A High-efficiency CRISPR Platform for Maize Improvement

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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 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]

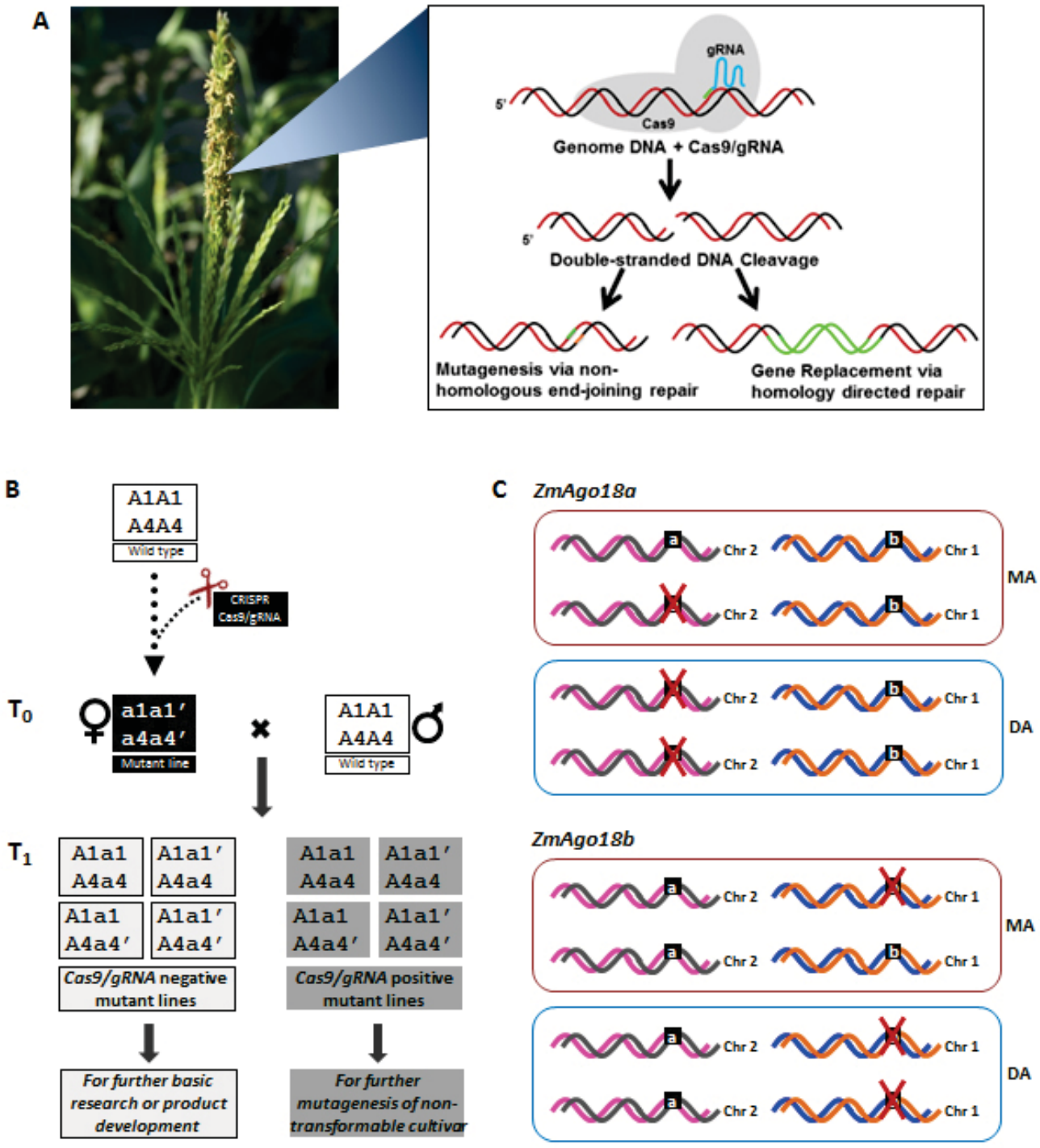

Figure 1. A. Diagram of CRISPR-mediated gene editing. **B.** Maize CRIPSR platform for targeted mutagenesis. **C.** Mono- (MA) and di- (DA) allelic mutations. Chr, chromosome.

(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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