DSB sites were recovered by DNA repair mechanisms that showed insertion or deletion at similar rates (Kim et al., 2013). However, for wider applications of this technology, one should overcome the limitations of low editing efficiency (0-24%), elevated design and optimization cost, and high off-target possibility (Kim, 2015). Many efforts have been conducted to overcome those barriers. For example, in order to enhance the cleavage activity of FokI cleavage domain, Gou and coworkers (2010) conducted direct evolution for optimizing a ZFN named as ‘Sharkey’. Several approaches were tested to reduce the off-target effect, e. g. extending the recognition length by using more zinc finger modules (Guo et al., 2010; Pattanayak et al., 2011).

TALEN is the second-generation molecular scissors, discovered during the studies of the plant immune system under the attack and hijack by pathogenic bacteria (Dangl and Jones, 2001). AvrBs3, an effector protein secreted by the plant pathogen Xanthomonas campestris is injected into host cells thereby binding to plant gene UPA-box and functioning as a transcription activator to modulate the host cells for its efficient colonization (Kay et al., 2009). The causal agents secreted by Xanthomonas were identified and named as transcription activator like effectors (TALEs). TALEs have highly conserved 33-35 amino acids except for those locate in the positions 12 and 13. These two hypervariable residues (namely repeat-variable di-residues (RVDs)) are oriented forward to outside of the protein and played a key role in recognizing a specific nucleotide (Moscou and Bogdanove, 2009). Common rules of RVDs nucleotide recognition for binding were validated as NG for thymine; HD for cytosine; NN for guanine or adenine; and NI for adenine (D = A or G or T). The first TALENs were introduced by fusing a DNA binding TAL type III effector with a FokI cleavage domain Figure 2C (Li et al., 2011). However, unlike ZFN which recognize 3bp per one zinc finger module, TALEN allows more precise recognition because each one RVD of TALE can recognize only one nucleotide. The TALENs were designed in pair with the 12-21nt distance in between two binding sites of them for the highest cutting activity (Miller et al., 2011). The combination of TAL effector AvrXa7, PthXo1 and FokI were demonstrated to function as a molecular scissors for cutting and hence modifying the binding sites of the TALs and subsequently resulting in resistance to rice blight disease (Li et al., 2011). The initial NN RVD repeat recognized either guanine or adenine raising concerns about its specificity (Moscou and Bogdanove, 2009). Ultimately, a NK RVD repeat that recognizes only guanine was discovered fulfilling the specificity requirement for the TALEN molecular scissors (Miller et al., 2011).

One of the weak points of TALEN approach is the big size of the binding domain as every nucleotide needs a repeat of ~34 amino acids to bind to it. As is, for one TALEN binding to 20 nt for assuring high specificity, its DNA binding domain requires 680 amino acid long. In addition, assemblies of the highly repeated modules were time-consuming and laborious. Thus, a well-designed modular RVD repeat library was highly demanded and eventually developed (Cermak et al., 2011; Kim et al., 2013; Zhang et al., 2011). TALENs have pros of high editing efficiency, low off target and lower designing cost than that of ZFN and cons of difficult constructions, no working on methylated cytosine (Kim et al., 2013), difficult introduction into cells owing to its big size (Kim, 2015).

#### Generation 3: CRISPR/Cas

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) was shown to be a DNA interference-based defense machinery of prokaryotes such as bacteria and archaea against phage infection (Barrangou et al., 2007; Brouns et al., 2008). CRISPR/Cas systems were classified into two classes according to the number of complexity of their effector modules (Makarova et al., 2011; Makarova et al., 2015). Class 1 systems involve effector complexes formed by multiple subunits whereas; in class 2 systems, single multi-domains proteins constitute the effector complexes. Furthermore, each class has been divided into several subtypes (class 1: types I, III and IV; and class 2: types II, V and VI) based on their effector architectures with unique signature proteins (Koonin and Makarova, 2019). Almost all of the CRISPR/Cas systems used in genome engineering till date are from class 2 thanks to the simplicity of their effector modules (Supplemental Table 1). The most widely used CRISPR/Cas systems are Cas9, Cas12a (Cpf1) and Cas13d.

In native CRISRP/Cas9 system, phage DNAs were shown to be cleaved by Cas9 effector complex which included Cas9 protein as a nuclease and a complexed RNA structure formed by a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA) as a probe. The two-component RNA secondary structure facilitated Cas9 assembly, searching and binding to dsDNA target sites by Watson-Crick complementarity to the 19-21nt of 5’ end of the crRNA (protospacer) and subsequently cleaving both the strands of the dsDNA at 3rd nucleotide proximal to an 5’-NGG-3’ protospacer-adjacent motif (PAM) site (Fig. 2D). Originally, crRNA:tracrRNA required maturation from a precursor crRNA:tracrRNA by RNase III processing activity making it more difficult for application. In the first application of CRISPR/Cas9 for genome editing, the crRNA and tracrRNA was engineered to make a single guide RNA molecule by connecting the 3’ crRNA repeat and 5’ tracrRNA anti-repeat thereby easing the uses of the system (Jinek et al., 2012). The Cas9 protein remains inactive until it binds to a guide crRNA:tracrRNA structure. The guide RNA-bound Cas9 complex undergoes conformational changes and then stochastically searches for potential target by PAM scanning and binding using it PAM-interacting motif. Then the Cas-sgRNA complex again changes conformation, and the guide RNA sequence is used to pair with sequence locating upstream of the PAM via Watson-Crick rule (Jinek et al., 2014; Sternberg et al., 2014; Zhu et al., 2019). The gRNA and its seed sequence (10-nucleotide RNA proximal to NGG PAM) should be fully complemented for R-loop formation and triggering Cas9 cleavage activities via its endonuclease domains (HNH and RuvC) (Hsu et al., 2013; Jiang et al., 2013; Jinek et al., 2012). The targeted and non-targeted strand of the dsDNA is cleaved by HNH and RuvC, respectively generating mostly blunt ends (Fig. 2D) (Anders et al., 2014; Nishimasu et al., 2014). Nickases Cas9 (nCas9) that cut either targeted strand or non-targeted strand and dead Cas9 (dCas9) were created by inactivating either the endonuclease domains or both domains for alternative gene editing and regulations.

Unlike Cas9, Cpf1 system does not require a tracrRNA to mature crRNA and to form effector complex for its cleavage activity. The Cpf1 protein was also shown to process the precursor crRNA (Zetsche et al., 2015). After assembly, Cpf1 effector complex recognizes a T-rich PAM for initiation of binding and searching for target sites. Its seed sequence was illustrated to be ranged from1-10 nt proximal to the PAM (Kim et al., 2016). Cpf1 protein has a Nuc nuclease domain that cleaves the target strand and a RuvC domain that cleaves the non-targeted strand (Makarova and Koonin, 2015; Schunder et al., 2013; Stella et al., 2017). The nuclease domains cut target dsDNAs at 18th nt on non-targeted strand and 23rd nt on targeted strand distal to the PAM generating 5’ overhang ends (Fig. 2D) (Zetsche et al., 2015).

### Precision editing

#### Base substitution

It is now well-known that the majority of genetic diseases resulted from point mutations but the potential DSB-based repair approaches for correcting the mutations somehow not applicable due to the inaccessibility and unsuitable principles of the repair mechanisms (Cox et al., 2015; Hilton and Gersbach, 2015). Therefore, a single base change technique is highly demanded and have been developed for at least transition fixation (C/G->T/A or A/T->G/C), so-called cytosine base editors (CBEs) or adenine base editors (ABEs) (Figure 3A) (Gaudelli et al., 2017; Komor et al., 2016). The basal principle behind the technique is the fusions of death or nickase Cas9 (d/nCas9) with a cytosine or adenine deaminase and introduction of the editor complex to the targeted site by CRISPR guide RNA structure. Deamination of C or A produces U or I, respectively, leading to lesion-by-pass replication and resulting in C/G->T/A or A/T->G/C transition, respectively. In addition, the CBEs and ABEs were shown to work in a framed window that was narrow (13rd to 17th nucleotides upstream of the 5’-NGG-3’ PAM) (Komor et al., 2016) or wide (4th to 20th nucleotides upstream of the 5’-NGG-3’ PAM) (Zong et al., 2018) at asymetric frequency distributions (Supplemental Table 2) depending on the types of deaminase used. This fact raises a possibility of controlably precise editing of every single base of interest by carefully calculating and evaluating editing frequency of base editors for a base of a given target. This could also help to avoid the possibility of bystander base changes and un-intended off-targets (Gehrke et al., 2018).

#### Oligonucleotide-directed mutagenesis (ODM)

Oligonucleotide-directed mutagenesis (ODM) or rapid trait development system (RTDS) are two common names of oligonucleotide-mediated targeted gene modification technique. This technique uses synthetic oligonucleotides or Gene Repair Oligonucleotides (GRON) which function as a template for endogenous DNA repair to form hetero-triplex with a targeted genome site via homology binding using their identical sequences to the site except the intentionally modified nucleotide(s) thereby triggering gene conversion and resulting in specific base changes (Figure 3B). The GRON itself is not inserted into the host genome and site-directed nuclease or double-strand breaks are not required for this technique. Therefore, ODM was classified as one of the precise gene editing technique (for review, see Sauer et al., 2016). The changes could be point mutations, multiple base changes, insertions or deletions. The GRON was subsequently degraded during cell divisions and the modified gene retains its normal pattern of expression and stability within the genome (Sauer et al., 2016).

The first application of synthetic nucleotide was shown in yeast in 1988 (Moerschell et al., 1988) and then in mammalian cells for correction of a faulty human β-globin that causes sickle cell anemia in 1996 (Allyson Cole-Strauss, 1996; Yoon et al., 1996). In plants, Beetham and co-workers used RNA/DNA chimeric molecules in a work known as chimeraplasty approach, to target tobacco acetoacetate synthase (ALS) (or aceto hydroxyl acid synthesis (AHAS)). Tobacco ALS is a biallelic gene (including alleles ALS1 and ALS2) due to its allotetraploid genome. Therefore, two chimeric ODM oligonucleotides were designed to engineer ALS1 and ALS2 as P196 (CCA) to CAA and to CTA, respectively. Particle bombardment of the oligos and subsequent selection on medium containing 200 ppb of chlorsulfuron revealed one out four ALS alleles with Pro-196 (CCA) to Thr-196 (ACA) modification. The efficiency was at two orders of magnitude higher than that of the control (Beetham et al., 1999). The ODM approach were also conducted in several studies in dicot such as canola (Gocal, 2015) and Arabidopsis (Sauer et al., 2016).

In monocots, ODM was used to target AHAS in maize (Zhu et al., 1999) and rice (Okuzaki and Toriyama, 2004). In maize, nucleotide changes were induced at two sites, S621A (AGT to AAT) for imidazolinone and sulfonylurea herbicide resistance and P165A mimicking the point mutation in tobacco in Beetham’s work (1999). The oligonucleotides were transformed to maize cells by bombardment and selected either by 7 µM imazethapyr for S612A or 20 bbp chlorsulfuron for P165A. The mutating frequencies were 1.0 x 10-4 to 1.4 x 10-4 approximately three orders of magnitude higher compared to that of spontaneous mutation and gene targeting by homologous recombination pathway in plants (Tong Zhu, 1999). In rice, three chimeric DNA/RNA oligonucleotides for targeted modification of ALS P171A, W548L and S627I were introduced into rice calli by bombardment. Screening by herbicide selection (chlorsulfuron for P171A and bispyribac-sodium for W548L and S627I) and Sanger sequencing obtained independent event for either P171A or W548L but not S627I, at 1x10-4 frequency. ODM approach was also demonstrated in wheat system using transient assay with GFP as a reporter. The authors claimed that using 2,4D in osmotic media boosted the gene targeting efficiency and the repair of point mutation had higher frequency than that of single base deletion in immature wheat embryos (Dong et al., 2006).

ODM products have been considered as a non-GMOs in a number of countries except EU thanks to it targeted point mutation mechanism and transgene-free outcomes (Eriksson, 2018). In 2011, the UK Advisory Committee of Releases into the Environment (ACRE) suggested that plants being developed by the ODM system should not be regulated as GMOs. Afterwards, Federal Ofﬁce of Consumer Protection and Food Safety of Germany decided that ODM products do not constitute GMOs in 2017. Based on the precisely modification and GMO regulation of this technology, ODM is potential for genome editing. However, its low efficiency is the main barrier for application research, thus improve the frequency is essential. Recently, ODM and SDN have been combined to enhance the efficiency with the range of precise editing from 0.09% to 0.23% in EPSPS target gene. In this study, it was also claimed that transgene targeting efficiency of CRISPR/Cas9 was nearly 3 times higher compare to TALEN (Sauer et al., 2016).

#### HR-based gene targeting

In 1988, gene targeting (GT) or HGT was first defined as the modification of the host genome that was achieved by the integration of foreign DNA via HR pathway (Paszkowski et al., 1988). This method provides a wide range of targeted genome modification such as precisely insertion, deletion or replacement of a gene or an allele. As a matter of fact, HR is an ideal mechanism that can precisely repair DSBs during S and G2 phase of the cell cycle while homologous sequences (sister chromatids or donor templates) are available (Tamura et al., 2002). However, its low frequency in higher plants is still a hurdle for practical applications (Puchta, 2005). The first application of HGT in a crop was performed to targeted knockout rice *Waxy* gene using positive/negative selection which helped to achieved approximately 1 % frequency but also left positive selection marker in the genome (Terada et al., 2007).

Since then, two other important achievements in plant gene targeting field regarding frequency enhancement have come to life: (1) the key finding of on-targeted DSB roles (Puchta et al., 1993) and (2) methods to introduce high doses of autonomously homologous donor templates to targeted cells (Baltes et al., 2014). By inducing DSBs at a specific locus using highly specific restriction enzyme I-*Sce* I, HDR efficiency can be enhanced from 10 to 100 times (Puchta et al., 1996). To further enhance the efficiency of HR for gene targeting, several approaches have been developed. Firstly, site-specific nucleases such as ZFNs, TALENs and CRISPR/Cas systems are applied to induce double-strand breaks at the target sequence (Belhaj et al., 2013; Voytas, 2013). The second approach is taking advantage of virus replicon system to increase the delivery ability and the number of donor template, hence the HGT efficiency is improved (Baltes et al., 2014). Apart from that, certain studies have demonstrated that over-expression of HR-involved genes or suppression of NHEJ pathway led to improvement of HGT frequency (Endo et al., 2016; Qi et al., 2013; Shaked et al., 2005).

## HGT in monocots

### HDR mechanism in plant

One of the principal questions regarding cell responses to DSBs is which repair consequences the cells favor, error-free or error-prone DNA products? In meiosis, error-prone crossing over (CO) or break induced repair (BIR) (or even NHEJ) is preferred for creating genetic diversity by exchanging genetic information between parental chromosomes, a key factor for adaptation to environmental changes. However, we can expect an opposite situation in mitotic cells that require a genetic stability instead of diversity. Then, should NHEJ be abolished from mitotic cells? The answer is absolutely no and one of the key reasons maybe the limitation of time since a single DSB persistence may induce programmed cell death after certain period of time (Nowsheen and Yang, 2012). What can the cells do? NHEJ is so abundant and efficient in mending the broken ends. What can we expect from the bulky HDR apparatus?

HDR have been extensively studied in yeasts and mammals for understanding mechanisms of genetic diseases caused by DNA DSB damages. Most of the components of plant HDR pathway can find their homologs in plant (Schuermann et al., 2005) but the regulation of DSB responses in the kingdoms maybe different (Yokota et al., 2005). Unlike in animal systems, HDR efficiency in plant somatic cells is extremely low (Puchta et al., 1996; Szostak et al., 1983) and over-dominated by NHEJ. Plant mitotic HDR is absent in G1 phase and limited to S/G2 phases while NHEJ is active throughout the cell cycle (Figure 4). HDR pathway is determined under the presence of sister chromatid as a homologous DNA donor which is normally produced by replication in S phase and prolonged until M phase. Even under the favorable cell cycle phases, the HDR pathway has to compete with the predominant NHEJ, and hence, in only certain conditions it can be chosen (Heyer et al., 2010; Jasin and Rothstein, 2013; Voytas, 2013). Therefore, a comprehensive knowledge of the conditions that favor HDR in plant somatic cells would offer key successes in plant gene targeting for crop improvement.

#### Sensing the DSBs and cell cycle arrest

In mammals, DSB formation induces cell cycle arrest that is necessary to help the cell repair the damage in a reasonable time (Kastan and Bartek, 2004). The process is initiated with the conformational changes of the ATM (ataxia telangiectasia mutated) homodimer resulted from sensing a chromatin structure change following DSB formation. Activation of human ATM by autophosphorylation of its serine 1981 disassociates its activated monomers (Bakkenist and Kastan, 2003). Monomeric ATM then phosphorylates all the members of MRE11 (meiotic recombination 11)/RAD50 (Radiation sensitive 50)/ NBS1 (Nijmegen breakage syndrome 1) (MRN) complex, a DSB sensor holoenzyme, and is additionally phosphorylated by MRE11 (Lamarche et al., 2010; Lee and Paull, 2005). Subsequently, ATM plays a central role in activating cell cycle checkpoint kinases, P53 and indirectly induces suppression of cyclin-dependent kinases that ultimately leads to cell cycle arrest (Harper and Elledge, 2007; Kastan and Bartek, 2004; Yata and Esashi, 2009). In *Arabidopsis*, SOG1 (suppressor of gamma response 1), activated by ATM, is responsible for regulation of multiple downstream proteins like CDKB1 (G2/M checkpoint) and CYCB1 (S phase checkpoint) or NAC-type transcription factors ANAC044 and ANAC085 (S/G2 checkpoints) or WEE1 kinase for cell cycle arrest (De Schutter et al., 2007; Takahashi et al., 2019; Weimer et al., 2016; Yoshiyama et al., 2013).

#### HDR pathway determination.

Post DSB formation, cell cycle arrest at S/G2 favors the essential condition for HDR pathway (Figure 3). In animals, HDR pathway is determined by recruitment competition between KU70/80-DNA-PK and MRN complex to the DSB ends and subsequent resection regulation by BRCA1/CtIP and 53BP1/RIF1 that favors HDR and NHEJ, respectively. However, only Ku complex but not DNA-PK is conserved in plants (Tamura et al., 2002; West et al., 2002) suggesting alternative regulation of activation by kinase in the kingdom. KU70 was shown to co-localize and interact with MRE11 in somatic cells and therefore, was proposed to be key player in determination of DSB repair pathway (Goedecke et al., 1999). Since majority of DNA end binding protein in a cell is KU70/80 (Gottlieb and Jackson, 1993), NHEJ becomes dominant and hence, HDR efficiency, especially in plant mitotic cells, is extremely low. Recently, it is increasingly accepted that DSB end resection plays a key role in determination of NHEJ- or HDR-mediated repair. NHEJ repair keeps the broken end resection at a limited range for its amendment but HDR needs DSB end resection to produce 3’-protruding ends those are long enough for template annealing and replicating homologous genetic information. NHEJ resection length usually ranges from 0-14 bp, very rare cases up to 25 bp and longer (Lieber, 2010).

ATM-dependent phosphorylation of RAD50, NBS1 and MRE11 of MRN complex plays important role in DSB end resection that determine ultimate repair pathway in a MRN-dependent manner. MRE11 acts as an endonuclease which nicks DNA upstream from the break and subsequently resects 3’->5’ toward the break and then the end is further resected by Endonuclease 1 and Dna 2 (Kijas et al., 2015). CtIP activated by ATM, acts in concert with MRN complex to enhance resection and HDR. It is shown that CtIP physically interacts with MRN complex and more importantly BRCA1 (Limbo et al., 2007), a protein that inactivate 53BP1 by dephosphorylation it (Isono et al., 2017) thereby supporting the DSB end resection for HDR determination. However, in a recent data, 53BP1 was shown to shield DSB ends from extensive resection that might result in a strong bias toward RAD52-dependent error-prone SSA (Ochs et al., 2016). The broken end resection is also controlled by phosphorylated MRE11 which keeps the exonuclease 1 from extensive resection by phosphorylating it (Kijas et al., 2015). In *Arabidopsis,* PHF11 (plant homeodomain finger 11) plays roles in binding and suppressing RPA, thereby enhancing Exo1 resection (Gong et al., 2017). Furthermore, the resection coordination activity of MRE11 and CtIP/Ctp1 may inactive KU70/80 and unloads it from the broken ends. Meanwhile, pre-defined resection length may deactivate the MRN complex and disassociate it from the ends (Langerak et al., 2011).

#### DSB amendment by HDR

Once being determined for HDR pathway, under the presence of homologous DNA templates, HDR can occur through gene conversion or synthesis-dependent strand annealing (SDSA), single-stranded annealing (SSA) or cross-over (CO, DSB repair (DSBR))/non-crossover (NCO) via double Holiday junction (dHj) formation (Holliday, 1977). Only the former HDR sub-pathway can produce precise sequence products (Figure 1). In plant somatic cells, SDSA was proved to be the major HDR mechanism to precisely repair damaged DNA (Puchta et al., 1996; Szostak et al., 1983; Voytas, 2013). The differentiation of HDR sub-pathways have been well studied in yeasts and mammals but still to be a matter of investigation in higher plants. In case of HDR, phosphorylation of H2AX histone protein by ATM or DNA-PKcs is important to open nucleosomes for strand annealing. As one H2AX presents at every 10 nucleosomes, efficient HDR requires relaxing up to thousands of base pairs (Lieber, 2010). The resection of broken ends at a controllable length of 3’ ssDNA overhangs would favor RAD51-dependent SDSA repair. RPA binds to the resected ssDNAs to prevent the formation of secondary loop for RAD51 loading. RAD51 loading is facilitated by BRCA2 through its BRC motif that plays dual roles as an ssDNA-dsDNA junction binding protein as well as RAD51 docking site provider (Dray et al., 2006; Heyer et al., 2010; Seeliger et al., 2012). The tight regulation of RAD51 loading and nucleofilament formation is shown to have involvement of a BRCA2-antagonistic protein called FIDGETIN-LIKE-1 (FIGL1) (Fernandes et al., 2018; Girard et al., 2015; Kumar et al., 2019). Extensive end resection by the involvement of Exonuclease 1 (Exo1) and/or Sgs1-Dna2 would lead to RPA disassociation facilitated by RAD52 which redirects to error-prone SSA repair pathway (Heyer et al., 2010) (Figure 1).

In *Arabidopsis*, INVOLVED IN DE NOVO2 (IDN2) was shown to help RAD51 loading by binding to RPA and unloaded them from DSB ends (Liu et al., 2017). RAD51 binds to the resected ssDNA overhang forming nucleoprotein filament or presynaptic filament. The filament structure invades donor template sequence, searches for, and anneals to complementary sequence followed by displacement loop (D-loop) formation (Rajanikant et al., 2008). RAD54 binds to and is required for supporting RAD51 strand invasion, annealing and disassociating RAD51 afterward (Klutstein et al., 2008; Osakabe et al., 2006). RAD54 formed DNA repair foci in living *Arabidopsis* cells depending on the ATM-SOG1 signaling and coincided with formation of the phosphorylated H2AX (Hirakawa et al., 2017). Subsequently, the free 3’ OH end of the invaded ssDNA primes donor template-dependent DNA synthesis. This process determines the outcomes of HDR with several sub-pathways (DSBR, dHJ and SDSA) depending on types of DNA synthesis and resolution of final products (Figure 1). In later stage of homologous template-dependent synthesis in somatic cells the D-loop may be processed and reannealed by the activity of RAD5A, REC4Q and MUS81 (Hartung et al., 2006; Mannuss et al., 2010). Only SDSA can generate precise repair products and is favored in mitotic cells (Heyer et al., 2010; Puchta, 2005).

### HGT in monocots

Plant gene targeting or HGT was defined by the homology-directed repair (HDR) of an endogenous gene by exogenously introduced homologous DNAs (Paszkowski et al., 1988). Obviously, the initial experiment obtained a very low frequency of homologous recombination (~10 -4 ) indicating difficulty but feasibility in engineering plant genome by site-specific gene targeting. Early in the 1990s, a transgenic approach using a pre-introduced yeast mitochondrial I-*Sce*I endonuclease as a DSB inducer was adopted in attempts to investigate the mechanisms of DSB repair in plants, especially the HDR pathway in plant somatic cells (Fauser et al., 2012; Puchta et al., 1993; Szostak et al., 1983). It became clear that HDR pathway employing homologous DNA templates to precisely repair DSB-damaged DNAs occurred mainly via SDSA mechanism (Figure 1), with an extremely low efficiency. Nonetheless, the induced DSBs could improve the HGT efficiency up to two orders of magnitudes (Puchta et al., 1996; Szostak et al., 1983), a big step in plant gene targeting research. Recently emerged CRISPR/Cas systems, being shown as powerful molecular scissors in the way they could be used for *in vivo* generation of site-specific DSBs, have revolutionized the plant gene targeting approach and bring hope for practical applications in crop improvement.

However, despite applying flexible approaches (i.e. particle bombardment, protoplast transfection and *Agrobacterium*-mediated transformation) for delivery and execution of HGT tools, gene targeting in most of the main crops is still challenging. As mentioned in the previous sections, most our knowledges about the principal mechanism of plant HDR were withdrawal from yeast and animal research studies and some of those increasingly unsuits to what we observed in plant kingdom. Therefore, plant genome engineering community should continuously focus on research for understanding plant-specific factor involving in DSB repair, especially via HDR pathway, the only approach providing precise gene targeting products. Using the background knowledge, one can propose approaches for improvement of gene targeting frequency. As far as we know, two most important factors affecting gene targeting efficiency in plant somatic cells are: 1) DSB formation at the targeted sites and 2) the amount of homologous DNA templates available at sites of breakage (Baltes et al., 2014; Endo et al., 2016; Puchta, 2005; Puchta et al., 1993; Townsend et al., 2009).

Since most of the early studies focused on gene targeting in model dicot plants like *Arabidopsis*, tobacco and tomato (for review, see (Puchta, 2005; Voytas, 2013), monocot gene targeting represented a big gap in the early reports indicating big challenges in monocot gene targeting. In this section, we aim to summarize recent knowledge regarding gene targeting in monocot plants that represent most of the major food crops for human-being. In addition, we are discussing about challenges and suggesting potential solutions for improvement of gene targeting frequency in monocots.

#### HGT without targeted DSBs

*In vivo* plant gene targeting without any assistance for selection was extremely low (Paszkowski et al., 1988; Puchta and Hohn, 1991). The first targeted knockout of an endogenous “*waxy*” allele via HGT was successfully generated in rice at 0.94% frequency by Terada and co-workers (2002) thanks to a smart positive (hygromycin phosphotransferase II (HptII)-based)/negative (using diphtheria toxin A (DT-A) subunit) selection (Table 1). Frequency of the gene targeted *waxy* and *xyl* (b1,2-xylosyltransferase) knockout alleles was further improved by experimental conditions (Ozawa et al., 2012). The weak side of the strategy is the obligatory use of an associated marker gene and hence the product is subjected to GMO category. Therefore, the Cre/loxP was applied to excise the marker out of the gene targeted allele (Terada et al., 2010). The approach was later successfully applied for functional genomic studies via tagging endogenous gene with visible marker(s) (Moritoh et al., 2012; Ono et al., 2012; Tamaki et al., 2015; Yamauchi et al., 2009). The positive/negative system using DT-A subunit might have posed risks to dicots since it was failed to apply in those plants. Therefore, an alternative positive/negative selection system was developed as alternatives based on caffeic acid O-methyltransferase (codA) D314A single-mutated version as the negative selection marker (Osakabe et al., 2014) or neomycin phosphotransferase II (NptII) (positive)/RNAi-based anti-NptII (negative) selection at much lower frequencies (Nishizawa-Yokoi et al., 2015b) that might be a result of less efficient G418 selection in rice. Nonetheless, the positive/negative selection strategy was shown to be unsuccessful in barley (Horvath et al., 2016) highlighting the extremely low efficiency in the absence of DSB and the high genome complexity in monocot gene targeting. In an herbicide selection-based gene targeting, Endo and coworkers successfully replaced WT allele by W548 L and S627I allele of the rice ALS and obtained hyper-tolerant homozygous T2 against an herbicide named bispyribac (BS). Under BS selection, gene targeting occurred at both the loci at ~3% (Endo et al., 2007). The frequency of targeting the OsALS for BS tolerance was enhanced to 6% by using the above mentioned HptII/DT-A selection system and the selection marker was subsequently excised by the use of *piggy*Bac system which can remove the marker gene without any DNA scar (Nishizawa-Yokoi et al., 2015a).

#### Targeted DSB-based HGT

DSBs induced at the gene targeting sites were shown to dramatically enhance efficiency at several orders of magnitude (Puchta et al., 1993). Since the introduced I-*Sce*I meganuclease-mediated DSBs showed significant enhancement of gene targeting frequency, ectopic recombination was tested in maize that revealed remarkably higher efficiencies compared to no DSB strategies using either particle bombardment or *Agrobacterium*-mediated transformation (Ayar et al., 2013; D'Halluin et al., 2008). However, since the pre-introduced homing nuclease targets a predefined sequence in the genome of the plant, gene targeting for a native gene/allele of interest in plant genomes was still far from expectation and a site-specific molecular scissors was highly demanded. Bearing that in mind, researchers engineered ZFNs, zinc finger motifs for DNA binding fused to the type IIS endonuclease *Fok*I, for efficiently specifically generating DSBs *in vivo* (Kim et al., 1996) and obtained significant enhancement of gene targeting efficiency at native loci in *Drosophila* (~1.5%) (Bibikova et al., 2003) and human cells (~18%) (Urnov et al., 2005). Subsequently, ZFNs were applied in plant gene targeting that obtained at an average of 17% HGT efficiency upon using a pre-integrated GUS:NPTII reporter system in tobacco protoplasts (Wright et al., 2005). A similar strategy also obtained ~10% HGT efficiency in restoring a pre-integrated defective herbicide tolerant gene (Cai et al., 2009). For targeting a multiple allelic loci, also acting as a herbicide tolerant selection marker, the efficiency were several folds lower at ~2% in tobacco (Townsend et al., 2009). In monocots, ZFN-base gene targeting was first shown to be efficient in maize via integrated insertion of a herbicide tolerant gene as selection marker into a native inositol-1,3,4,5,6-pentakisphosphate 2-kinase (IPK) gene (Shukla et al., 2009). Even though ZFNs offered a very much advantage over the meganucleases in plant gene targeting; their design, validation and specificity optimization processes were so much time-consuming and laborious (Puchta and Hohn, 2010).

TALENs, the second generation of sequence specific nuclease, also used protein-based DNA binding domain for targeting sites of interest. The highly specific and modular binding repeats offered an easier alternative for plant gene targeting. The first plant gene targeted events via HGT using TALENs were tobacco calli regenerated from protoplasts at 3.5 % efficiency without any selection marker (Zhang et al., 2013). 3.5% calli showed HGT events without antibiotic selection, however, it is not clear how many protoplasts were used for transfection. The TALEN approach was first applied in monocot for demonstrating the feasibility of gene targeting that reached 2-3% post bombardment of leaves with TALENs plus donors (Budhagatapalli et al., 2015). In rice, the similar range (1.4-6.3 %) of gene targeting frequencies was obtained with the OsALS herbicide tolerant allele (Li et al., 2016).

With the advent of CRISPR/Cas, a revolutionized molecular scissors for DSB formation, plant gene targeting is in theory applicable to any gene/ crop of interest thanks to the simplicity, flexibility and versatility of the system (Jinek et al., 2012; Zetsche et al., 2015). The CRISPR/Cas tools have been adapted for wide use in genome engineering studies in various kingdoms including Plantae (Barrangou and Doudna, 2016; Hsu et al., 2014; Jinek et al., 2012). The first attempt to modify a monocot genome via HGT using CRISPR/Cas9 was shown in 2013 by Shan and coworkers. Conducting a transient experiment, OsPDS was modified by HGT at 6.9 % frequency in rice protoplasts using CRISPR/Cas9 for DSB formation and single-stranded oligos as donor templates (Shan et al., 2013). Gene targeting in maize was shown with efficiency comparison between *Agrobacterium*-mediated delivery and particle bombardment, and between mega-nuclease and CRISPR/Cas9 in two loci, ALS and LIG1 (Svitashev et al., 2015). Several herbicide tolerant events were obtained from bombardment approach only indicating the very low targeting efficiency in maize and the requirement of high dose of donor template and editing tools for enhancing it. The herbicide-tolerant ALS allele was also shown in another CRISPR/Cas9-based gene targeting work using short dsDNA donor delivered as linearized or plasmid forms by bombardment or agrobacteria. Using hygromycin and BS herbicide for double selection, the total frequency of HGT events was obtained at 22.5-25%. In details, most of the HGT lines (42/52) obtained from bombardment showed a range of diversity with mixtures of perfect W548L and imperfect S627I. In *Agrobacterium*-mediated delivery, most of HGT lines (30/40) were perfect but heterozygous and the HGT alleles co-located at the loci with NHEJ alleles (Sun et al., 2016). The highly chimeric HGT patterns indicate prolonged activities of CRISPR/Cas9 and/or un-synchronized states of the cells used in the experiments and/or predominance of organogenesis during shoot formation post editing. Therefore, to synchronize the DSB formation and HDR, Endo and co-workers (2016) used a SpCas9-stably expressed calli and sequential transformation of sgRNAs and repair templates for OsALS gene targeting. The HGT frequency was too low and difficult to obtain targeted plants. However, when the DNA ligase 4 (LIG4) gene, a key player in NHEJ pathway was pre-targeted knocking-out, up to 1% frequency gene targeting of total herbicide-tolerant calli was observed indicating an competition between NHEJ and HDR pathways (Endo et al., 2016). In an attempt to modify a nitrate transporter gene NRT1.1B using CRISPR/Cas9-based tools, Li and coworkers obtained 6.72 % of precise replacement of 4 SNPs in the gene sequence without additional allele-associated selection marker (Li et al., 2018a). The gene targeted lines might contain inserted DNAs in their genome due to the high frequency of DNA integration of the bombardment system, but it was not determined.

An alternative of Cas9 system is the Cpf1-based molecular scissors. The latter cut dsDNAs using T-rich PAM for binding initiation and usually form 5’-protruding-broken ends at their distal ends relative to the PAM (Zetsche et al., 2015). CRISPR/Cpf1 was also used for gene targeting in monocot and showed precise SDSA-based gene replacement at the OsALS loci at comparable frequencies (0.66-1.22 %) (Li et al., 2018b) to those of Cas9 systems (Endo et al., 2016).

#### Replicon-based HDR

Because of the highly efficient replication of geminivirus genomes and their single-stranded DNA nature, these genomes have been used as perfect DNA template cargo for gene targeting in plants. Geminiviral genomic DNAs have been reconstructed to overexpress foreign proteins in plants at up to 80-fold higher levels compared to those of conventional T-DNA (Mor et al., 2003; Needham et al., 1998; Zhang and Mason, 2006) systems, due to their highly autonomous replication inside host nuclei and the ability to reprogram cells (Gutierrez, 1999; Hanley-Bowdoin et al., 2013). Furthermore, Rep/RepA has been reported to promote a cell environment that is permissive for HR to stimulate the replication of viral DNA (Baltes et al., 2014). Interestingly, it has been reported that somatic HR is promoted by geminiviral infection (Richter et al., 2014). The above characteristics of geminiviral replicons have been shown to make them perfect delivery tools for introducing large amounts of homologous donor templates to plant nuclei. Likewise, the movement and coat proteins of a bean yellow dwarf virus (BeYDV)–based replicon were removed and replaced with Cas9 or TALEN to improve gene targeting in plants (Baltes et al., 2014; Butler et al., 2016; Cermak et al., 2015; Dahan-Meir et al., 2018).

In monocots, wheat dwarf virus (WDV) was first engineered for CRISPR/Cas9-based genome editing and gene targeting in wheat (Gil-Humanes et al., 2017). More importantly, the work showed feasibility of multiplexed gene targeting of multiple homeoalleles of the wheat genome at 1 % frequency. A similar approach using WDV was also applied in rice for targeted insertion of GFP-2A-NPTII to the C terminals of ACT1 and GST gene in a WT of Cas9-overexpressed background. The WDV replicon-based tools showed significantly higher targeted knock-in efficiencies compared to that of conventional T-DNA tools (Wang et al., 2017).

#### Present challenges

Despite higher success rates in gene targeting in plants, most of the above mentioned cases required markers associated- or selectable loci, while the selection and regeneration of HGT events from edited cells are still challenging (Butler et al., 2016; Gil-Humanes et al., 2017; Hummel et al., 2018). As we could notice, most effective delivery methods for HGT tools were reported to be particle bombardment with relatively high frequencies of gene targeting (see Table 1) thanks to high doses of introduced donor DNAs but it also resulted in multiple DNA integration and/or regeneration difficulties. In addition, compared to the other delivery methods like *Agrobacterium*-mediated transformation, particle bombardment requires special equipment and costly consumables that are not popularly available in every research lab. *Agrobacterium*-mediated transformation is a very common and cost-effective method for plant gene targeting but it showed too low frequencies with conventional T-DNA cargos (Table 1). There has been one solution for delivery of high copy numbers of donor DNAs but not facilitating multiple DNA integration is using autonomous DNA replicons (Baltes et al., 2014; Cermak et al., 2015) but still challenging in monocots if it was not in combination with bombardment (Wang et al., 2017) or using stable Cas9-overexpressed background and selectable marker (Gil-Humanes et al., 2017). The frequencies dramatically reduced if multiple allelic loci and/or polyploid plants were targeted (Table 1). Further, the effective application of replicon cargos in plant gene targeting has been shown to be limited by their size (Baltes et al., 2014; Gil-Humanes et al., 2017; Suarez-Lopez and Gutierrez, 1997). Therefore, plant gene targeting, especially in cases of un-selectable alleles, is still a matter of improvement.

## Potential solutions and perspectives of monocot HGT

To improve plant gene targeting frequency, understanding HDR mechanism and finding optimal conditions for the HDR are the most important subjects in the field. The initial data on DSB-based gene targeting was led to an important conclusion that in plant somatic cells the majority of HGT-based products were formed via SDSA pathway (Figure 1) (D'Halluin et al., 2008; Puchta, 1998; Voytas, 2013; Vu et al., 2017). Since it has been well known that DSB formation is one of the key factors of gene targeting and that viral replicons are used as efficient delivery systems for HDR donor templates we are only discussing and proposing other factors regarding monocot gene targeting.

### *The role of homologous donor templates*

The initial experiments for understanding plant homologous recombination were mostly conducted in a transient manner using newly introduced homologous DNAs/plasmids in plant protoplasts/cells. Baur and coworkers (1990) reported extrachromosomal homologous recombinations between two plasmids in tobacco mesophyll protoplasts. The most favorable donor plasmid was in linearized forms that obtained 15 to 88-folds higher in recombination efficiency and were proportional to homologous zone size. The closer break sites to homologous zones the higher recombination frequencies were shown (Table 2) (Baur et al., 1990). Puchta and Hohn also confirmed that the homologous zone sizes (456 bp to 1200 bp) have direct correlation with extrachromosomal recombination frequencies in *Nicotiana plumbaginifolia* protoplasts. The frequency was significantly reduced when the homologous zone size was 456 bp or lower (Puchta and Hohn, 1991). Single-stranded DNA templates were shown to be efficient substrates for extrachromosomal recombination since they could directly facilitate the initial annealing step between donor and targeted DNAs. The double-stranded circular DNAs were the least efficient templates for the recombination mode (Bilang et al., 1992; de Groot et al., 1992).

### *Positive-negative selection*

Selection and/or regeneration of gene targeted events are critical to the successes of the approach. The dual mode of selection strongly enhanced the possibility in obtaining gene targeting events in monocots (see Table 1) even without the involvement of the revolutionized CRISPR/Cas molecular scissors. The positive-negative selection system provides big advantage in rice HGT and may help us in crop improvement by HGT (Terada et al., 2007). The hurdles in the removal of the associated positive selection markers have been solved by using a smart transposon-based excision system (Dang et al., 2013; Nishizawa-Yokoi et al., 2015a). It is exciting to combine the positive-negative selection system with the high DSB performance of CRISPR/Cas complexes for monocot gene targeting.

### *Overexpression of genes involving in HDR pathway*

A good number of HDR-related protein homologs were identified among prokaryotes and eukaryotes. Attempts were also made to study and/or improve HDR in somatic cells by overexpressing the proteins in targeted organisms. We are discussing the approaches in this part, thereby highlighting important points for improvement of plant gene targeting frequency.

*Escherichia coli* RecA protein (EcRecA) was shown to involve in HR in the bacterium by facilitating ssDNA searching and annealing to its homologous DNA repair templates and subsequently sequence exchanging and displacing (Chen et al., 2008; Muniyappa et al., 1984; Radding, 1981). Overexpression of EcRecA in tobacco protoplasts enhanced the DNA repair efficiency at 3 folds upon treatment with inter-strand DNA crosslinking agent (mitomycin C) (Table 2). Intrachromosomal HR frequency was also shown to be 10 times higher in the cells expressing the protein (Reiss et al., 1996). However, SpCas9-EcRecA fusion was shown to enhance indel mutation via supporting the SSA repair mode (Figure 1) and hence suppressed homology-directed gene conversion at 33 % in mammalian cells (Lin et al., 2017). By contrast, Cai and coworkers showed 1.7 folds increment of HGT frequency, when co-transfected CRISPR/Cas9 complex and EcRecA into human embryonic kidney (HEK) 293FT cells (Cai et al., 2019). In *E. coli* EcRecA acted in concert with RuvC protein that resolved Holiday junctions in late stage of DNA recombination (Iwasaki et al., 1991). By introducing the RuvC into nuclei of tobacco plants, Shalev and coworkers obtained strong enhancement of somatic crossover (12 folds); intrachromosomal recombination (11 folds), and extrachromosomal recombination (56 folds) (Shalev et al., 1999). The improvement may also be useful and applicable for DSB formation-based plant gene targeting approaches.

Activities of helicases were shown in the initiation of homologous recombination. A transgenic approach using *E. coli* RecQ (EcRecQ) revealed positive effects on extrachromosomal recombination of a two-vector system co-introduced into rice leaves. The EcRecQ transient expression driven by a monocot specific promoter induced a 4-fold increment of extrachromosomal gene targeting. The stimulation was much higher, at 20-40 folds in cases of EcRecQ stable expression (Li et al., 2004). This report confirmed the importance of helicase activities in HDR and suggested another potential approach for enhancement of monocot HGT frequency.

As discussed earlier, RAD54 plays roles in concert with the activities of RAD51 during HDR-mediated DSB amendment stage. Overexpression of yeast RAD54 in *Arabidopsis* was reported to increase gene targeting frequencies up to 27 folds indicating importance of strand invasion and/or chromatin remodeling in HDR pathway (Shaked et al., 2005). Developmental stages of explants used for monocot gene targeting may differentially support HDR pathway. It was shown that the highest recombination happened in embryogenic cells and explained by higher expression levels of OsRAD51 mRNA in the cells (Yang et al., 2010). Enhancement of the resection of the broken ends by overexpressing OsRecQl4 (BLM counterpart) and/or OsExo1 (Exo1 homolog) might positively support gene targeting in rice (Kwon et al., 2012).

### *Knockout of genes relating to HDR pathway*

As discussed above, RAD50 plays a central role in MRN/MRX complex for the resection of broken ends of dsDNAs. Knock out mutations of RAD50 led to lethal development in mice (Roset et al., 2014) and suppression of gene targeting in moss (Kamisugi et al., 2012). Surprisingly, a homozygous rad50 KO *A. thaliana* showed hyper-recombination in somatic cells as it supported 8-10 folds higher in gene conversion frequencies of an inverted repeat substrate (Table 2) (Gherbi et al., 2001). It led to an important conclusion that MRN/MRX activities are required by NHEJ more than HR does. The data suggests a strategy that transiently suppresses plant RAD50 during a gene targeting experiment for achieving high frequencies.

Sequence divergence between homologous DNA templates and targeted loci were shown to affect plant HGT frequency. The HGT frequencies were dramatically reduced at 4.1-, 9.6-, 11.7- or 20.3-fold when the levels of sequence divergence were increased at 0.5%, 2%, 4% or 9%, respectively. The sequence divergence might trigger nucleotide mismatch repair (NMR) mechanism with the involvement of NMR key protein AtMSH2 and hence, disturbing HDR process (Emmanuel et al., 2006; Li et al., 2006). AtMLH1, a homolog of E. coli MutL that involves in NMR, was shown to be required for homologous recombination and homeologous recombination. AtMHL1 mutation led to strong HDR reduction but less severe on homeologous recombination (Dion et al., 2007). The data indicates potential in regulation of MSH2 and/or MLH1 expression for enhancement of HGT in monocots, especially when we use homologous DNA templates with obligated mismatches.

Chromosome accessibility is a key factor determining DSB formations and subsequent repair of the broken DNAs. During replication or transcription, chromatin is loosened and nucleosomes are opened for assessment of related proteins involving in the processes. *Arabidopsis thaliana* chromatin assembly factor 1 (CAF‐1) complex involving in nucleosome assembly is formed by AtFAS1, AtFAS2 and AtMSI1 subunits. Endo and coworkers showed that knockout mutations of either AtFAS1 or AtFAS2 led to enhancement of somatic homologous recombination (HR) at 40‐folds potentially thanks to opened nucleosomes for accessibility, cell cycle synchronization favoring HDR conditions, and high expression of HDR-related genes in the mutant backgrounds (Endo et al., 2006). The data suggest a potential gene targeting enhancement with transient AtFAS1/2 knockdown by RNAi while introducing editing tools in somatic cells of monocots.

Another approach was tested in several studies that showed the positive effects on HGT by suppression of important genes that involve in NHEJ pathway such as KU70/80 or Lig4 (Endo et al., 2016; Nishizawa-Yokoi et al., 2012). The approach also showed reduction of stable integration of T-DNA in the KU70/80 and Lig4 suppression conditions suggesting a mechanism of T-DNA integration in genome.

### *Favorable tissue culture conditions for gene targeting*

Polyamines accumulated in cells with induced DSBs and were subsequently shown to improve HGT by promoting RAD51- mediated DNA strand exchange. Proving by in vitro assays, polyamines facilitated the capture of duplex DNA by the RAD51 pre-synaptic filament (Lee et al., 2019). Physical support of the substances may be a good approach for enhancing the activity of RAD51, a key protein in SDSA sub-pathway for gene targeting in monocot somatic cells. Chemicals that suppress genes involving in NHEJ pathway were used for testing HGT enhancement effects. Some chemicals inhibited DNA-PK (Robert et al., 2015) or KU70/80 or Lig4 (Table 2) (Chu et al., 2015; Maruyama et al., 2015) thereby enhanced HGT frequency in mammalian cell lines (Yu et al., 2015). It is still not clear whether we can achieve the similar gene targeting enhancement in plants. Data obtained from our lab showed nearly no effects of SCR7 and/or RS-1 on tomato gene targeting using geminiviral replicons in combination with CRISPR/Cpf1 (unpublished data). Temperature is an important factor enhancing CRISPR/Cas9-based targeted mutagenesis in plants (LeBlanc et al., 2018), and CRISPR/Cpf1-based HDR in zebrafish and *Xenopus* by controlling genome accessibility (Moreno-Mateos et al., 2017). Recently we re-engineered geminiviral replicon vectors in combination with CRISPR/Cpf1 and showed enhancement of HGT frequency at high temperatures and under lighting conditions (Vu et al., 2019).

### *Cell cycle synchronization*

One of the reason that HDR is limited to S-G2 phases is the availability of sister chromatids to be used as donor templates. As a consequence, majority of HDR genes might have evolved to be specifically expressed in the phases. The ideas are to artificially favor cellular conditions (S and G2 phases) that HDR is more efficient and that also limit NHEJ in blocking the targeted sites in other phases (M and G1), especially in case of Cas9s since they make cut in the core sequences proximal to their PAM. To that end, cell cycle synchronization at S/G2 phase using chemical (hydroxyurea) or molecular approach could be applied (Gutschner et al., 2016; Tsakraklides et al., 2015). Cas9 fused with N-terminal (110a.a) of human Gemini, a replication licensing factor that is a direct target of an M/G1-restricted E3 ubiquitin ligase for proteolysis, synchronized Cas9 expression in S/G2 phase thereby enhanced HGT up to 87% compared to only Cas9 (Table 2) (Gutschner et al., 2016).

### *In planta gene targeting*

Gene targeting in maize may be performed during fertilization as it provides permissive environment for sequence exchange by HGT (Djukanovic et al., 2006). Targeted mutagenesis using CRISPR/Cas9 been shown to be a highly valuable-*in planta* approach for crop improvement (Kelliher et al., 2019). The approach could also be applied for monocot gene targeting and it is also omitting the laborious, time consuming and complexed tissue culture process. In planta gene targeting could reduce mutation rate compared to tissue culture system that accompanies many mutations. However, the targeting tools should be re-designed to match with the conditions (pollen specific and/or ovule specific) they work within a short time period of pollination and fertilization

## HDR-based monocot events and regulation aspects

Genome edited crops including those created by CRISPR/Cas-based targeted mutagenesis and HGT approaches with or without the uses of DNA cargos are referred to as products of “new breeding techniques (NBTs)” (Laaninen, 2016; Lusser et al., 2011) or “new genetic modification techniques (nGMs)” (Eckerstorfer et al., 2019). In most of the genome edited events, foreign genetic editing tools could be excluded from the events after finishing their roles except that exotic DNA sequence(s) need to be introduced to specific site(s) in their genome(s). Likewise, most of the genome edited events could not be distinguished among the other mutated crops generated by conventional mutagens or natural mutations (Friedrichs et al., 2019; Grohmann et al., 2019), and hence, should not be regulated. The regulation legislation seems to be more complicated for HGT events since they have been regulated either as non-GMOs or GMOs by the US, EU, Japan, Australia… (For extensive review, see Eckerstorfer et al., 2019). In this part we are summarizing and discussing about regulation aspects as the critical hurdle for commercialization of HGT crops including monocots. We would also attempt to propose a regulation principle that could be useful for countries in the legislation process.

### Current status in regulation policies for genome edited crops

The US is the leading country in commercialization of GM crops till date with 75 Mha of planted biotech crops in 2018 (ISAAA, 2019). In the same year, the US was also the leading country to release policies for regulation of genome edited crops. The USDA announced that “Under its biotechnology regulations, USDA does not currently regulate, or have any plans to regulate plants that could otherwise have been developed through traditional breeding techniques as long as they are developed without the use of a plant pest as the donor or vector and they are not themselves plant pests” (USDA\_Press, 2018). This means that genome modifications like deletions, base substitutions and plant DNA modifications being similar to that potentially generated by conventional cross-breeding are all deregulated by USDA policies (NatPlants/Editorial, 2018). In Japan, the Ministry of Environment released its final policy on environmental safety on Feb. 8, 2019. According to the decision, creating food items using genome editing is not considered as GMOs, under the conditions that any DNA from nucleases required to edit the target organism are not left within the genome and the resulting gene edits could have also occurred naturally. The Japan Ministry of Health, Labor and Welfare announced a nearly identical assessment with regard to food safety on March 27, 2019 (USDA/JA9050, 2019). Brazil, Argentina, Canada, Chile and Colombia have decided to regulate genome edited event at a similar levels to that of the US (Ledford, 2019). Australia government adopted a middle level of regulation as SDN-1 products would not be regulated (Mallapaty, 2019). By contrast, on July 25, 2018, The European Court of Justice decided that genome edited crops would be subject to the same rules as transgenic plants or animals (ECJ, 2018). Other Governments of Republic of Korea, China, Russia and India are still making their determinations on how to regulate this technology.

### SDNs declaration

In fact, according to the released ruling policies of the governments except EU, not all of the genome edited events are considered as non-GMs. In principle, the genome edited crops were initially divided under classification of the so-called Site Directed Nucleases (SDN) by European Food Safety Authority (EFSA) in 2012: “In SDN-1 applications, only the SDNs are introduced into plant cells (stably or transiently), generating site-specific mutations by non-homologous end-joining (NHEJ). In SDN-2 applications, homologous repair DNA (donor DNA) is introduced together with the SDN complex to create specific nucleotide sequence changes by homologous recombination (HR) or homology-directed repair (HDR). The SDN-2 technique can introduce substantial changes to the nucleotide sequences of the target gene, but more precise changes according to bioengineer’s plan. SDN-2 techniques can provide unlimited SNP alleles that can boost innovative crop breeding. In the SDN-3 technique a large stretch of donor DNA (up to several kilobases) is introduced together with the SDN complex to target DNA insertion into a predefined genomic locus. The predefined locus may or may not have extensive similarity to the DNA to be inserted. The insertion can take place either by HR or by NHEJ. In the case of insertion by means of NHEJ, the technique is denominated the SDN-3–NHEJ technique” (EFSA, 2012). The classification is now generally accepted as the basal information for genome edited crop regulation. On August 20, 2018, Japan’s Ministry of Environment (MOE) released a draft of its regulation policies adding some detailed requirement of SDNs to be excluded from the Cartagena Protocol regulation (USDA/JA8064, 2018). The levels of regulation are decided based on the presence/absence of foreign genetic carriers, the levels of modification and the natural existence of the modification in genome edited events. In another point of view they are case-by-case basis (see Table 3). From the released regulations, it is now clear that HGT will be regulated as either non-GMOs (some cases of SDN-2) or GMOs (some cases of SDN-2 and SDN-3).

The classification and regulation consideration have given plant gene targeting approaches a big challenge to be commercialized even its efficacy would be enhanced to a practical level. The gene targeting for modifying SNPs is deregulated by “relaxed” governments like the US and Japan (case-by-case) but not Australia. HGT products subjected to SDN-3 category containing inserted sequence(s) that could not potentially formed in nature will all be regulated as transgenic products (Table 3).

### A regulation proposal for NBPT products

Many governments seem to be trying to create sufficient oversight to protect the public interest and at the same time not create new obstacles to technical innovation. Genome editing-based precision breeding is an innovative technology, but the technologies will evolve continuously. Particularly HDR-based precision breeding technologies are the most cutting-edge technology among genome editing techniques since it can produce both precise SDN-1 and SDN-2/SDN-3 products. HDR-based precision breeding products are generally classified as SDN-2 or SDN3 that use DNA donor template during gene editing process, thus regulated as GMO in Australia and Japan, with potential exceptions. Mechanical classification of HDR-based genome-edited products in GMO category might pose the most unreasonable obstacle to this plant breeding innovative. In fact, HDR-based precision breeding can come true long waiting dream of breeders, which precisely introduce beneficial gene alleles from crossable relatives without other trait compromise such as linkage drag (mixed-up of targeted beneficial traits and unintentional undesirable traits by a linkage effect). What might be the solution? We must now again remind ourselves of the purpose of the regulation of biotechnology products. Regulations exist to prevent new products from harming human health or the environment. Many various technologies can be used to produce the same or similar indistinguishable products to tradition breeding products; therefore, the consistent risk-based regulatory approach is to treat similar products identically. In this view, it worth to refer the Canadian regulation policy that regulates only products, but only plants with novel traits (PNTs), irrespective of used technologies (Ellens et al., 2019). According to Canadian regulation, some SDN-1 products or even chemically mutagenized products can be regulated. But this regulation policy provides more open possibility to use various innovative technologies including genome editing or GMO etc. In the end, fruiting from the NPBT crops will mainly dependent on the level of regulation on the NPBT products.

# Conclusions

Development of novel traits for monocot crops is crucial for coping with the future challenges in crop production for feeding approximately 10 billion of people in 2050 (Hickey et al., 2019; Ray et al., 2013). Recent development of NPBT has paved ways for crop improvement to cope with the difficult mission. Targeted mutagenesis approaches (Figure 1, error-prone approaches and Figure 2) of NBPT have been gaining significant succeeds in targeting a wide range of crop plants including monocots thanks to the ease and high frequencies of the revolutionized molecular scissors, especially the CRISPR/Cas complexes (Figure 2 and Supplemental Table 1). Similar outcomes could also be expected with the base editing techniques (Figure 3B and Supplemental Table 2). However, precision editing approaches like ODM (Figure 3A) and especially plant HGT approaches are still facing hurdles in practical applications due to their low efficiencies and complexities in editing event regeneration (Figure 1. HR and SDSA approaches and Table 1). A significant number of studies have been conducted in order to unveil the mechanisms of HDR pathway (Figure 1) as well as to overcome the obstacles in HGT frequency (Table 2). The major HGT supporting strategies till date are (1) appropriate selection of HGT events; (2) enhancement of DSB formation frequency and specificity at the targeted site; (3) high dose delivery of homologous donors; (4) Over-expression/interference of genes involve in HR/C-NHEJ; (5) chemical-based activation/suppression of gene involving in HR/C-NHEJ; and (6) chemical/biological-based cell cycle synchronization (Table 2). Though it is still not clear, we realize of the potency of the in planta HGT approach which help in omitting the laborious and time consuming tissue culture process. In addition, it may reduce un-intended effects due to the high performance of CRISPR/Cas system as well as genetic variation in calli-mediated plant regeneration. Appropriate applications of every of the strategies or in combinations, and with the uses of CRISPR/Cas complexes may offer better way to overcome the low efficiency and regeneration concerns. More works still need to be done for practical customization of monocot crop traits using HGT technique.

Recently negative predictions about food productions have forced several governments to accept NPBTs as the only way to sustain our future. Regulation legislation has been more relaxed with NPBT products produced by SDN-1 and SDN-2 (case-by-case) in USA, Japan and Australia but not the EU. Regulation pending countries are China, India, and Republic of Korea. Based on the background and understanding, we attempt to propose a few principles for upcoming regulation policies for the same.

# Funding

This work was supported by the National Research Foundation of Korea (Grant NRF 2017R1A4A1015515) and by the Next-Generation BioGreen 21 Program (SSAC, Grant PJ01322601), Rural Development Administration (RDA), Republic of Korea.

# Acknowledgements

# Authors’ Contributions

All the authors contribute in writing the manuscript. Vu TV and Kim JY revised the manuscript. Kim JY finalized the manuscript.

# Competing Interest

The authors declare that they have no competing interests

**FIGURE LEGENDS:**

**Figure 1. DSB repair pathways.**.

In C-NHEJ pathway, DSB formation induces binding to broken ends by KU70/80 heterodimers that subsequently recruit DNA-PK complex. DNA-PK then activate 53BP1/RIF1 complex that plays role in shielding the broken ends from resection by antagonizing BRCA1/CtIP activity. DNA-PK also activates other KU-recruited protein like XLF, XRCC4 and Lig4 for ligating the broken ends. In HDR pathway, DSB formation induces cell cycle arrest initiated with the activation of ATM resulted from sensing a chromatin structure change. Activated monomeric ATM then phosphorylates MRN complex and P53/SOG1 which regulates cell cycle checkpoint and arrest. MRN/MRX activation support end resection for HDR. Limited resection leads to MMEJ and if substantial level of resection is formed but in the absence of donor template, SSA is likely to be used for repairing. MMEJ requires PARP and Pol Q for its processes and SSA requires the role of RAD52. Both MMEJ and SSA need ssDNA flapping endonuclease FEN1 and Lig3/Lig1:XRCC1 for ligating final products. Extensive resection of the broken ends is facilitated by Exonuclease 1 (Exo1) and/or Sgs1-Dna2. Under the presence of donor template, the 3’ overhangs of resected ends could be protected by RPA binding and then recruiting RAD51 to the ssDNA with the support and control by BRCA2. RAD51 binds to the resected ssDNA overhang forming nucleoprotein filament or presynaptic filament. With the support by RAD54, the filament structure invades donor template sequence, searches for, and anneals to complementary sequence followed by displacement loop (D-loop) formation. Subsequently, the free 3’ OH end of the invaded ssDNA primes donor template-dependent DNA synthesis. This process determines the outcomes of HDR with several sub-pathways (DSBR with dHJ and SDSA) with the supportive activity of RAD5A, REC4Q and MUS81. The representative DNA sticks and protein structures are not proportional to their reality. The potential proteins involving in the processes of each pathway or sub-pathway are denoted in adjacent to their approaching lines. XRCC: X-ray repair cross-complementing protein; XLF: XRCC4-like factor; Lig4: DNA ligase 4; PARP: poly-ADP-ribose polymerase; Pol Q: DNA polymerase theta.

**Figure 2. The four generations of molecular scissors.**

The first, second and third generations of molecular scissors, Homing nuclease (A); ZFN (B); and TALEN (C), are characterized as nucleases relying on DNA binding domains to recognize DNA target sites. Homing nucleases recognize long DNA sequences of 14-40 bp by its DNA binding domain. A ZFN or TALEN is designed by connecting 3-6 zinc finger motifs or 17-20 TALE modules, respectively for DNA binding and an endonuclease domain of FokI restriction enzyme for cutting. FokI works only in homodimer form so usually one has to design a pairs of ZFNs or TALENs for targeting a DNA site. FokI activity usually produces DSB with 4nt overhangs. The fourth generation, CRISPR/Cas (D), is also the most powerful one that uses guide RNA components to form active complex thereby interrogating and searching for target DNA site based on Watson-Crick base pairing between the guide RNA and targeted strand. The representative DNA sticks and protein structures are not proportional to their reality.

**Figure 3. non-DSB precise gene targeting approaches.**

A. Base editing approaches. Cytidine base editors (CBEs) and Adenosine base editors (ABEs) are two types of base editors published till date**.** CBEs. Dead Cas9 (blue) binds with the target C (green) in a guide RNA (purple), which mediates local DNA strand separation. A tethered APOBEC1 enzyme (brown) through cytidine deamination converts the single-stranded target C to U. Through DNA repair or replication, the original G:C is replaced by an A:T base pair at the target site. ABEs. A hypothetical deoxyadenosine deaminase and a catalytically impaired nCas9 (Cas9 D10A nickase) bind target DNA in a guide RNA to expose a small bubble of single-stranded DNA, which catalyze to convert A to I within this bubble. The final result can be converted I:T base pair to a G:C base pair following DNA replication or DNA repair. B. Oligonucleotide directed mutagenesis process. A Gene Repair Oligonucleotide (GRON) which contains designed modification is delivered and paired with the target DNA sequence. The GRON creates a mismatch at target site and triggers DNA repair mechanism. DNA repair enzymes detect the mismatch and repair target DNA sequence using the GRON as a template. Once repair process is completed during cell division and multiplication, the GRON is removed and degraded. The target sequence is modified with designed changes. The representative DNA sticks and protein structures are not proportional to their reality.

**Figure 4. Homology-directed repair pathway determination and its favorable cell contexts.**

Activation of MRN complex and P53/SOG1 triggers the activation of cell cycle checkpoint proteins like CDKB1 (G2/M checkpoint) and CYCB1 (S phase checkpoint) or NAC-type transcription factors ANAC044 and ANAC085 (S/G2 checkpoints) or WEE1 kinase for cell cycle arrest

**TABLE LEGENDS:**

**Table 1. Major HGT studies in monocots**

**Table 2. Potential approaches for improvement of HGT in monocots**

**Table 3. Gene edited crop regulation status on the SDN basis**

**SUPPLEMENTAL TABLE LEGENDS:**

**Supplemental Table 1. Targeted mutagenesis-genome editing in monocots using CRISPR/Cas**

**Supplemental Table 2. Major applications of base editing approaches in plants**

# References

Allyson Cole-Strauss, K.Y., Yufei Xiang, Bruce C. Byrne, Michael C. Rice, Jeff Gryn, William K. Holloman, Eric B. Kmiect (1996). Correction of the Mutation Responsible for Sickle Cell Anemia by an RNA-DNA Oligonucleotide.

Anders, C., Niewoehner, O., Duerst, A., and Jinek, M. (2014). Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature *513*, 569-573.

Asano, K., Yamasaki, M., Takuno, S., Miura, K., Katagiri, S., Ito, T., Doi, K., Wu, J., Ebana, K., Matsumoto, T.*, et al.* (2011). Artificial selection for a green revolution gene during japonica rice domestication. Proc Natl Acad Sci U S A *108*, 11034-11039.

Ayar, A., Wehrkamp-Richter, S., Laffaire, J.B., Le Goff, S., Levy, J., Chaignon, S., Salmi, H., Lepicard, A., Sallaud, C., Gallego, M.E.*, et al.* (2013). Gene targeting in maize by somatic ectopic recombination. Plant Biotechnol J *11*, 305-314.

Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature *421*, 499-506.

Baltes, N.J., Gil-Humanes, J., Cermak, T., Atkins, P.A., and Voytas, D.F. (2014). DNA replicons for plant genome engineering. Plant Cell *26*, 151-163.

Barrangou, R., and Doudna, J.A. (2016). Applications of CRISPR technologies in research and beyond. Nat Biotechnol *34*, 933-941.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., and Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science *315*, 1709-1712.

Baur, M., Potrykus, I., and Paszkowski, J. (1990). Intermolecular homologous recombination in plants. Mol Cell Biol *10*, 492-500.

Beetham, P.R., Kipp, P.B., Sawycky, X.L., Arntzen, C.J., and May, G.D. (1999). A tool for functional plant genomics: chimeric RNA/DNA oligonucleotides cause in vivo gene-specific mutations. Proc Natl Acad Sci U S A *96*, 8774-8778.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., and Nekrasov, V. (2013). Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods *9*, 39.

Berg, J.M. (1988). Proposed structure for the zinc-binding domains from transcription factor IIIA and related proteins. Proc Natl Acad Sci U S A *85*, 99-102.

Bibikova, M., Beumer, K., Trautman, J.K., and Carroll, D. (2003). Enhancing gene targeting with designed zinc finger nucleases. Science *300*, 764.

Bilang, R., Peterhans, A., Bogucki, A., and Paszkowski, J. (1992). Single-stranded DNA as a recombination substrate in plants as assessed by stable and transient recombination assays. Mol Cell Biol *12*, 329-336.

Bitinaite, J., Wah, D.A., Aggarwal, A.K., and Schildkraut, I. (1998). FokI dimerization is required for DNA cleavage. Proc Natl Acad Sci U S A *95*, 10570-10575.

Bortesi, L., and Fischer, R. (2015). The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv *33*, 41-52.

Brouns, S.J., Jore, M.M., Lundgren, M., Westra, E.R., Slijkhuis, R.J., Snijders, A.P., Dickman, M.J., Makarova, K.S., Koonin, E.V., and van der Oost, J. (2008). Small CRISPR RNAs guide antiviral defense in prokaryotes. Science *321*, 960-964.

Budhagatapalli, N., Rutten, T., Gurushidze, M., Kumlehn, J., and Hensel, G. (2015). Targeted Modification of Gene Function Exploiting Homology-Directed Repair of TALEN-Mediated Double-Strand Breaks in Barley. G3 (Bethesda) *5*, 1857-1863.

Butler, N.M., Baltes, N.J., Voytas, D.F., and Douches, D.S. (2016). Geminivirus-Mediated Genome Editing in Potato (Solanum tuberosum L.) Using Sequence-Specific Nucleases. Front Plant Sci *7*, 1045.

Cai, C.Q., Doyon, Y., Ainley, W.M., Miller, J.C., Dekelver, R.C., Moehle, E.A., Rock, J.M., Lee, Y.L., Garrison, R., Schulenberg, L.*, et al.* (2009). Targeted transgene integration in plant cells using designed zinc finger nucleases. Plant Mol Biol *69*, 699-709.

Cai, Y., Cheng, T., Yao, Y., Li, X., Ma, Y., Li, L., Zhao, H., Bao, J., Zhang, M., Qiu, Z.*, et al.* (2019). In vivo genome editing rescues photoreceptor degeneration via a Cas9/RecA-mediated homology-directed repair pathway. Sci Adv *5*, eaav3335.

Cermak, T., Baltes, N.J., Cegan, R., Zhang, Y., and Voytas, D.F. (2015). High-frequency, precise modification of the tomato genome. Genome Biol *16*, 232.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., and Voytas, D.F. (2011). Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res *39*, e82.

Chen, Z., Yang, H., and Pavletich, N.P. (2008). Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. Nature *453*, 489-484.

Chevalier, B.S., Kortemme, T., Chadsey, M.S., Baker, D., Monnat, R.J., and Stoddard, B.L. (2002). Design, activity, and structure of a highly specific artificial endonuclease. Mol Cell *10*, 895-905.

Chevalier, B.S., and Stoddard, B.L. (2001). Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. Nucleic Acids Res *29*, 3757-3774.

Chu, V.T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K., and Kuhn, R. (2015). Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotechnol *33*, 543-548.

Cox, D.B., Platt, R.J., and Zhang, F. (2015). Therapeutic genome editing: prospects and challenges. Nat Med *21*, 121-131.

D'Halluin, K., Vanderstraeten, C., Stals, E., Cornelissen, M., and Ruiter, R. (2008). Homologous recombination: a basis for targeted genome optimization in crop species such as maize. Plant Biotechnol J *6*, 93-102.

Dahan-Meir, T., Filler-Hayut, S., Melamed-Bessudo, C., Bocobza, S., Czosnek, H., Aharoni, A., and Levy, A.A. (2018). Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. Plant J *95*, 5-16.

Dang, T.T., Shimatani, Z., Kawano, Y., Terada, R., and Shimamoto, K. (2013). Gene editing a constitutively active OsRac1 by homologous recombination-based gene targeting induces immune responses in rice. Plant Cell Physiol *54*, 2058-2070.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature *411*, 826-833.

de Groot, M.J., Offringa, R., Does, M.P., Hooykaas, P.J., and van den Elzen, P.J. (1992). Mechanisms of intermolecular homologous recombination in plants as studied with single- and double-stranded DNA molecules. Nucleic Acids Res *20*, 2785-2794.

De Schutter, K., Joubes, J., Cools, T., Verkest, A., Corellou, F., Babiychuk, E., Van Der Schueren, E., Beeckman, T., Kushnir, S., Inze, D.*, et al.* (2007). Arabidopsis WEE1 kinase controls cell cycle arrest in response to activation of the DNA integrity checkpoint. Plant Cell *19*, 211-225.

Dion, E., Li, L., Jean, M., and Belzile, F. (2007). An Arabidopsis MLH1 mutant exhibits reproductive defects and reveals a dual role for this gene in mitotic recombination. Plant J *51*, 431-440.

Djukanovic, V., Orczyk, W., Gao, H., Sun, X., Garrett, N., Zhen, S., Gordon-Kamm, W., Barton, J., and Lyznik, L.A. (2006). Gene conversion in transgenic maize plants expressing FLP/FRT and Cre/loxP site-specific recombination systems. Plant Biotechnol J *4*, 345-357.

Dong, C., Beetham, P., Vincent, K., and Sharp, P. (2006). Oligonucleotide-directed gene repair in wheat using a transient plasmid gene repair assay system. Plant Cell Rep *25*, 457-465.

Doudna, J.A., and Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science *346*, 1258096.

Dray, E., Siaud, N., Dubois, E., and Doutriaux, M.P. (2006). Interaction between Arabidopsis Brca2 and its partners Rad51, Dmc1, and Dss1. Plant Physiol *140*, 1059-1069.

ECJ (2018). JUDGMENT OF THE COURT (Grand Chamber).

Eckerstorfer, M.F., Engelhard, M., Heissenberger, A., Simon, S., and Teichmann, H. (2019). Plants Developed by New Genetic Modification Techniques-Comparison of Existing Regulatory Frameworks in the EU and Non-EU Countries. Front Bioeng Biotechnol *7*, 26.

EFSA (2012). Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function. European Food Safety Authority (EFSA). EFSA Journal *10*, 2943.

Ellens, K.W., Levac, D., Pearson, C., Savoie, A., Strand, N., Louter, J., and Tibelius, C. (2019). Canadian regulatory aspects of gene editing technologies. Transgenic Res *28*, 165-168.

Emmanuel, E., Yehuda, E., Melamed-Bessudo, C., Avivi-Ragolsky, N., and Levy, A.A. (2006). The role of AtMSH2 in homologous recombination in Arabidopsis thaliana. EMBO Rep *7*, 100-105.

Endo, M., Ishikawa, Y., Osakabe, K., Nakayama, S., Kaya, H., Araki, T., Shibahara, K., Abe, K., Ichikawa, H., Valentine, L.*, et al.* (2006). Increased frequency of homologous recombination and T-DNA integration in Arabidopsis CAF-1 mutants. EMBO J *25*, 5579-5590.

Endo, M., Mikami, M., and Toki, S. (2016). Biallelic Gene Targeting in Rice. Plant Physiol *170*, 667-677.

Endo, M., Osakabe, K., Ono, K., Handa, H., Shimizu, T., and Toki, S. (2007). Molecular breeding of a novel herbicide-tolerant rice by gene targeting. Plant J *52*, 157-166.

Eriksson, D. (2018). The Swedish policy approach to directed mutagenesis in a European context. Physiol Plant *164*, 385-395.

FAO (2019a). Food Outlook - Biannual Report on Global Food Markets. . Rome Licence: CC BY-NC-SA 30 IGO.

FAO (2019b). The state of food security nutrition in the world. <http://wwwfaoorg/state-of-food-security-nutrition/en/>.

Fauser, F., Roth, N., Pacher, M., Ilg, G., Sanchez-Fernandez, R., Biesgen, C., and Puchta, H. (2012). In planta gene targeting. Proc Natl Acad Sci U S A *109*, 7535-7540.

Fernandes, J.B., Duhamel, M., Seguela-Arnaud, M., Froger, N., Girard, C., Choinard, S., Solier, V., De Winne, N., De Jaeger, G., Gevaert, K.*, et al.* (2018). FIGL1 and its novel partner FLIP form a conserved complex that regulates homologous recombination. PLoS Genet *14*, e1007317.

Friedrichs, S., Takasu, Y., Kearns, P., Dagallier, B., Oshima, R., Schofield, J., and Moreddu, C. (2019). Meeting report of the OECD conference on "Genome Editing: Applications in Agriculture-Implications for Health, Environment and Regulation". Transgenic Res *28*, 419-463.

Gaudelli, N.M., Komor, A.C., Rees, H.A., Packer, M.S., Badran, A.H., Bryson, D.I., and Liu, D.R. (2017). Programmable base editing of A\*T to G\*C in genomic DNA without DNA cleavage. Nature *551*, 464-471.

Gehrke, J.M., Cervantes, O., Clement, M.K., Wu, Y., Zeng, J., Bauer, D.E., Pinello, L., and Joung, J.K. (2018). An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities. Nat Biotechnol *36*, 977-982.

Gherbi, H., Gallego, M.E., Jalut, N., Lucht, J.M., Hohn, B., and White, C.I. (2001). Homologous recombination in planta is stimulated in the absence of Rad50. EMBO Rep *2*, 287-291.

Gil-Humanes, J., Wang, Y., Liang, Z., Shan, Q., Ozuna, C.V., Sanchez-Leon, S., Baltes, N.J., Starker, C., Barro, F., Gao, C.*, et al.* (2017). High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. Plant J *89*, 1251-1262.

Girard, C., Chelysheva, L., Choinard, S., Froger, N., Macaisne, N., Lemhemdi, A., Mazel, J., Crismani, W., and Mercier, R. (2015). AAA-ATPase FIDGETIN-LIKE 1 and Helicase FANCM Antagonize Meiotic Crossovers by Distinct Mechanisms. PLoS Genet *11*, e1005369.

Gocal, G. (2015). Non-Transgenic Trait Development in Crop Plants Using Oligo-Directed Mutagenesis: Cibus’ Rapid Trait Development System.

Goedecke, W., Eijpe, M., Offenberg, H.H., van Aalderen, M., and Heyting, C. (1999). Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. Nat Genet *23*, 194-198.

Gong, Y., Handa, N., Kowalczykowski, S.C., and de Lange, T. (2017). PHF11 promotes DSB resection, ATR signaling, and HR. Genes Dev *31*, 46-58.

Gottlieb, T.M., and Jackson, S.P. (1993). The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. Cell *72*, 131-142.

Grohmann, L., Keilwagen, J., Duensing, N., Dagand, E., Hartung, F., Wilhelm, R., Bendiek, J., and Sprink, T. (2019). Detection and Identification of Genome Editing in Plants: Challenges and Opportunities. Front Plant Sci *10*, 236.

Guo, J., Gaj, T., and Barbas, C.F., 3rd (2010). Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases. J Mol Biol *400*, 96-107.

Gutierrez, C. (1999). Geminivirus DNA replication. Cell Mol Life Sci *56*, 313-329.

Gutschner, T., Haemmerle, M., Genovese, G., Draetta, G.F., and Chin, L. (2016). Post-translational Regulation of Cas9 during G1 Enhances Homology-Directed Repair. Cell Rep *14*, 1555-1566.

Hanley-Bowdoin, L., Bejarano, E.R., Robertson, D., and Mansoor, S. (2013). Geminiviruses: masters at redirecting and reprogramming plant processes. Nat Rev Microbiol *11*, 777-788.

Harper, J.W., and Elledge, S.J. (2007). The DNA damage response: ten years after. Mol Cell *28*, 739-745.

Hartung, F., Suer, S., Bergmann, T., and Puchta, H. (2006). The role of AtMUS81 in DNA repair and its genetic interaction with the helicase AtRecQ4A. Nucleic Acids Res *34*, 4438-4448.

Heyer, W.D., Ehmsen, K.T., and Liu, J. (2010). Regulation of homologous recombination in eukaryotes. Annu Rev Genet *44*, 113-139.

Hickey, L.T., A, N.H., Robinson, H., Jackson, S.A., Leal-Bertioli, S.C.M., Tester, M., Gao, C., Godwin, I.D., Hayes, B.J., and Wulff, B.B.H. (2019). Breeding crops to feed 10 billion. Nat Biotechnol *37*, 744-754.

Hilton, I.B., and Gersbach, C.A. (2015). Enabling functional genomics with genome engineering. Genome Res *25*, 1442-1455.

Hirakawa, T., Hasegawa, J., White, C.I., and Matsunaga, S. (2017). RAD54 forms DNA repair foci in response to DNA damage in living plant cells. Plant J *90*, 372-382.

Holliday, R. (1977). Recombination and meiosis. Philos Trans R Soc Lond B Biol Sci *277*, 359-370.

Horvath, M., Steinbiss, H.H., and Reiss, B. (2016). Gene Targeting Without DSB Induction Is Inefficient in Barley. Front Plant Sci *7*, 1973.

Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell *157*, 1262-1278.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O.*, et al.* (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol *31*, 827-832.

Hummel, A.W., Chauhan, R.D., Cermak, T., Mutka, A.M., Vijayaraghavan, A., Boyher, A., Starker, C.G., Bart, R., Voytas, D.F., and Taylor, N.J. (2018). Allele exchange at the EPSPS locus confers glyphosate tolerance in cassava. Plant Biotechnol J *16*, 1275-1282.

ISAAA (2019). Global Status of Commercialized Biotech/GM Crops: 2018. Brief *54*.

Isono, M., Niimi, A., Oike, T., Hagiwara, Y., Sato, H., Sekine, R., Yoshida, Y., Isobe, S.Y., Obuse, C., Nishi, R.*, et al.* (2017). BRCA1 Directs the Repair Pathway to Homologous Recombination by Promoting 53BP1 Dephosphorylation. Cell Rep *18*, 520-532.

Iwasaki, H., Takahagi, M., Shiba, T., Nakata, A., and Shinagawa, H. (1991). Escherichia coli RuvC protein is an endonuclease that resolves the Holliday structure. EMBO J *10*, 4381-4389.

Jasin, M. (1996). Genetic manipulation of genomes with rare-cutting endonucleases. Trends Genet *12*, 224-228.

Jasin, M., and Rothstein, R. (2013). Repair of strand breaks by homologous recombination. Cold Spring Harb Perspect Biol *5*, a012740.

Jiang, W., Bikard, D., Cox, D., Zhang, F., and Marraffini, L.A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol *31*, 233-239.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science *337*, 816-821.

Jinek, M., Jiang, F., Taylor, D.W., Sternberg, S.H., Kaya, E., Ma, E., Anders, C., Hauer, M., Zhou, K., Lin, S.*, et al.* (2014). Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science *343*, 1247997.

Kamisugi, Y., Schaefer, D.G., Kozak, J., Charlot, F., Vrielynck, N., Hola, M., Angelis, K.J., Cuming, A.C., and Nogue, F. (2012). MRE11 and RAD50, but not NBS1, are essential for gene targeting in the moss Physcomitrella patens. Nucleic Acids Res *40*, 3496-3510.

Kastan, M.B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Kay, S., Hahn, S., Marois, E., Wieduwild, R., and Bonas, U. (2009). Detailed analysis of the DNA recognition motifs of the Xanthomonas type III effectors AvrBs3 and AvrBs3Deltarep16. Plant J *59*, 859-871.

Kelliher, T., Starr, D., Su, X., Tang, G., Chen, Z., Carter, J., Wittich, P.E., Dong, S., Green, J., Burch, E.*, et al.* (2019). One-step genome editing of elite crop germplasm during haploid induction. Nat Biotechnol *37*, 287-292.

Khush, G.S. (2001). Green revolution: the way forward. Nat Rev Genet *2*, 815-822.

Kijas, A.W., Lim, Y.C., Bolderson, E., Cerosaletti, K., Gatei, M., Jakob, B., Tobias, F., Taucher-Scholz, G., Gueven, N., Oakley, G.*, et al.* (2015). ATM-dependent phosphorylation of MRE11 controls extent of resection during homology directed repair by signalling through Exonuclease 1. Nucleic Acids Res *43*, 8352-8367.

Kim, D., Kim, J., Hur, J.K., Been, K.W., Yoon, S.H., and Kim, J.S. (2016). Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. Nat Biotechnol *34*, 863-868.

Kim, E.J.K.a.J.S. (2015). Genome editing. IBS Center for Genome Engineering *16*.

Kim, Y., Kweon, J., Kim, A., Chon, J.K., Yoo, J.Y., Kim, H.J., Kim, S., Lee, C., Jeong, E., Chung, E.*, et al.* (2013). A library of TAL effector nucleases spanning the human genome. Nat Biotechnol *31*, 251-258.

Kim, Y.G., Cha, J., and Chandrasegaran, S. (1996). Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc Natl Acad Sci U S A *93*, 1156-1160.

Klutstein, M., Shaked, H., Sherman, A., Avivi-Ragolsky, N., Shema, E., Zenvirth, D., Levy, A.A., and Simchen, G. (2008). Functional conservation of the yeast and Arabidopsis RAD54-like genes. Genetics *178*, 2389-2397.

Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., and Liu, D.R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature *533*, 420-424.

Koonin, E.V., and Makarova, K.S. (2019). Origins and evolution of CRISPR-Cas systems. Philos Trans R Soc Lond B Biol Sci *374*, 20180087.

Kumar, R., Duhamel, M., Coutant, E., Ben-Nahia, E., and Mercier, R. (2019). Antagonism between BRCA2 and FIGL1 regulates homologous recombination. Nucleic Acids Res *47*, 5170-5180.

Kwon, Y.I., Abe, K., Osakabe, K., Endo, M., Nishizawa-Yokoi, A., Saika, H., Shimada, H., and Toki, S. (2012). Overexpression of OsRecQl4 and/or OsExo1 enhances DSB-induced homologous recombination in rice. Plant Cell Physiol *53*, 2142-2152.

Laaninen, T. (2016). New plant-breeding techniques Applicability of GM rules. EPRS *PE 582.018*.

Laity, J.H., Lee, B.M., and Wright, P.E. (2001). Zinc finger proteins: new insights into structural and functional diversity. Curr Opin Struct Biol *11*, 39-46.

Lamarche, B.J., Orazio, N.I., and Weitzman, M.D. (2010). The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett *584*, 3682-3695.

Langerak, P., Mejia-Ramirez, E., Limbo, O., and Russell, P. (2011). Release of Ku and MRN from DNA ends by Mre11 nuclease activity and Ctp1 is required for homologous recombination repair of double-strand breaks. PLoS Genet *7*, e1002271.

LeBlanc, C., Zhang, F., Mendez, J., Lozano, Y., Chatpar, K., Irish, V.F., and Jacob, Y. (2018). Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. Plant J *93*, 377-386.

Ledford, H. (2019). CRISPR conundrum: Strict European court ruling leaves food-testing labs without a plan. Nature *572*, 15.

Lee, C.Y., Su, G.C., Huang, W.Y., Ko, M.Y., Yeh, H.Y., Chang, G.D., Lin, S.J., and Chi, P. (2019). Promotion of homology-directed DNA repair by polyamines. Nat Commun *10*, 65.

Lee, J.H., and Paull, T.T. (2005). ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science *308*, 551-554.

Li, H.Q., Terada, R., Li, M.R., and Iida, S. (2004). RecQ helicase enhances homologous recombination in plants. FEBS Lett *574*, 151-155.

Li, J., Zhang, X., Sun, Y., Zhang, J., Du, W., Guo, X., Li, S., Zhao, Y., and Xia, L. (2018a). Efficient allelic replacement in rice by gene editing: A case study of the NRT1.1B gene. J Integr Plant Biol *60*, 536-540.

Li, L., Jean, M., and Belzile, F. (2006). The impact of sequence divergence and DNA mismatch repair on homeologous recombination in Arabidopsis. Plant J *45*, 908-916.

Li, S., Li, J., Zhang, J., Du, W., Fu, J., Sutar, S., Zhao, Y., and Xia, L. (2018b). Synthesis-dependent repair of Cpf1-induced double strand DNA breaks enables targeted gene replacement in rice. J Exp Bot *69*, 4715-4721.

Li, T., Huang, S., Jiang, W.Z., Wright, D., Spalding, M.H., Weeks, D.P., and Yang, B. (2011). TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. Nucleic Acids Res *39*, 359-372.

Li, T., Liu, B., Chen, C.Y., and Yang, B. (2016). TALEN-Mediated Homologous Recombination Produces Site-Directed DNA Base Change and Herbicide-Resistant Rice. J Genet Genomics *43*, 297-305.

Lieber, M.R. (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem *79*, 181-211.

Limbo, O., Chahwan, C., Yamada, Y., de Bruin, R.A., Wittenberg, C., and Russell, P. (2007). Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. Mol Cell *28*, 134-146.

Lin, L., Petersen, T.S., Jensen, K.T., Bolund, L., Kuhn, R., and Luo, Y. (2017). Fusion of SpCas9 to E. coli Rec A protein enhances CRISPR-Cas9 mediated gene knockout in mammalian cells. J Biotechnol *247*, 42-49.

Liu, M., Ba, Z., Costa-Nunes, P., Wei, W., Li, L., Kong, F., Li, Y., Chai, J., Pontes, O., and Qi, Y. (2017). IDN2 Interacts with RPA and Facilitates DNA Double-Strand Break Repair by Homologous Recombination in Arabidopsis. Plant Cell *29*, 589-599.

Lusser, M., Parisi, C., Plan, D., and Rodriguez-Cerezo, E. (2011). New plant breeding techniques: state-of-the-art and prospects for commercial development. In Joint Research Centre Technical Report EUR 24760 (European Commission Joint Research Centre, Brussels).

Makarova, K.S., Haft, D.H., Barrangou, R., Brouns, S.J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F.J., Wolf, Y.I., Yakunin, A.F.*, et al.* (2011). Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol *9*, 467-477.

Makarova, K.S., and Koonin, E.V. (2015). Annotation and Classification of CRISPR-Cas Systems. Methods Mol Biol *1311*, 47-75.

Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders, S.J., Barrangou, R., Brouns, S.J., Charpentier, E., Haft, D.H.*, et al.* (2015). An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol *13*, 722-736.

Mallapaty, S. (2019). Australian gene-editing rules adopt ‘middle ground’. Nature.

Mannuss, A., Dukowic-Schulze, S., Suer, S., Hartung, F., Pacher, M., and Puchta, H. (2010). RAD5A, RECQ4A, and MUS81 have specific functions in homologous recombination and define different pathways of DNA repair in Arabidopsis thaliana. Plant Cell *22*, 3318-3330.

Maruyama, T., Dougan, S.K., Truttmann, M.C., Bilate, A.M., Ingram, J.R., and Ploegh, H.L. (2015). Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol *33*, 538-542.

Meyer, R.S., DuVal, A.E., and Jensen, H.R. (2012). Patterns and processes in crop domestication: an historical review and quantitative analysis of 203 global food crops. New Phytol *196*, 29-48.

Meyer, R.S., and Purugganan, M.D. (2013). Evolution of crop species: genetics of domestication and diversification. Nat Rev Genet *14*, 840-852.

Miller, J., McLachlan, A.D., and Klug, A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J *4*, 1609-1614.

Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J.*, et al.* (2011). A TALE nuclease architecture for efficient genome editing. Nat Biotechnol *29*, 143-148.

Moerschell, R.P., Tsunasawa, S., and Sherman, F. (1988). Transformation of yeast with synthetic oligonucleotides. Proc Natl Acad Sci U S A *85*, 524-528.

Mor, T.S., Moon, Y.S., Palmer, K.E., and Mason, H.S. (2003). Geminivirus vectors for high-level expression of foreign proteins in plant cells. Biotechnol Bioeng *81*, 430-437.

Moreno-Mateos, M.A., Fernandez, J.P., Rouet, R., Vejnar, C.E., Lane, M.A., Mis, E., Khokha, M.K., Doudna, J.A., and Giraldez, A.J. (2017). CRISPR-Cpf1 mediates efficient homology-directed repair and temperature-controlled genome editing. Nat Commun *8*, 2024.

Moritoh, S., Eun, C.H., Ono, A., Asao, H., Okano, Y., Yamaguchi, K., Shimatani, Z., Koizumi, A., and Terada, R. (2012). Targeted disruption of an orthologue of DOMAINS REARRANGED METHYLASE 2, OsDRM2, impairs the growth of rice plants by abnormal DNA methylation. Plant J *71*, 85-98.

Moscou, M.J., and Bogdanove, A.J. (2009). A simple cipher governs DNA recognition by TAL effectors. Science *326*, 1501.

Muniyappa, K., Shaner, S.L., Tsang, S.S., and Radding, C.M. (1984). Mechanism of the concerted action of recA protein and helix-destabilizing proteins in homologous recombination. Proc Natl Acad Sci U S A *81*, 2757-2761.

NatPlants/Editorial (2018). A CRISPR definition of genetic modification. Nat Plants *4*, 233.

Needham, P.D., Atkinson, R.G., Morris, B.A.M., Gardner, R.C., and Gleave, A.P. (1998). GUS expression patterns from a tobacco yellow dwarf virus-based episomal vector. Plant Cell Rep *17*, 631-639.

Nishimasu, H., Ran, F.A., Hsu, P.D., Konermann, S., Shehata, S.I., Dohmae, N., Ishitani, R., Zhang, F., and Nureki, O. (2014). Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell *156*, 935-949.

Nishizawa-Yokoi, A., Endo, M., Ohtsuki, N., Saika, H., and Toki, S. (2015a). Precision genome editing in plants via gene targeting and piggyBac-mediated marker excision. Plant J *81*, 160-168.

Nishizawa-Yokoi, A., Nonaka, S., Osakabe, K., Saika, H., and Toki, S. (2015b). A Universal Positive-Negative Selection System for Gene Targeting in Plants Combining an Antibiotic Resistance Gene and Its Antisense RNA. Plant Physiol *169*, 362-370.

Nishizawa-Yokoi, A., Nonaka, S., Saika, H., Kwon, Y.I., Osakabe, K., and Toki, S. (2012). Suppression of Ku70/80 or Lig4 leads to decreased stable transformation and enhanced homologous recombination in rice. New Phytol *196*, 1048-1059.

Nowsheen, S., and Yang, E.S. (2012). The intersection between DNA damage response and cell death pathways. Exp Oncol *34*, 243-254.

Ochs, F., Somyajit, K., Altmeyer, M., Rask, M.B., Lukas, J., and Lukas, C. (2016). 53BP1 fosters fidelity of homology-directed DNA repair. Nat Struct Mol Biol *23*, 714-721.

Okuzaki, A., and Toriyama, K. (2004). Chimeric RNA/DNA oligonucleotide-directed gene targeting in rice. Plant Cell Rep *22*, 509-512.

Ono, A., Yamaguchi, K., Fukada-Tanaka, S., Terada, R., Mitsui, T., and Iida, S. (2012). A null mutation of ROS1a for DNA demethylation in rice is not transmittable to progeny. Plant J *71*, 564-574.

Osakabe, K., Abe, K., Yoshioka, T., Osakabe, Y., Todoriki, S., Ichikawa, H., Hohn, B., and Toki, S. (2006). Isolation and characterization of the RAD54 gene from Arabidopsis thaliana. Plant J *48*, 827-842.

Osakabe, K., Nishizawa-Yokoi, A., Ohtsuki, N., Osakabe, Y., and Toki, S. (2014). A mutated cytosine deaminase gene, codA (D314A), as an efficient negative selection marker for gene targeting in rice. Plant Cell Physiol *55*, 658-665.

Ozawa, K., Wakasa, Y., Ogo, Y., Matsuo, K., Kawahigashi, H., and Takaiwa, F. (2012). Development of an efficient agrobacterium-mediated gene targeting system for rice and analysis of rice knockouts lacking granule-bound starch synthase (Waxy) and beta1,2-xylosyltransferase. Plant Cell Physiol *53*, 755-761.

Paszkowski, J., Baur, M., Bogucki, A., and Potrykus, I. (1988). Gene targeting in plants. EMBO J *7*, 4021-4026.

Pattanayak, V., Ramirez, C.L., Joung, J.K., and Liu, D.R. (2011). Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. Nat Methods *8*, 765-770.

Puchta, H. (1998). Repair of genomic double-strand breaks in somatic plant cells by one-sided invasion of homologous sequences. The Plant Journal *13*, 331–339.

Puchta, H. (2005). The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. J Exp Bot *56*, 1-14.

Puchta, H., Dujon, B., and Hohn, B. (1993). Homologous recombination in plant cells is enhanced by in vivo induction of double strand breaks into DNA by a site-specific endonuclease. Nucleic Acids Res *21*, 5034-5040.

Puchta, H., Dujon, B., and Hohn, B. (1996). Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proc Natl Acad Sci U S A *93*, 5055-5060.

Puchta, H., and Hohn, B. (1991). A transient assay in plant cells reveals a positive correlation between extrachromosomal recombination rates and length of homologous overlap. Nucleic Acids Res *19*, 2693-2700.

Puchta, H., and Hohn, B. (2010). Breaking news: plants mutate right on target. Proc Natl Acad Sci U S A *107*, 11657-11658.

Qi, Y., Zhang, Y., Zhang, F., Baller, J.A., Cleland, S.C., Ryu, Y., Starker, C.G., and Voytas, D.F. (2013). Increasing frequencies of site-specific mutagenesis and gene targeting in Arabidopsis by manipulating DNA repair pathways. Genome Res *23*, 547-554.

Radding, C.M. (1981). Recombination activities of E. coli recA protein. Cell *25*, 3-4.

Rajanikant, C., Melzer, M., Rao, B.J., and Sainis, J.K. (2008). Homologous recombination properties of OsRad51, a recombinase from rice. Plant Mol Biol *68*, 479-491.

Ray, D.K., Mueller, N.D., West, P.C., and Foley, J.A. (2013). Yield Trends Are Insufficient to Double Global Crop Production by 2050. PLoS One *8*, e66428.

Rees, H.A., and Liu, D.R. (2018). Base editing: precision chemistry on the genome and transcriptome of living cells. Nat Rev Genet *19*, 770-788.

Reiss, B., Klemm, M., Kosak, H., and Schell, J. (1996). RecA protein stimulates homologous recombination in plants. Proc Natl Acad Sci U S A *93*, 3094-3098.

Richter, K.S., Kleinow, T., and Jeske, H. (2014). Somatic homologous recombination in plants is promoted by a geminivirus in a tissue-selective manner. Virology *452-453*, 287-296.

Robert, F., Barbeau, M., Ethier, S., Dostie, J., and Pelletier, J. (2015). Pharmacological inhibition of DNA-PK stimulates Cas9-mediated genome editing. Genome Med *7*, 93.

Roset, R., Inagaki, A., Hohl, M., Brenet, F., Lafrance-Vanasse, J., Lange, J., Scandura, J.M., Tainer, J.A., Keeney, S., and Petrini, J.H. (2014). The Rad50 hook domain regulates DNA damage signaling and tumorigenesis. Genes Dev *28*, 451-462.

Sauer, N.J., Mozoruk, J., Miller, R.B., Warburg, Z.J., Walker, K.A., Beetham, P.R., Schopke, C.R., and Gocal, G.F. (2016). Oligonucleotide-directed mutagenesis for precision gene editing. Plant Biotechnol J *14*, 496-502.

Schuermann, D., Molinier, J., Fritsch, O., and Hohn, B. (2005). The dual nature of homologous recombination in plants. Trends Genet *21*, 172-181.

Schunder, E., Rydzewski, K., Grunow, R., and Heuner, K. (2013). First indication for a functional CRISPR/Cas system in Francisella tularensis. Int J Med Microbiol *303*, 51-60.

Seeliger, K., Dukowic-Schulze, S., Wurz-Wildersinn, R., Pacher, M., and Puchta, H. (2012). BRCA2 is a mediator of RAD51- and DMC1-facilitated homologous recombination in Arabidopsis thaliana. New Phytol *193*, 364-375.

Shaked, H., Melamed-Bessudo, C., and Levy, A.A. (2005). High-frequency gene targeting in Arabidopsis plants expressing the yeast RAD54 gene. Proc Natl Acad Sci U S A *102*, 12265-12269.

Shalev, G., Sitrit, Y., Avivi-Ragolski, N., Lichtenstein, C., and Levy, A.A. (1999). Stimulation of homologous recombination in plants by expression of the bacterial resolvase ruvC. Proc Natl Acad Sci U S A *96*, 7398-7402.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J.J., Qiu, J.L.*, et al.* (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol *31*, 686-688.

Shukla, V.K., Doyon, Y., Miller, J.C., DeKelver, R.C., Moehle, E.A., Worden, S.E., Mitchell, J.C., Arnold, N.L., Gopalan, S., Meng, X.*, et al.* (2009). Precise genome modification in the crop species Zea mays using zinc-finger nucleases. Nature *459*, 437-441.

Stella, S., Alcon, P., and Montoya, G. (2017). Structure of the Cpf1 endonuclease R-loop complex after target DNA cleavage. Nature *546*, 559-563.

Sternberg, S.H., Redding, S., Jinek, M., Greene, E.C., and Doudna, J.A. (2014). DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature *507*, 62-67.

Suarez-Lopez, P., and Gutierrez, C. (1997). DNA replication of wheat dwarf geminivirus vectors: effects of origin structure and size. Virology *227*, 389-399.

Sun, Y., Zhang, X., Wu, C., He, Y., Ma, Y., Hou, H., Guo, X., Du, W., Zhao, Y., and Xia, L. (2016). Engineering Herbicide-Resistant Rice Plants through CRISPR/Cas9-Mediated Homologous Recombination of Acetolactate Synthase. Mol Plant *9*, 628-631.

Svitashev, S., Young, J.K., Schwartz, C., Gao, H., Falco, S.C., and Cigan, A.M. (2015). Targeted Mutagenesis, Precise Gene Editing, and Site-Specific Gene Insertion in Maize Using Cas9 and Guide RNA. Plant Physiol *169*, 931-945.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J., and Stahl, F.W. (1983). The double-strand-break repair model for recombination. Cell *33*, 25-35.

Takahashi, N., Ogita, N., Takahashi, T., Taniguchi, S., Tanaka, M., Seki, M., and Umeda, M. (2019). A regulatory module controlling stress-induced cell cycle arrest in Arabidopsis. Elife *8*.

Tamaki, S., Tsuji, H., Matsumoto, A., Fujita, A., Shimatani, Z., Terada, R., Sakamoto, T., Kurata, T., and Shimamoto, K. (2015). FT-like proteins induce transposon silencing in the shoot apex during floral induction in rice. Proc Natl Acad Sci U S A *112*, E901-910.

Tamura, K., Adachi, Y., Chiba, K., Oguchi, K., and Takahashi, H. (2002). Identification of Ku70 and Ku80 homologues in Arabidopsis thaliana: evidence for a role in the repair of DNA double-strand breaks. Plant J *29*, 771-781.

Terada, R., Johzuka-Hisatomi, Y., Saitoh, M., Asao, H., and Iida, S. (2007). Gene targeting by homologous recombination as a biotechnological tool for rice functional genomics. Plant Physiol *144*, 846-856.

Tong Zhu, D.J.P., Laura Tagliani, Grace ST.Clair, Chris L. Baszczynski and Ben Bowen (1999). Targeted manipulation of maize genes in vivo using chimeric.

Townsend, J.A., Wright, D.A., Winfrey, R.J., Fu, F., Maeder, M.L., Joung, J.K., and Voytas, D.F. (2009). High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature *459*, 442-445.

Tsakraklides, V., Brevnova, E., Stephanopoulos, G., and Shaw, A.J. (2015). Improved Gene Targeting through Cell Cycle Synchronization. PLoS One *10*, e0133434.

UN (2019). World Population Prospects 2019. UN Department Global Communications.

Urnov, F.D., Miller, J.C., Lee, Y.L., Beausejour, C.M., Rock, J.M., Augustus, S., Jamieson, A.C., Porteus, M.H., Gregory, P.D., and Holmes, M.C. (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature *435*, 646-651.

Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D. (2010). Genome editing with engineered zinc finger nucleases. Nat Rev Genet *11*, 636-646.

USDA (2019). World Agricultural Production. Circular Series *WAP 8-19*.

USDA/JA8064 (2018). Japan Holds Second Meeting to Discuss Genome Editing

Technology.

USDA/JA9050 (2019). Japanese Health Ministry Finalizes Genome Edited Food Policy.

USDA\_Press (2018). Secretary Perdue Issues USDA Statement on Plant Breeding Innovation.

Voytas, D.F. (2013). Plant genome engineering with sequence-specific nucleases. Annu Rev Plant Biol *64*, 327-350.

Vu, G.T.H., Cao, H.X., Fauser, F., Reiss, B., Puchta, H., and Schubert, I. (2017). Endogenous sequence patterns predispose the repair modes of CRISPR/Cas9-induced DNA double-stranded breaks in Arabidopsis thaliana. Plant J *92*, 57-67.

Vu, T.V., Sivankalyani, V., Kim, E.J., Tran, M.T., Kim, J., Sung, Y.W., Doan, D.T.H., and Kim, J.Y. (2019). Highly efficient homology-directed repair using transient CRISPR/Cpf1-geminiviral replicon in tomato. bioRxiv; <https://doiorg/101101/521419>.

Wang, M., Lu, Y., Botella, J.R., Mao, Y., Hua, K., and Zhu, J.K. (2017). Gene Targeting by Homology-Directed Repair in Rice Using a Geminivirus-Based CRISPR/Cas9 System. Mol Plant *10*, 1007-1010.

Weimer, A.K., Biedermann, S., Harashima, H., Roodbarkelari, F., Takahashi, N., Foreman, J., Guan, Y., Pochon, G., Heese, M., Van Damme, D.*, et al.* (2016). The plant-specific CDKB1-CYCB1 complex mediates homologous recombination repair in Arabidopsis. EMBO J *35*, 2068-2086.

West, C.E., Waterworth, W.M., Story, G.W., Sunderland, P.A., Jiang, Q., and Bray, C.M. (2002). Disruption of the Arabidopsis AtKu80 gene demonstrates an essential role for AtKu80 protein in efficient repair of DNA double-strand breaks in vivo. Plant J *31*, 517-528.

Wright, D.A., Townsend, J.A., Winfrey, R.J., Jr., Irwin, P.A., Rajagopal, J., Lonosky, P.M., Hall, B.D., Jondle, M.D., and Voytas, D.F. (2005). High-frequency homologous recombination in plants mediated by zinc-finger nucleases. Plant J *44*, 693-705.

Yamauchi, T., Johzuka-Hisatomi, Y., Fukada-Tanaka, S., Terada, R., Nakamura, I., and Iida, S. (2009). Homologous recombination-mediated knock-in targeting of the MET1a gene for a maintenance DNA methyltransferase reproducibly reveals dosage-dependent spatiotemporal gene expression in rice. Plant J *60*, 386-396.

Yang, Z., Tang, L., Li, M., Chen, L., Xu, J., Wu, G., and Li, H. (2010). Monitoring homologous recombination in rice (Oryza sativa L.). Mutat Res *691*, 55-63.

Yata, K., and Esashi, F. (2009). Dual role of CDKs in DNA repair: to be, or not to be. DNA Repair (Amst) *8*, 6-18.

Yokota, Y., Shikazono, N., Tanaka, A., Hase, Y., Funayama, T., Wada, S., and Inoue, M. (2005). Comparative radiation tolerance based on the induction of DNA double-strand breaks in tobacco BY-2 cells and CHO-K1 cells irradiated with gamma rays. Radiat Res *163*, 520-525.

Yoon, K., Cole-Strauss, A., and Kmiec, E.B. (1996). Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA.DNA oligonucleotide. Proc Natl Acad Sci U S A *93*, 2071-2076.

Yoshiyama, K.O., Kobayashi, J., Ogita, N., Ueda, M., Kimura, S., Maki, H., and Umeda, M. (2013). ATM-mediated phosphorylation of SOG1 is essential for the DNA damage response in Arabidopsis. EMBO Rep *14*, 817-822.

Yu, C., Liu, Y., Ma, T., Liu, K., Xu, S., Zhang, Y., Liu, H., La Russa, M., Xie, M., Ding, S.*, et al.* (2015). Small molecules enhance CRISPR genome editing in pluripotent stem cells. Cell Stem Cell *16*, 142-147.

Zaidi, S.S., Vanderschuren, H., Qaim, M., Mahfouz, M.M., Kohli, A., Mansoor, S., and Tester, M. (2019). New plant breeding technologies for food security. Science *363*, 1390-1391.

Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., van der Oost, J., Regev, A.*, et al.* (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell *163*, 759-771.

Zhang, F., Cong, L., Lodato, S., Kosuri, S., Church, G.M., and Arlotta, P. (2011). Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat Biotechnol *29*, 149-153.

Zhang, X., and Mason, H. (2006). Bean Yellow Dwarf Virus replicons for high-level transgene expression in transgenic plants and cell cultures. Biotechnol Bioeng *93*, 271-279.

Zhang, Y., Zhang, F., Li, X., Baller, J.A., Qi, Y., Starker, C.G., Bogdanove, A.J., and Voytas, D.F. (2013). Transcription activator-like effector nucleases enable efficient plant genome engineering. Plant Physiol *161*, 20-27.

Zhu, T., Peterson, D.J., Tagliani, L., St Clair, G., Baszczynski, C.L., and Bowen, B. (1999). Targeted manipulation of maize genes in vivo using chimeric RNA/DNA oligonucleotides. Proc Natl Acad Sci U S A *96*, 8768-8773.

Zhu, X., Clarke, R., Puppala, A.K., Chittori, S., Merk, A., Merrill, B.J., Simonovic, M., and Subramaniam, S. (2019). Cryo-EM structures reveal coordinated domain motions that govern DNA cleavage by Cas9. Nat Struct Mol Biol *26*, 679-685.

Zong, Y., Song, Q., Li, C., Jin, S., Zhang, D., Wang, Y., Qiu, J.L., and Gao, C. (2018). Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. Nat Biotechnol.