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Prospects for CRISPR-Assisted Plant Breeding: The Necessity to Enhance Gene Knock-in Efficiency

Cécile Collonnier, François Maclot, Kostlend Mara, Anouchka Guyon-Debast, Florence Charlot, Fabien Nogué

Genome editing for breeding new plant varieties

Genome editing via the CRISPR/Cas9 system provides new means of creating and manipulating genetic diversity in plants. Mutations can be created either by error-prone non-homologous end joining (NHEJ)-mediated repair of the double-strand breaks (DSBs) induced at the target (knock-out of genes), or by insertion of an exogenous donor DNA at the cleavage site driven or not by homology (knock-in of genes). The donor DNA can contain a modified version of the targeted locus leading to allele replacement, or be totally foreign to the recipient genome for targeted transgene insertion. On one hand, these approaches can be used to progress in the understanding of the mechanisms underlying the traits of interest and in the validation of candidate genes. On the other hand, they provide the means of integrating the desired alleles of these genes in any transformable material in a controlled and targeted way.

Many applications of the CRISPR/Cas9 system are envisioned for plant breeding. One of them, called Variation Induced by Genome Editing (VIGE), consists in the production of new portfolios of synthetic alleles for genes of agronomic interest. Unlike methods based on random mutagenesis (such as TILLING) that require the screening of large populations of mutants, VIGE generates mutations directly into the selected genes, and a large range of alleles can be produced in a limited number of individuals. However, gene editing can also be combined with large phenotypic screens to systematize the search for new interesting phenotypes after induction of various mutations in a set of genes selected for their potential implication in a given trait (TIGV, Targeted Induced Gene Variation)[1]. By challenging multi-targeted mutants in different environmental conditions, new alleles of resistance to biotic and abiotic stresses could eventually be identified and combined, as well as alleles adapted to specific cultural contexts for tailored breeding.

Gene editing could also greatly facilitate genes/QTLs pyramiding in elite lines in the following ways: first, by avoiding long and costly crossing plans; second, by helping to evaluate the effect of the genetic background and of the interactions between genes/QTLs; and third, by avoiding recombination between the genes of interest located on the same chromosomal fragments. Furthermore, the CRISPR/Cas9 system could be applied to specific breeding goals, such as engineering of polyploid species, breaking of linkage drags, selection of recessive alleles, and removal of unfavorable alleles either to optimize a given trait or to get rid of certain mutations accumulated during domestication and selection (a process called “rewilding”)[2].

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Virginia Tech
1900 Kraft Drive, Suite 103
Blacksburg, VA 24061

Tel. 540-231-3747
Fax 540-231-4434

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Editor:
Ruth Irwin
rirwin@vt.edu

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The management of genetic resources, which is a key element of plant breeding as these accessions represent a reservoir of genetic diversity for the creation of new varieties, may also be impacted by genome editing. Indeed, using the CRISPR/Cas9 system to create libraries of elite clones bearing mutated alleles for a range of useful genes could decrease the interest in maintaining large collections of genetic resources and narrow even more the genetic basis of crop varieties. However, "breeding without sex" applies only to the traits underlined by genes that have already been identified, and the potential of the collections of genetic resources needs to be preserved to answer future needs that are still unknown. Therefore, the libraries of edited elite lines will mainly contribute to enlarge the germplasms used by the breeders. The alleles potentially discovered by VIGE will also participate to create new diversity. Moreover, by bypassing sexual barriers and avoiding the genetic burden due to hybridization, gene editing may better exploit genetic resources. It could allow the direct transfer of new alleles of interest into breeding populations or elite lines, as well as the edition, in the crops, of homologous genes identified in relative species.

Gene editing is particularly adapted to the breeding of mono- or oligogenic traits, but can also be applied to polygenic traits underlined by a limited number of major genes. Depending on the number of targeted loci and the method of transformation, multiplexing by using different guide RNAs simultaneously should lead to the production of the desired assortments of genes in a limited number of generations[3]. For all these reasons, the CRISPR/Cas9 system should rapidly be integrated into selection schemes where it may accelerate the breeding process and facilitate the combination of new sets of alleles of interest, even when identified in sexually incompatible species.

So far, the CRISPR/Cas9 system has successfully been applied to various crop species, including rice, maize, wheat, barley, sorghum, soybean, *Brassica oleracea*, tomato, potato, lettuce, sweet orange, poplar, and grapevine[4,5,6] The first edited agronomic traits reported in the literature relate to disease tolerance (powdery mildew in wheat[7], bacterial blight in rice[8] and potyviruses in cucumber[9], modified ripening profiles in tomato[10], male sterility[11], and factors involved in yield in rice[12,13] and drought tolerance in maize[14]. The first CRISPR varieties that will be marketed will probably present already well characterized traits inserted in optimized backgrounds (waxy starch, tolerance to herbicides) but, in the long term, innovative properties could be developed in relation with biotic and abiotic stresses, yield, quality (nutritional compounds), physiology (improved biomass, accelerated flowering time), molecular pharming (therapeutic molecules), or even genome remodeling (e.g., bananas without endogenous banana streak virus sequences)[15].

A critical step: optimizing CRISPR-induced gene knock-in

Applying the CRISPR/Cas9 system to crops depends on the availability of efficient transformation and regeneration protocols. It also relies on the existence of assembled and, if possible, annotated versions of reference genomes for the design of sgRNAs with limited numbers of potential off-target sites. In a given experimental context, all selected sgRNAs may not trigger cleavage by the Cas9

protein as effectively but, in general, the knock-out of endogenous plant loci by illegitimate recombination-mediated (IR) targeted mutagenesis is relatively easy to achieve, with efficiencies varying from 2%[16] to sometimes 100% of regenerated individuals presenting mutations at the target site[5]. Conversely, CRISPR-induced gene targeting, corresponding to the knock-in of a donor DNA presenting homology to the target and resulting in gene replacement by homology-driven repair (HDR), is much more difficult to achieve in higher plants where it rarely reaches the percentage range[14,16,17,18,19,20,21]. This is likely due to the fact that gene targeting (GT) efficiency is usually very low in these species, ranging from 0.01 to 0.1 % of the effectively transformed plants[22]. Thus, even if inducing DSBs by using the CRISPR-Cas9 system is a way to increase gene targeting in crops[23], gene knock-in remains very challenging compared to gene knockout, which strongly limits the development of many potential applications for plant breeding.

Apart from optimizing the protocols for better transformation and regeneration efficiencies, different technical factors can be worked on to try to increase the rate of CRISPR-induced GT in plants. The type of cell delivery of the sgRNAs and the Cas9 nuclease can be adjusted by performing stable or transient transformation, and by using plasmids, RNAs, or ribonucleic complexes[24,25]. The level of expression of the system can be increased in the germlines with cell-specific promoters[26,27,28]. The sequences coding for the CRISPR/Cas9 system can also be inserted into disarmed viral replicons of geminivirus to strongly increase their copy number in the transformed cells[29,30,31,32]. Finally, the timing of delivery of the donor template can be optimized by using the "*in planta* gene targeting" strategy, which consists in cloning the donor cassette next to the sequences coding for the CRISPR/Cas9 system and in between synthetic target sites for simultaneous cleavage of the target and release of the cassette[20]. In addition to these parameters, another determinant way of improving the knock-in strategy in crops would be to progress the understanding of the mechanisms involved in this phenomenon by studying the very few species/cell lines that show a highly efficient HDR of genomic DSBs, such as budding yeast[33] and the model plant *Physcomitrella patens*[34]. The high gene targeting frequencies naturally achieved by these species seems to be associated with the fact that homologous recombination (HR) is for them the principal mechanism for DSBs repair. However, things may be more complex, and unravelling the roles of the different DNA repair pathways potentially implicated could be very useful for shaping the genetic background of breeding materials in order to increase their suitability for gene knock-in.

Using *P. patens* for deciphering the mechanisms involved in CRISPR-induced gene knock-in in plants

P. patens is the only plant naturally capable of performing gene targeting at high frequencies[35]. This unique property, combined with the availability of a fully sequenced genome and of fast and simple in vitro culture protocols[36], makes it a model of choice to study the mechanisms involved in gene targeting and more particularly in CRISPR-induced gene knock-in in plants.

The first report on the use of the CRISPR/Cas9 system in *P. patens* was recently published by our team[37]. We demonstrated the efficiency of the cleavage by targeting an endogenous reporter gene coding for adenine phosphoribosyl transferase (*PpAPT*), whose loss of function confers resistance to the toxic compound 2-fluoroadenine (2-FA). The mutations observed included a diversity of deletions, insertions, and/or substitutions, mainly resulting from classical NHEJ (c-NHEJ), but a bias was observed in favor of a certain type of deletion resulting from an alternative end joining reaction (alt-EJ, also called microhomology-mediated end joining, MMEJ) between micro-homologies located on both sides of the cleavage sites.

To study gene targeting when inducing a DSB at the target site with the CRISPR/Cas9 system, we used a donor template bearing an antibiotic resistance gene framed by homologies to the reporter gene *PpAPT*. Classical GT has been well described in *P. patens*[38,39,40,41]. When a homologous donor DNA template is provided to the cell, different types of insertions are possible: targeted gene replacement (TGR) can be obtained if the template is integrated by homologous recombination on

both sides; and targeted gene insertions (TGI), upstream or downstream of the targeted locus, are observed if the insertion involves homology-driven repair (HDR) on one side and illegitimate recombination (IR) on the other. Such patterns of integration are also frequently observed in other plants, including crops[42]. For both these types of events, the insertions frequently contain multiple copies of the donor template[43,35]. When a cleavage is induced at the target site, it can be repaired either by insertion of the donor template or by NHEJ, potentially inducing mutations which lead in both cases to the disruption of the target gene. To discriminate between these events and evaluate GT frequency, we followed the procedure described in Figure 1. With the CRISPR/Cas9 system, we found that the GT efficiency was significantly increased, since HDR-mediated integration of the donor DNA occurred in almost 100% of the transformed plants (compared to 54% in the classical approach) (Cf. Figure 2). This means that, when using CRISPRs, if the donor template is integrated in the genome, it will always be at the cleavage site. However, 40% of Cas9-induced DSBs are not repaired by HDR, but by mutagenic IR or End Joining reactions. This indicates that even in the presence of a homologous donor template both homology-driven and illegitimate recombination pathways are equally proficient to repair Cas9-induced chromosomal DSBs in *P. patens*. The ratio between TGR and TGI events was not significantly different between the two methods, but the proportion of plants with a single copy replacement was significantly higher with the CRISPRs (40.5%) than without (15%). Gene targeting in *P. patens* has recently been associated with the classical RAD51-mediated HR repair pathway[44,38,39]. Thus, we assessed CRISPR-Cas9 mediated GT efficiency in the *Pprad51-1-2* double mutant. Interestingly, we revealed that HDR-mediated GT was reduced but not abolished in the mutant, reaching approximately 30% of the wild type level. This observation implies that other types of DNA repair pathways are involved in the integration of the donor template when a DSB is induced at the chromosomal target gene.

Thus, the CRISPR/Cas9 system significantly improves GT efficiency and precision in *P. patens*. Further work will be needed in order to decipher the mechanism controlling the choice for DSB repair pathways and to elucidate their respective contributions. But already, this study uncovers novel features of CRISPR-induced HDR-mediated GT that could improve the efficiency of such approach in GT non-competent organisms like crops.

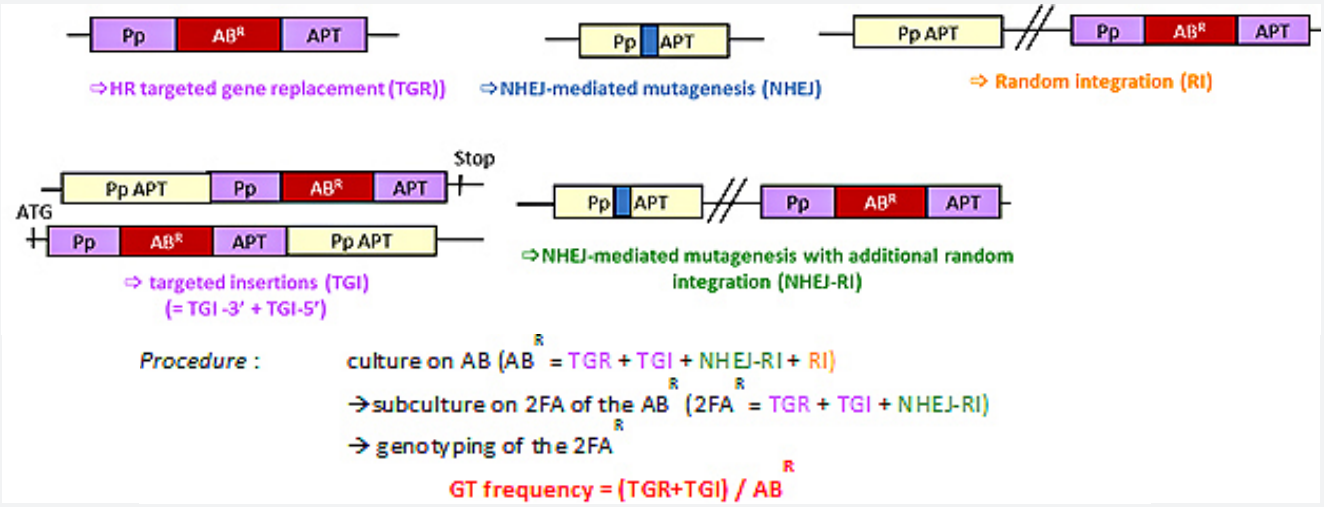

Figure 1: Theoretical types of gene targeting (GT) events in *P. patens* using the CRISPR/Cas9 system, and procedure used to detect them and evaluate the frequency of gene targeting. (AB = antibiotic, 2-FA = 2-fluoroadenine).

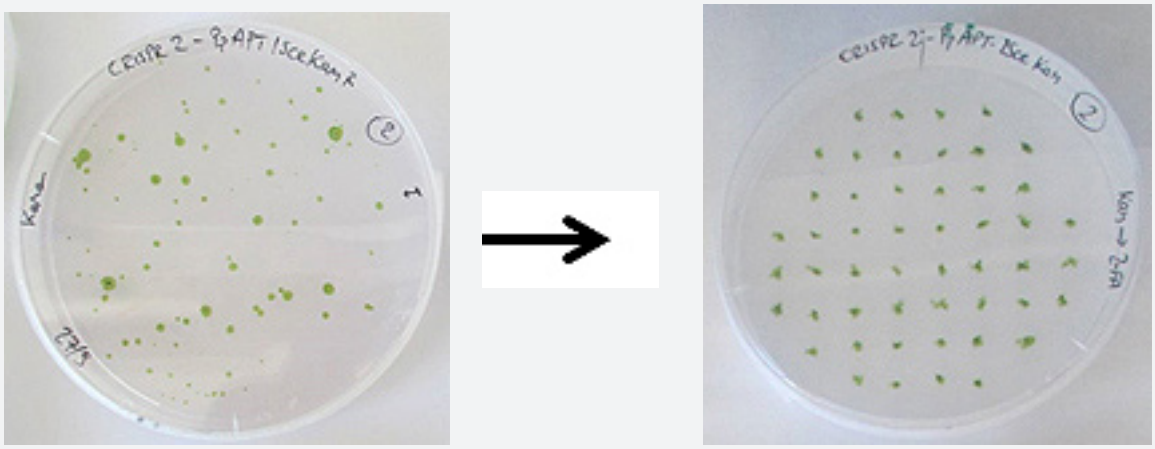

Figure 2: *P. patens* clones sequentially subcultured on selective media after transformation using the CRISPR/Cas9 system. Clones resistant to antibiotic have stably integrated the donor template. Among them, those that are also resistant to 2-FA have integrated it at the target locus (*PpAPT*). In our conditions, 100% of the plants having integrated the donor template (AB^R) presented targeted gene replacement ($2\text{-}FA^R$).

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Cécile Collonnier[1], François Maclot[1], Kostlend Mara[1], Anouchka Guyon-Debast[1], Florence Charlot[1], Fabien Nogué[1]

1 INRA Centre Versailles-Grignon, IJPB (UMR1318), Lab. 'DNA repair and Genome editing', route de St-Cyr, 78026 Versailles cedex, France.

A High-efficiency CRISPR Platform for Maize Improvement

Kan Wang and Bing Yang

Crop breeders frequently employ mutagenic technologies such as radiation (gamma rays or fast neutron) or chemical (ethyl methanesulfonate or EMS) treatment to accelerate crop improvement. This mutation breeding process exposes plants or seeds to mutagens that cause damage to plant DNA. During the DNA repair process that is natural for plant cells, genetic changes such as mutations are introduced into their genetic makeup including genes. These genetic changes are completely random across the whole genome and can result in both positive and negative outcomes; too often one positive change is accompanied by many unintended changes. For example, a new variety may be more resistant to disease but has a reduced essential amino acid content. Therefore, stringent screening/selection and multigenerational crosses are usually required before any new variety can be marketed. Mutation breeding has brought us over 3200 plant varieties as food, feed, or ornamentals from 1930 to 2014[1].

Biotechnologists have been searching for tools for modifying genomes to alter gene expressions in a precise manner—the so-called gene or genome editing. The purpose of targeted gene editing is to obtain the desired modification without collateral damage caused by random mutagenesis. In recent decades, a number of site-directed mutagenesis or recombination methods have been developed. These methods introduce a double stranded DNA break at a preselected genome site and induce the desired DNA changes by exploiting the cell's natural ability to repair the DNA break. Most widely reported methods involve engineered nucleases with the ability to recognize and cleave specific DNA sequences, such as Zinc Finger Nucleases (ZFNs), meganucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat/CRISPR-associated Cas9 (CRISPR/Cas9). Recently, the modified CRISPR/Cas9 system, consisting of a nuclear localized endonuclease and a small single guide RNA, referred to here as Cas9/gRNA or popularly called CRISPR technology, has become the most promising and popular genetic tool for basic and applied research in eukaryotes (Figure 1A).

To realize the advances of precision and high efficiency of CRISPR technology in crop improvement, we need to nurture the individual mutagenized plant cells into mature plants and ensure the genetic changes are passed on to subsequent generations. This process is called plant tissue culture and transformation. Through this technology, the mutagenic reagent (e.g., Cas9/gRNA) is delivered into plant cells or embryogenic cells. Under a suite of artificial medium and plant hormones, the transformed plant cells can be grown into plantlets that contain heritable mutations in the genes of interest. Eventually, the CRISPR reagents in the regenerated plants can be purposely sorted out, resulting in transgene-free breeding strains with desired genes/traits (Figure 1B).

Compared to ZFNs and TALENs, CRISPR technology is much simpler in design and construction of reagents and much more efficient in mutagenesis. However, it still must be optimized for each plant species to obtain the desired site-specificity and high efficiency. In addition, it has to take into consideration which type of plant tissue to transform and what method to use for delivery of Cas9/gRNA in the form of DNA or as premixed ribonucleoprotein. To this end, one must test different polymerase II-based promoters for driving Cas9 and polymerase III-type promoters to express gRNAs. In the system described in our work[2], we used a strong and constitutive promoter of the maize ubiquitin gene for driving the expression of a rice codon-optimized Cas9. For gRNA expression, we used two rice U6 small nuclear RNA gene promoters. We chose the *Agrobacterium*-mediated transformation method for delivery of the CRISPR reagent, because it gives a high frequency of inserting single or low copy number of transgenes in the plant genome. Moreover, compared to biolistic gun-mediated gene delivery, *Agrobacterium*-mediated transformation

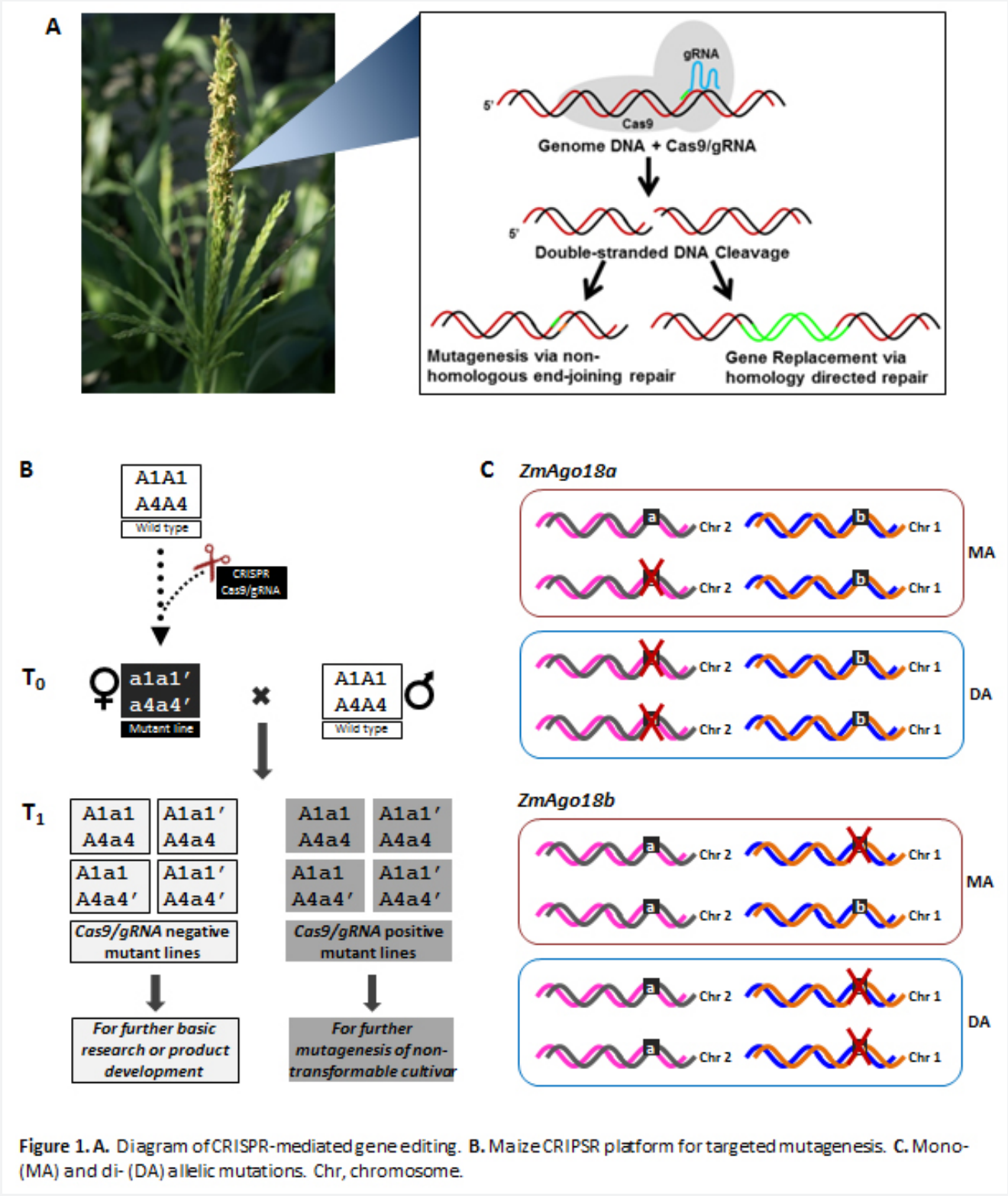

Figure 1. A. Diagram of CRISPR-mediated gene editing. **B.** Maize CRIPSR platform for targeted mutagenesis. **C.** Mono- (MA) and di- (DA) allelic mutations. Chr, chromosome.

does not require expensive supplies and equipment for performing transformation experiments, such as a particle gun apparatus.

For genome editing to be successful, one has to know the genome sequence to ensure target-site specificity in designing guide RNAs and to minimize any undesired off-target effect. In our work, the gRNA spacer sequences are chosen based on the maize B73 reference genome sequence using the CRISPR Genome Analysis Tool developed by the Iowa State University Crop Bioengineering Consortium[3] (http://cbc.gdcb.iastate.edu/cgat/). The construction of a binary construct for *Agrobacterium*-mediated transformation includes two-step cloning; i.e., (1) introduce gRNA sequences into the gRNA vectors[4] by inserting into BtgZ1 or BsaI restriction sites, and (2) mobilize the gRNA cassette through Gateway recombination to the destination vector pGW-Cas9. The final binary construct containing both gRNAs and Cas9 can then be transferred to *Agrobacterium* strains (e.g., EHA101) for further plant transformation.

It is worth mentioning that as many as four gRNA sequences can be cloned simultaneously into the gRNA receiving vector used in this work. The advantages of introduction and expression of multiple gRNAs include the simultaneous mutagenesis of multiple genes or loci, and the increase of mutagenesis frequency of a single gene.

Agrobacterium strains containinging the maize CRISPR binary constructs were used to infect immature embryos of two transformable maize genotypes—Hi-II, a hybrid genotype with high transformation and regeneration frequencies, and B104, an inbred that has high sequence homology with the reference genome B73. To ensure targeting specificity and efficiency, the relevant DNA regions in these genotypes were sequenced and verified. The *Agrobacterium*-based maize transformation was performed at the Iowa State University Plant Transformation Facility that provides maize transformation service to public researchers (http://agron-www.agron.iastate.edu/ptf/).

For a typical project, we first identify 20 to 30 herbicide bialaphos resistant callus lines for genotyping, using the T7 endonuclease I (T7E1) assay and subsequently Sanger sequencing. We then select 10 independent mutation-positive callus lines for regeneration of plantlets. Multiple (usually 2 to 5) plantlets are produced from each callus line and are further confirmed for mutation using T7E1 assay and sequencing of the site-specific PCR amplicons. Mutation positive plantlets are then grown to maturity in the greenhouse in order to produce transgenic seeds. The total time duration for the initiation of the project until the harvesting of seeds is roughly 7 to 8 months, including 2 weeks for design and construction of Cas9/sRNA, 8 to 10 weeks for maize transformation, including screening for mutation-positive callus lines, 4 to 5 weeks for plant regeneration, including further mutation confirmation, and 12 weeks for plant maturation, crosses, and seed harvest.

When performing crosses to obtain seeds, we used wild type B73 pollen to pollinate Hi-II transgenic mutant lines and wild type B104 pollen to pollinate B104 transgenic mutant lines (Figure 1B). This outcrossing generated T_1 seeds of two major mutant populations; 50% of the seed carries Cas9/gRNA transgene and 50% is free of the transgene. The transgene-free mutant seeds can be used as intended research materials. The seeds carrying the Cas9/gRNA transgene can be used for controlled crossing to any non-transformable maize genotype to induce mutagenesis in the intended genes.

While the Cas9/gRNA seeds are useful for mutagenizing maize genotypes that cannot be transformed, the pollination process should be properly controlled and closely monitored. Maize is a wind pollinated plant. Inadvertent hybridization of Cas9/gRNA pollen with any non-target maize plant can result in "Gene Drive," in which the gene modified by the gRNA may be preferentially inherited through sexual reproduction and altered for entire population[5].

We tested the efficacy of our CRISPR system, termed the ISU maize CRISPR platform, for targeted mutagenesis by applying it to two duplicated gene pairs. The first pair was the Argonaute genes (*ZmAgo*), which function within the small RNA pathway. We designed two gRNAs targeting the 2nd exon of *ZmAgo18a* located on chromosome 2 and another two gRNAs targeting the 5th exon of *ZmAgo18b* located on chromosome 1. The reason for using two gRNAs for one exon was to enhance the targeted mutagenesis efficiency and to generate small deletions in the protein-

coding regions of the genes. Out of 23 transgenic lines generated for each gene, 17 lines were positive for the *ZmAgro18a* mutation and 16 lines were positive for the *ZmAgo18b* mutation. Therefore in the two single-gene targeting experiments, the mutation frequencies were 74% (17/23) for *ZmAgo18a* and 70% (16/23) for *ZmAgo18b*. Both mono-allelic mutations (MA) and di-allelic mutations (DA) (Figure 1C) could be identified from these lines. Sixty-five percent (65%) and fifty-six percent (56%) of the mutants were MA mutations for *ZmAgo18a* and *ZmAgo18b*, respectively. The rest were di-allelic mutant lines.

Another CRISPR construct was made to target *dihydroflavonol 4-reductase* or anthocyanin biosynthesis gene *a1* (*anthocyaninless1*) and its homolog *a4*, which help to regulate endogenous brassinosteroid hormone levels in plants. The maize a4 and a1 protein sequences shared 88.3% similarity with each other[6]. The four gRNAs (2x for each gene) were designed to target the conserved sites of both genes with a perfect match to *a4* (on chromosome 8) but with a mismatch to *a1* (on chromosome 3) at position 3 at the 5' end of each guide RNA. We performed transformation experiments for duplex gene targeting and generated 47 transgenic lines. Thirty-seven out of 47 (79%) transgenic lines had targeted mutations either on *a1* (15%), or on *a4* (49%) or on both (15%). The lower mutation efficiency in *a1* is likely attributable to the 1-bp mismatch between the target sequence of *a1* and each of two gRNAs. However, the overall mutation frequency was similar to what we have seen with *ZmAgo18* genes.

Selected mutant lines were evaluated for their mutation inheritance in subsequent generations. All mutations that were identified in the T_0 plantlet stage could pass on to T_1 and T_2 generations, indicating that the mutations were stable and heritable. The mutant lines that also carried the Cas9/gRNA transgene not only passed on the transgene to the next generation, but also induced new heritable mutations in the wild-type alleles, indicating that constitutively expressing Cas9/gRNA transgenes continue to be active after being mobilized into another maize genotype in the progeny (Figure 1B). On the other hand, when the Cas9 gene was silenced in a Cas9-positive line, no new mutation in the targeted gene could be detected. This indicates that continuous mutagenesis requires the presence of an actively expressed Cas9 gene.

In summary, the ISU maize CRISPR platform, a robust and highly efficient public system, can be used for targeted mutagenesis in maize using the *Agrobacterium*-mediated transformation method. We reported the evaluation of this system on four maize genes in two duplicated pairs with over 60% mutagenesis frequency in combined results. This robustness has been further confirmed by experiments with an additional 27 constructs targeting 30 maize genes performed and analyzed in the Iowa State University Plant Transformation Facility (unpublished data).

The ISU maize CRISPR platform is much simpler to construct and more efficient in inducing mutations when compared to a TALEN maize system previously reported by our groups[7]. Using the same maize transformation procedure, the frequency of mutagenesis by TALENs was about 10% in Hi-II and 3.7% in B104, though with different target genes[7]. The mutagenesis frequency of the ISU maize CRISPR platform is also much higher than reported in other maize studies[8-12]. By pollinating transgenic mutant plants with wild type pollen, we can readily generate transgene-free lines with targeted mutations on genes of interest in one generation. Because these mutant lines do not contain any foreign DNA sequences, it is anticipated that they would be treated the same as mutation lines generated by using conventional mutation breeding methods such as radiation or chemical treatment.

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Kan Wang
Department of Agronomy & Crop Bioengineering Consortium
Bing Yang
Department of Genetics, Development and Cell Biology &
Crop Bioengineering Consortium
Iowa State University, Ames, IA 50011
kanwang@iastate.edu; byang@iastate.edu

Register for the Forum of Scientific Society Leaders on Genetically Engineered Crops: Experiences and Prospects

On December 7, 2016, representatives of more than 12 scientific societies will meet to explore the findings, conclusions, and recommendations of the report, Genetically Engineered Crops: Experiences and Prospects, which was released earlier this year. The report has quickly become one of the most popular reports from the National Academies, having been downloaded more than 25,000 times since May. In addition to commenting on the reports' conclusions and recommendations, panelists will explore how the report might be used in the societies' academic education and public communication activities. The forum agenda is organized around several panels that will focus on different sections of the report presented by members of the study committee.

View the full agenda here: http://dels.nas.edu/Upcoming-Event/Forum-Scientific-Society-Leaders/AUTO-5-80-52-G?bname=banr

The forum will be held in the Auditorium of the National Academy of Sciences Building and will be webcast live.

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