CHAPTER 3: CRISPR-Cas9 editing gene over expression of genes involved in resistance to *Fusarium verticilloides* in maize

Introduction

Developing more pathogen-tolerant crops in a sustainable manner is one means to meet the demand of an increasing human population that will require more food, feed and fuel. In addition to a transgenic approach, natural genetic variation for traits that impact drought tolerance has also been used in maize breeding programs to improve grain yield. By applying precision phenotyping and molecular markers as well as understanding the genetic architecture of quantitative traits, maize breeders developed hybrids with increased grain yield under drought stress conditions (Cooper et al., 2014; Gaffney et al., 2015). The drought tolerance in these hybrids is governed by multiple genes which individually have small effects. Potentially, some of these key genes could be identified and altered to generate new alleles to produce a larger effect, thus enhancing the breeding process. However, until recently, generating such allelic variation with physically or chemically induced mutagenesis was a random process, which made it difficult to produce intended DNA sequence changes at a target locus. In the past few years, efficient genome editing technologies have emerged, enabling rapid and precise manipulation of DNA sequences, and setting the stage for developing pathogen-tolerant germoplasm by editing major genes in their natural chromosomal context.

Four genome editing tools, meganucleases, zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease protein (Cas) system, have provided targeted gene modification in plants (Gao et al., 2010; Li et al., 2012, 2013; Shukla et al., 2009). However, CRISPR/Cas9 has largely overtaken other gene- editing techniques. The CRISPR-Cas9 system is easiest to implement and is highly efficient. The system consists of a Cas9 endonuclease derived from *Streptococcus pyogenes* and a chimeric single guide RNA that directs Cas9 to a target DNA sequence in the genome (Figure 1). CRISPR-Cas9 genome editing is accomplished by introducing a DNA double-strand break in the target locus via Cas9, followed by DNA repair through either the endogenous imprecise non homologous end-joining (NHEJ) or the high-fidelity homology-directed repair (HDR) pathways. NHEJ can induce small insertions or deletions at the repair junction while HDR stimulates precise sequence alterations, including programmed sequence correction as well as DNA fragment insertion and swap, when a DNA repair template is exogenously supplied (Figure 2).

The system has been successfully tested in staple crops, such as maize, wheat, rice and soybean (Du et al., 2016; Jacobs et al., 2015; Jiang et al., 2013; Li et al., 2015; Shan et al., 2015; Sun et al., 2016; Svitashev et al., 2015; Wang et al., 2014; Zhang et al., 2014; Zhou et al., 2014, 2015).

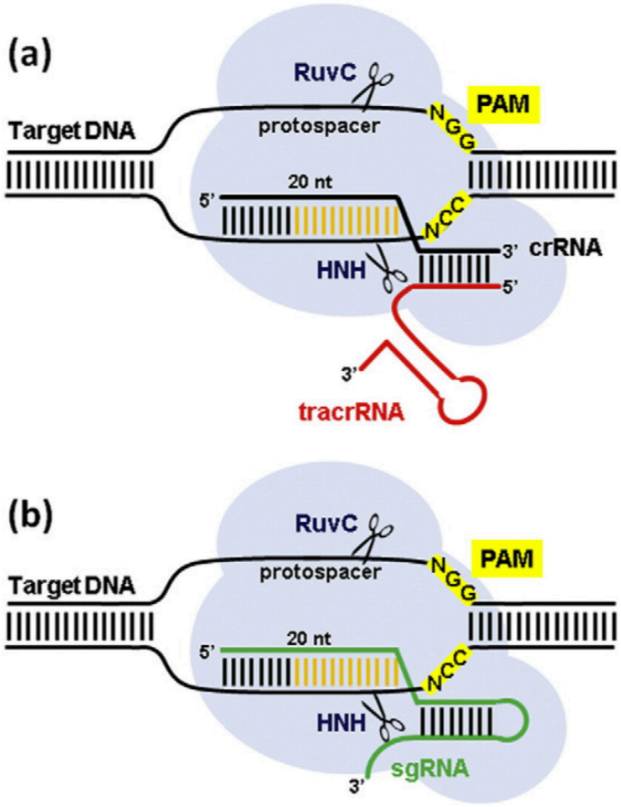


Figure 1. RNA-guided DNA cleavage by Cas9. (a) In the native system, the Cas9 protein (light blue) is guided by a structure formed by a CRISPR RNA (crRNA , in black), which contains a 20 - nt segment determining target specificity, and a trans-activating CRISPR RNA (tracrRNA, in red), which stabilizes the structure and activates Cas9 to cleave the target DNA (protospacer). The presence of a protospacer-adjacent motif (PAM, in yellow), i.e., an NGG (or less frequently NAG) sequence directly downstream from the target DNA, is a prerequisite for DNA cleavage by Cas9. Among the 20 RNA nucleotides determining tar- get specificity, the so-called seed sequence of approximately 12 nt (in orange) upstream of the PAM is thought to be particularly important for the pairing between RNA and target DNA. (b) Cas9 can be reprogrammed to cleave DNA by a single guide RNA molecule (gRNA, in green), a chimera generated by fusing the 3′ end of the crRNA to the 5′ end of the tracrRNA.

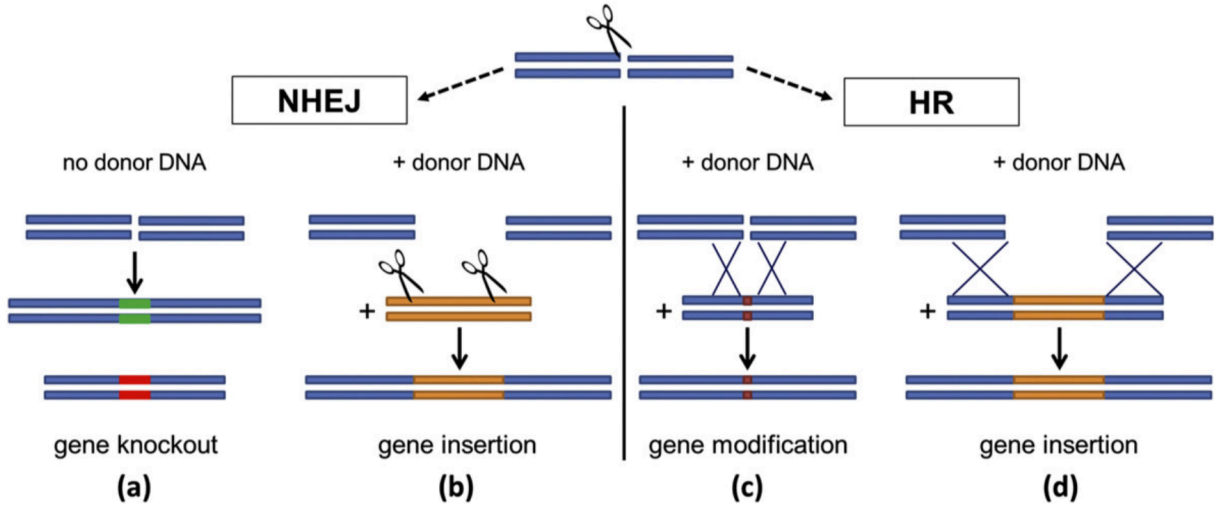


Figure 2. Genome editing with site-specific nucleases. Double-strand breaks induced by a nuclease at a specific site can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). (a) Repair by NHEJ usually results in the insertion (green) or deletion (red) of random base pairs, causing gene knockout by disruption. (b) If a donor DNA is available, which is simultaneously cut by the same nuclease leaving compatible overhangs, gene insertion by NHEJ can also be achieved. (c) HR with a donor DNA template can be exploited to modify a gene by introducing precise nucleotide substitutions or (d) to achieve gene insertion.

In maize, the unique example of CRISPR-Cas9 edited plant belong to stress tolerance and is based on endogenous ARGOS8 mRNA expression which is relatively low and spatially nonuniform. Previous field testing showed that constitutive over-expression of ARGOS8 in transgenic plants increases grain yield under drought stress conditions without yield penalty in non-stress environments (Shi et al., 2015). Aiming at creating novel ARGOS8 variants which would confer beneficial traits for maize breeding, the genomic sequence of ARGOS8 was edited using CRISPR-Cas9-enabled advanced breeding technology to produce ubiquitous and elevated expression across multiple tissues and at different developmental stages. Replacement of the ARGOS8 promoter with a maize GOS2 promoter (GOS2 PRO), or insertion of a GOS2 PRO into the 5^0-UTR of the ARGOS8 gene, led to a change in the ARGOS8 expression pattern from tissue preferred to ubiquitous, and from relatively low mRNA expression levels to significantly increased ARGOS8 expression levels. A field study showed that compared to the wild-type, the ARGOS8 variants increased grain yield by five bushels per acre under stress conditions at flowering and had no yield loss under well-watered conditions. ARGOS8 genome editing led to multiple possibilities in maize resistance improvement, sustaining CRISPR-Cas9 approach in biotic and abiotic stress tolerance.

Stoking disease resistance

Plant pathogens, which deliver disease-causing molecules known as effectors to their hosts, can devastate a farmer's crop, often causing financial ruin or food insecurity within a region. While the plant's immune system works to clear these effector molecules (TAL effectors are are one example of these plant pathogen effectors), conserved sequences within species plant genes can prove to be weak points, and the pathogen's effectors can exploit them to cause disease. Once established within the plant's genome, such sequences are known as susceptibility genes.

Removing the targets of effectors, then the pathogen would struggle in causing disease and modifying the plant to make it susceptible. CRISPR offers a convenient tool for both identifying such genes and producing plants resistant to the disease. Kamoun et al. (2017) removed a portion of a susceptibility factor in a tomato plant using CRISPR. The resulting non-transgenic plants, which were fully resistant to the fungal disease powdery mildew, were developed quickly, within 10 months. Xie and Yang (2013) who have been a key in the development of CRISPR technology in plants, are focused on bacterial blight in rice. This severe disease in South Asia and Africa takes advantage of binding to the promoter of sucrose trans- porter genes, SWEET genes, to induce susceptibility. Using CRISPR, Yang was able to make multiple changes to these promoters to produce the equivalent of a plant vaccine. Jia et al. (2017) at the University of Florida Citrus Research and Education Center have successfully modi ed yet another known susceptibility gene, for a bacterial disease citrus canker, in a species of grapefruit. They are currently looking for susceptibility genes in another destructive citrus disease, citrus greening, also known as Huanglongbing.

Cassava is hearty tuberous root vegetable that serves as a food security crop in sub-Saharan Africa, South American, and Asia, Bart et al. (2017) screened Cassava for bacterial disease and looked at various mutations that would protect these plants from a bacterial disease and two viral diseases. To date, they've successfully screened for mutations that abolish susceptibility genes for two of the diseases, and they've regenerated plants with mutations that they'll soon be testing for disease tolerance (Bart et al. 2017). As with diseases in other organisms, pathogens are constantly adapting and changing. CRISPR could provide a way to outpace those mutations or to generate plants with broad-spectrum resistance.

Targeted mutagenesis strategy: CRISPR-Cas9 double cloning

A schematic of the ISU Maize CRISPR plasmids currently used for *Agrobacterium*-mediated Cas9/ gRNA introduction into maize is shown in Figure 3. The gRNA vectors are based on pENTR-gRNA1 and pENTR-gRNA2 described previously (Zhou et al., 2014). In each intermediate vector, two different rice U6 small nuclear RNA gene promoters (PU6.1 and PU6.2) are used to express the gRNA genes. The first gRNA scaffold (85 nucleotides) is preceded by a cloning site containing two BtgZI sites in a tail-to-tail orientation downstream of PU6.1. The second gRNA scaffold follows a pair of tail-to-tail-oriented BsaI sequences downstream of PU6.2. Two sequential rounds of cloning permit the insertion of custom double-stranded gRNA spacer DNA sequences into these double BtgZ1 and double BsaI restriction enzyme sites in the vectors to generate intermediate constructs pgRNA-IM1 or pgRNA-IM2 (Figure 3).

As described in an earlier publication (Zhou et al., 2014), these two vectors differ by one feature: pENTR-gRNA1 possesses two HindIII sites near the Gateway recombination sites attL1 and attL2, while pENTR-gRNA2 has only one HindIII site near the attL1 site (Figure 3).

This feature allows pgRNA-IM2 to receive the gRNA cassettes from pgRNA-IM1 via HindIII digestion and subcloning. Therefore, this strategy can be used to construct up to four gRNAs, simultaneously targeting up to four DNA sequences in the maize genome.

The guide RNA spacer sequences were designed based on the maize B73 reference genome sequence (Schnable et al., 2009) using the CRISPR Genome Analysis Tool (Brazelton et al., 2015; http://cbc.gdcb.iastate.edu/cgat/). The relevant target regions in Hi-II and B104 genotypes were PCR-amplified and confirmed by sequencing. All pgRNA-IM constructs were confirmed for sequence accuracy at the insertion sites and flanking regions by Sanger sequencing. The confirmed gRNA cassette can be mobilized through Gateway recombination to the destination vector pGW-Cas9. The vector is built on the backbone of pMCG1005 this vector contains a rice codon-optimized Cas9 with the maize ubiquitin 1 gene promoter and the bar gene with a 49 CaMV 35S promoter used as transformation selectable marker (Figure 3). The binary plasmid is mobilized into *Agrobacterium* strain EHA101 for the transformation of maize immature embryos.

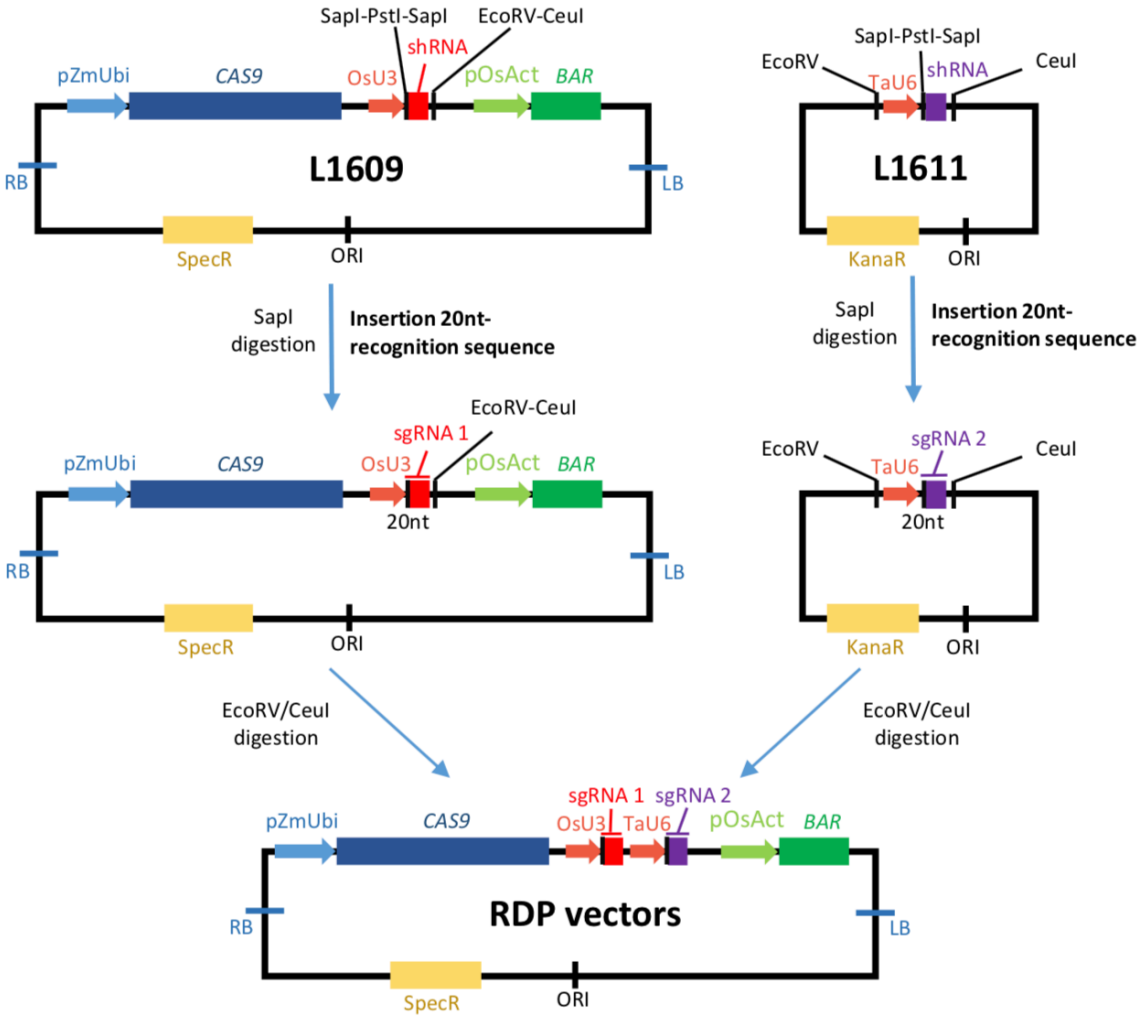


Figure 3. Schematic diagram of Cas9/gRNA construction. The final RDP plasmids contain two small guide RNAs (sgRNA1 and sgRNA2) and are generated by assembly of the two initial plasmids L1609 and L1611. First the 20 nt corresponding to the recognition sequences are synthesized as oligonucleotides with SapI compatible ends and inserted between the U3 or U6 promoter and the scaffold RNA (shRNA) after SapI digestion in both plasmids, forming sgRNA1 and sgRNA2. Then the TaU6::sgRNA2 cassette is transferred by EcoRV/CeuI digestion into the plasmid already containing the OsU3::sgRNA1 cassette. BAR = Basta® resistance gene, Cas9 = rice codon optimized Cas9 gene, LB = T-DNA left border, OsU3 = rice U3 promoter, pActUbi = maize ubiquitin promoter, pOsAct = rice actin promoter, RB = T-DNA right border, shRNA = short hairpin RNA, sgRNA = small guide RNA, TaU6 = wheat (*Triticum aestivum*) U6 promoter, 20 nt = recognition sequence of 20 nucleotides inserted before the shRNA.

***Zea mays* transformation**

Transgenic maize plants were first obtained from protoplasts by an electroporation method (Rhodes et al. 1988) but fertile plants have never been produced by this method. Other direct gene transfer methods, which did not require the prior culture of protoplasts, were then tried (Gordon- Kamm et al. 1990) and microprojectile bombardment (Koziel et al. 1993) of cells in suspension cultures or immature embryos became quite popular in basic and applied studies. Efficiency of transformation by microprojectile bombardment has been higher than other direct methods, and quite a few fertile plants have been generated (Armstrong et al. 1999). Microprojectile bombardment is also useful for the analysis of the transient expression of foreign genes in intact, fully developed tissues. However, high copy numbers and extensive rearrangement of the foreign DNA have frequently been found in plants transformed with direct gene transfer methods (Shou et al 2004).

For the last two decades, dicotyledonous plants have been transformed using the soil phytopathogen *A. tumefaciens*. *A. tumefaciens* is first transformed with the DNA construct of interest (T-DNA); this modified bacterial strain is then used to introduce the T-DNA into plants. A major advantage of *Agrobacterium*- mediated transformation is that a small number of copies (often one or two) of relatively large segments (can be larger than 10 kb) of T-DNA with defined ends are integrated into the plant genome with minimal rearrangement, resulting in transgenic plants of high quality. Initially, it was not clear if this technology could be extended to monocotyledonous plants, as they are not natural hosts of *A. tumefaciens*. However, highly efficient method of transformation have been reported of important cereals such as maize (Ishida et al. 1996), wheat (Cheng et al. 1996), and sorghum (Zhao et al. 2000) by *A. tumefaciens*. Key factors in these achievements include the optimization of types of plant material for infection with *A. tumefaciens*, choice of vectors, choice of strains of *A. tumefaciens* and optimization of tissue culture techniques. Transformation mediated by *A. tumefaciens* is now highly recommended for maize varieties with good tissue culture responses.

For the successful production of transgenic plants in any species, foreign genes must be delivered to undifferentiated, dedifferentiated or dedifferentiating cells that are actively dividing or about to divide and that are capable of regenerating plants. In maize, the material of choice is immature embryos (Figure 4), and all protocols mediated by particle bombardment or *A. tumefaciens* for efficient production of transgenic maize have solely employed immature embryos. Thus, the primary determinants of a successful transformation are the response of immature embryos in tissue culture, the types of cells that grow from immature embryos and subsequent characteristics in

growth and regeneration. Unfortunately, many genotypes of maize, especially so-called elite varieties, are poor in these aspects, and thus only a limited number of genotypes have been efficiently transformed so far.

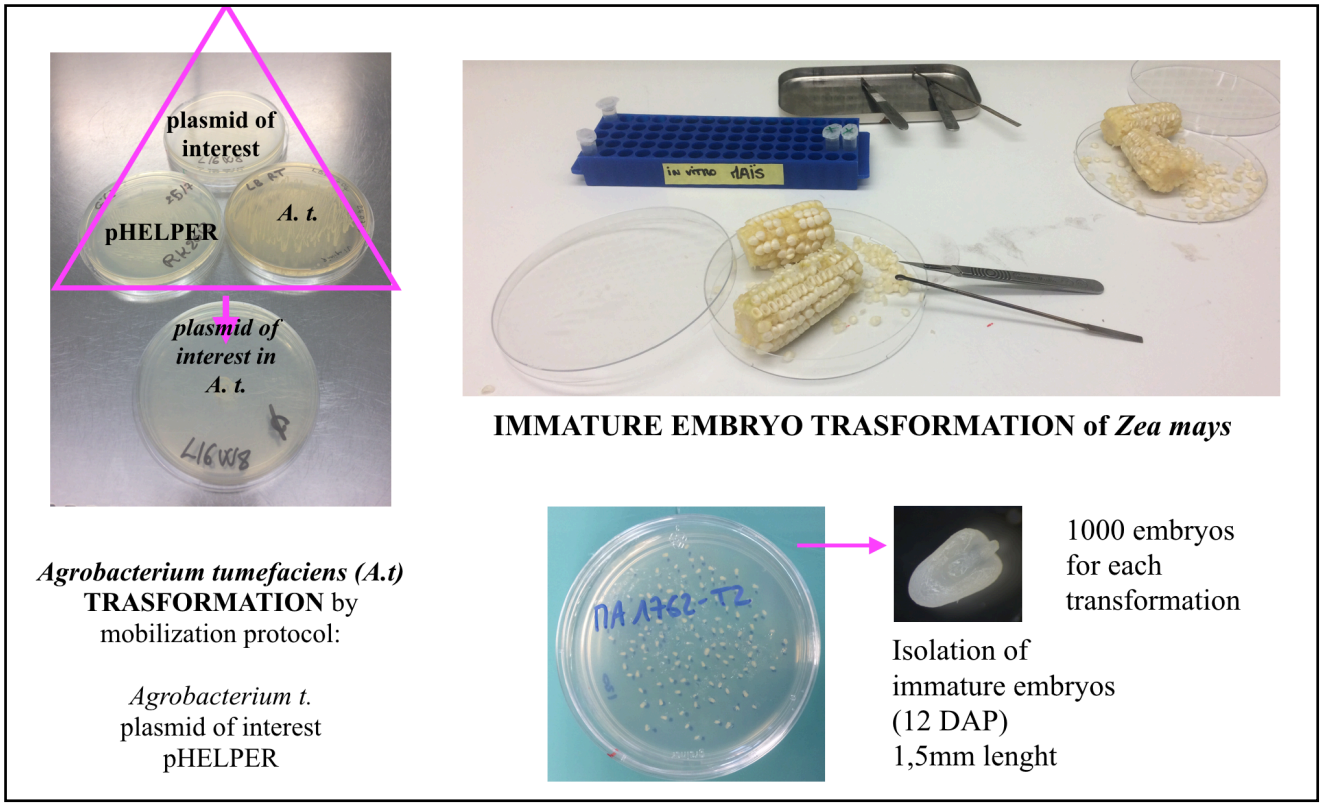


Figure 4. Schematic summary of *A.tumefaciens* transformation by mobilization protocol and immature embryos isolation used in this thesis. Details are described in Methods.

In the original protocol of Ishida et al. (1996), transgenic plants were obtained from between 5% and 30% of *A. tumefaciens*-infected immature inbred A188 embryos. The protocol was successfully employed in a number of studies in molecular biology and biotechnology. Since then, the methods have been greatly improved, and a highly optimized protocol routinely used is presented in this thesis. The modifications made to the protocol include pretreatment by heat and centrifugation addition of silver and copper ions to the co-cultivation medium and extension of the co-cultivation period from 3 to 7 days. The effects of heat, centrifugation and ions are evident (Hiei et al. 2006) but the mechanisms are not understood. In protocols described by other authors, a resting culture, which is a non- selective incubation of embryos on a medium that contains an antibiotic to kill bacteria, is performed after co-cultivation (Zhao et al. 2001). In practice, the growth of transformed cells was better in the selection culture if the resting culture was performed; however, the same effect was produced by an extended period of co-cultivation. Thus, if the co-cultivation is extended, no resting culture is necessary. In general, the bar gene (De Block et al 1987), which confers

resistance to the herbicide phosphinothricin, is a more effective selection agent than the hpt gene (van den Elzen et al. 1985) which confers resistance to the antibiotic hygromycin. Further, use of vectors that carry additional virB and virG genes from pTiBo542 (Hood et al. 1984; Komari et al. 1986) gives much higher transformation frequencies. The function of virB is related to formation of a transmembrane channel between the bacterium and the plant cell and the function of virG is related to the activation of the other vir genes (Sheng et al. 1996).

Transgenic plants may be routinely obtained from more than 50% of the immature embryos from the A188 genotype, and the range of transformable genotypes has been extended to inbreds A634, H99 and W117 and hybrids between pairs of these varieties.

***Zea mays* calli cultivation and plant growth**

Figure 5 shows the procedure used for transformation and regeneration. It should be noted that two types of embryogenic callus, type I and type II, may proliferate from the scutellum of immature embryos, depending on the genotypes of maize (Bajaj 1994). The type I callus is a relatively hard and compact embryogenic callus, whereas the type II callus is relatively soft and friable.

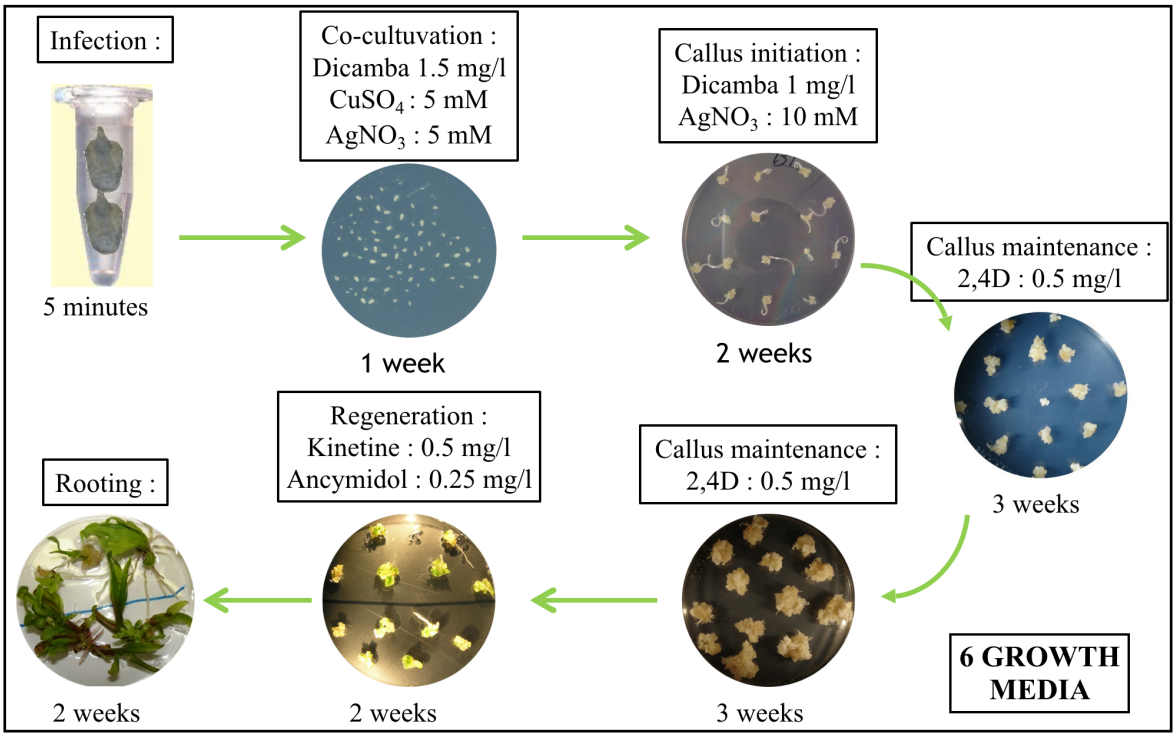


Figure 5. Scheme of *Zea mays* calli cultivation from *A.tumefaciens* transformation to plant rooting.

A type I callus is usually obtained from inbreds A188, A634, H99 and W117 on the media described in this work and based on LS medium. Hi-II and some other genotypes have been adopted by other groups (De Block et al. 1987) using media based mostly on the N6 medium. In addition, Frame et al. (2002) used media that contained cysteine for co-cultivation.

Elimination of selection marker genes from transgenic plants for commercialization is an important task because the presence of selection marker genes, which are unnecessary once transgenic plants are established, is of high public concern. A simple approach to remove selection markers is to perform *A. tumefaciens*-mediated co-transformation of plants with two T-DNA segments, one with a selection marker and the other with genes of interest, followed by segregation of marker-free progeny (Komari et al. 1986). Because the frequency of co-transformants among initial transformants is never 100% and about a half of the co-transformants do not segregate marker-free progeny, this approach requires a high frequency of transformation, desirably more than 20% (independent transgenics/immature embryo). With the highly optimized protocol presented here, production of marker-free transgenics has become a realistic option in maize; selection-marker-free transformants may be obtained from about 50% of co-transformed plants when co-transformation vectors are employed.

Candidate genes for CRISPR-Cas9 genome editing

Genes belonging to LOX pathway resulted strongly induced after *Fv* inoculation in a resistant (R) inbred at 3 and 7 dpi, whilst in susceptible (S) inbred the induction was reduced or delayed at 14 dpi (Maschietto et al. 2015). In addition, all *LOX* genes were induced in resistant (R) kernels already at 3 dpi in control condition (water inoculation), suggesting that this line activated earlier and more efficiently the transcription of defence responses. Moreover among 9-LOX genes, *ZmLOX6* and *ZmLOX4* resulted strongly up-regulated after pathogen inoculation at 7 dpi in the R line and their expression values were not significantly different among genotypes in mock kernels, suggesting that these two genes are related to resistance and exclusively responsive to pathogen inoculation. These results collectively suggest that resistance in maize may depend on an over-expression of LOX pathway genes and highlighted the central role of JA in *Fv* resistance. Because *Zmlox4* mutant has already been obtained as transposon insertion, as shown in chapter 2, we move the attention to *ZmLOX6* editing approach while ZmLOX4 will be over-express in the susceptible A188 background.

In addition, three genes were found associated with the difference in infection between control and inoculated inbred lines in a GWAS experiment performed by Stagnati et al. (2018) SNPs associated with this trait are reported in Manhattan plot (Figure 6).

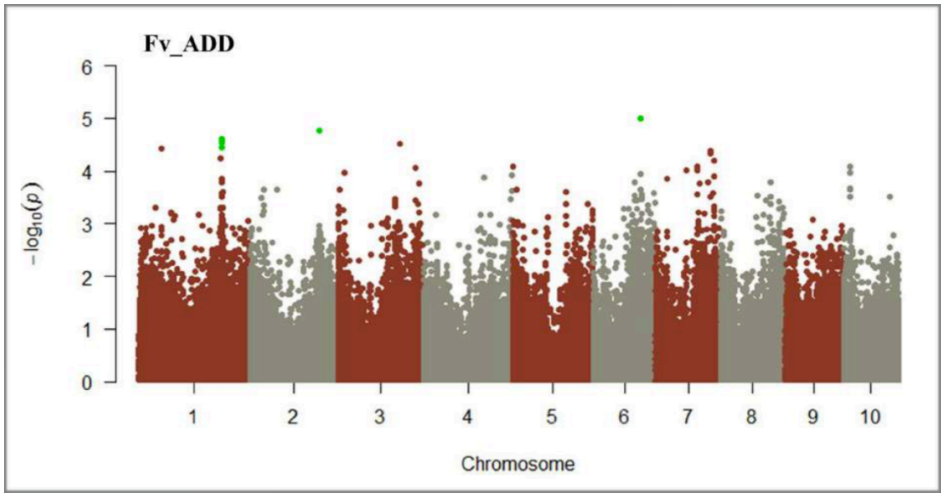


Figure 6. Manhattan plot of the SNP associated with *Fv* resistance (Fv_ADD).

The SNP on chromosome 2 (number 6 in Tables 2 and 4) was closed to the gene GRMZM2G113257. This gene encodes for bHLH-transcription factor 169. In this protein is present the ACT domain that characterize ACR proteins. In Arabidopsis and rice these ACR proteins are proposed to function as novel regulatory or sensor proteins in plants (Marchler-Bauer et al., 2015). On chromosome 6 the SNP (number 7 in Tables 2 and 4) was located inside GRMZM2G163054 (number 7 in Tables 2 and 4), which encodes for a putative WRKY125 DNA-binding domain superfamily protein (number 6 in Tables 2 and 4). These transcription factors are involved in the regulation of various physiological programs that are unique to plants, including pathogen defense, senescence and trichome development (Marchler-Bauer et al., 2015). WRKY proteins have a regulatory function in plant response against pathogens. It is reported that levels of WRKY mRNA increase after viral, bacterial or fungal infection (Marchler-Bauer et al., 2015). Targets of WRKY proteins are W-boxes, WRKY genes, and defense related genes of the PR type. WRKY proteins are also involved in gibberellin and JA response (Eulgem et al., 2000). Regarding biotic stresses, WRKY genes are involved in transcriptional reprogramming associated with plant immune response. They are key components of the innate plant-immune system comprising microbe-triggered immunity, PTI, ETI, basal defense and SAR. WRKY transcription factors are involved also in abiotic stress response (Rushton et al., 2010). In developing maize kernels of the resistant genotype CO441 WRKY transcription factors were observed highly expressed 72 hours after *F. verticillioides* infection (Lanubile et al., 2014b). Moreover, WRKY125 was found up-regulated in

maize kernels infected with *A. flavus* or *F. verticillioides* 72 hours after infection (Shu et al., 2014). As *ZmWRKY125* was found associated with *Fv* resistance it was decided to edit *ZmWRKY125* to further investigate its role in *Fv* defense.

Aim of the work

To date, *LOX* and *WRKY* genes have been implicated in playing important roles in a variety of developmental processes and defense responses to insects and pathogens. While the role of these and other *LOXs* have been studied in dicotyledonous species, their role in maize pathogen resistance and other monocotyledonous species remains unclear. Therefore, the major goal of this PhD study was to broaden this knowledge base and probe CRISPR-Cas9 and transformation in maize. The information gleaned from this research is expected to improve our understanding of plant transformation and defense mechanisms and may help design novel strategies to enhance resistance to harmful pathogens as *Fv*.

The line A188 was transformed to pursue two specific objectives:

1). *LOX6* and *ZmWRKY125* editing to determinate their role in *Fv* resistance

2). *LOX4* over expression to test the increased resistance to *Fv*

All of the work performed in this chapter have been done in collaboration with Peter Rogowsky group of École Normale Supérieure (ENS), Lyon. The data obtained have been part of the paper by Doll et al. Single and multiple gene knockout by CRISPR-Cas9 in maize, submitted to Plant Cell Reports, and reported at the end of this chapter.

Experimental design and results

sgRNA Design for CRISPR-Genome editing

Identification of target sequences:

In a first instance, the target sites in the genomic DNA sequence of the genes of interest were identified. For knockout the presence of 5'-A-N(20)-GG-3' or 5'-G-N(20)-GG-3' were verified as suitable for U3 or U6 promoters, respectively. Other criteria were sequences without AG termination and successions of more than 3 T in the sequence. All recent web sites such as http://cbi.hzau.edu.cn/cgi-bin/CRISPR or http://cropbioengineering.iastate.edu/cgat take into account these criteria.

The strategy was to target the same gene with two sgRNAs sequences that are approximately 40 bp to 100 bp from each other. If both sgRNA work, this leads to a deletion that can easily be detected by PCR. Moreover, because the reference genome is from genotype B73 whereas the genotype used for transformation is A188, the absence of SNP in the target site was verified in order to minimize the risk to come across a SNP in the target sequence.

CRISPR design prior to cloning: how the cloning works

The system described hereafter uses the basic elements (rice optimized Cas9 gene, OsU3 promoter) of the rice system published by Miao et al (2013). It allows the direct cloning of an oligonucleotide representing the target in the genome into the SapI site of the transformation vector L1609 (described in example 1) to obtain a first sgRNA under the control of the rice OsU3 promoter. It allows also the addition of a second sgRNA under the control of the wheat TaU6 promoter after cloning of an oligonucleotide representing the second target in the genome in the SapI site (see example 2) of the small plasmid L1611 and transfer of this second sgRNA into the transformation vector by cloning with EcoRV et I-CeuI (described in example 3). U3 and U6 promoters are transcribed by RNA polymerase III, consequently, these transcripts do not carry a polyA tail.

The size of the oligonucleotide cloned into the sgRNA was of 20 nt, even if in plants sizes between 19 and 22 nt seem to work.

Example 1: vector L1609

>L1609_OsU3_sgRNA

AAGGGATCTTTAAACATACGAACAGATCACTTAAAGTTCTTCTGAAGCAACTTAAAGTTATCAGGCATGCATGGATCTTG
GAGGAATCAGATGTGCAGTCAGGGACCATAGCACAGGACAGGCGTCTTCTACTGGTGCTACCAGCAAATGCTGGAAGCCG
GGAACACTGGGTACGTTGGAAACCACGTGATGTGGAGTAAGATAAACTGTAGGAGAAAAGCATTTCGTAGTGGGCCATGA
AGCCTTTCAGGACATGTATTGCAGTATGGGCCGGCCCATTACGCAATTGGACGACAACAAAGACTAGTATTAGTACCACC
TCGGCTATCCACATAGATCAAAGCTGGTTTAAAAGAGTTGTGCAGATGATCCGTGGCAAGAAGAGCACTGCAGAGCTCTT
CAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
TTTTTT

rice U3 promoter | SapI recognition site | PstI | sgRNA scaffold | terminator

Example 2: vector L1609 after SapI digestion

In example 2 and 3 SapI produces sticky ends and the GC nucleotides need to be present in the primer design:

GCAGATGATCCGTG GCA-N19- GTTTTAGAGCTATGC
CGTCTACTAGGCACCGT -N19-CAA AATCTCGATACG

Example 3: vector L1611

The following is the DNA sequence around the cloning site for the U6 promoter pENTR-gRNA1 published in Char et al. 2017:

>L1611_TaU6_sgRNA

GACCAAGCCCGTTATTCTGACAGTTCTGGTGCTCAACACATTTATATTTATCAAGGAGCACATTGTTACTCACTGCTAGG
AGGGAATCGAACTAGGAATATTGATCAGAGGAACTACGAGAGAGCTGAAGATAACTGCCCTCTAGCTCTCACTGATCTGG
GTCGCATAGTGAGATGCAGCCCACGTGAGTTCAGCAACGGTCTAGCGCTGGGCTTTTAGGCCCGCATGATCGGGCTTTTG
TCGGGTGGTCGACGTGTTCACGATTGGGGAGAGCAACGCAGCAGTTCCTCTTAGTTTAGTCCCACCTCGCCTGTCCAGCA
GAGTTCTGACCGGTTTATAAACTCGCTTGCTGCATCAGACTTGGGAAGAGCAGATATCAGCTCTTCAGTTTTAGAGCTAG
AAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT

wheat U6 promoter | SapI recognition site | EcoRV | sgRNA scaffold | terminator

GCAGATGATCCGTG GCA-N19- GTTTTAGAGCTATGC
CGTCTACTAGGCACCGT -N19-CAA AATCTCGATACG

To summarize the cloning of two sgRNA in a transformation vector according to Char et al. (2017), includes to the following steps:

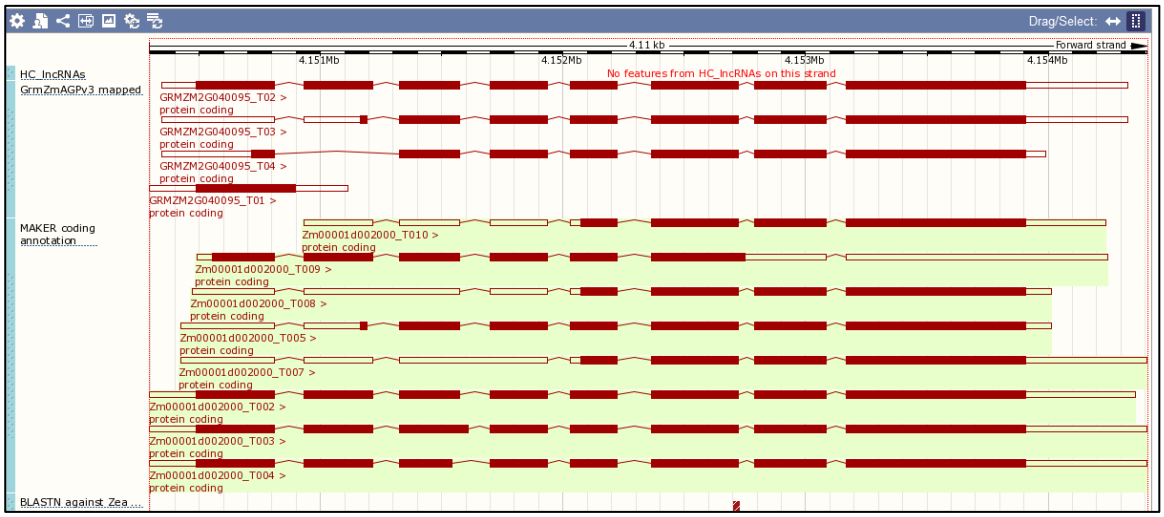
1) Cloning of the sgRNA A in plasmid L1609 (rice OsU3 promoter, unique SapI cloning site), which an integrative transformation vector carrying a SpcR marker. It has to be used with *Agrobacterium* strain LBA4404pSB1 to allow integration of L1609 into pSB1.

2) Cloning of the sgRNA B in plasmid L1611 (wheat TaU6 promoter, unique SapI cloning site), which is a small plasmid with a KanR marker.

3) Transfer of the cassette "sgRNA B" from step 2 into the plasmid obtained in step 1 by digestion with EcoRV et I-CeuI.

sgRNA Design for ZmLOX6 (GRMZM2G040095)

1. Choice of the mRNA between the different splicing sequences for ZmLOX6; the mRNA corresponds to Zm00001d002000_T02.



2. SNPs study to avoid differences in sequence between the inbred used for the transformation and the template used for sgRNA. The SNPs in *ZmLOX6* are highlighted in yellow and green.

Zm00001d002000_T02

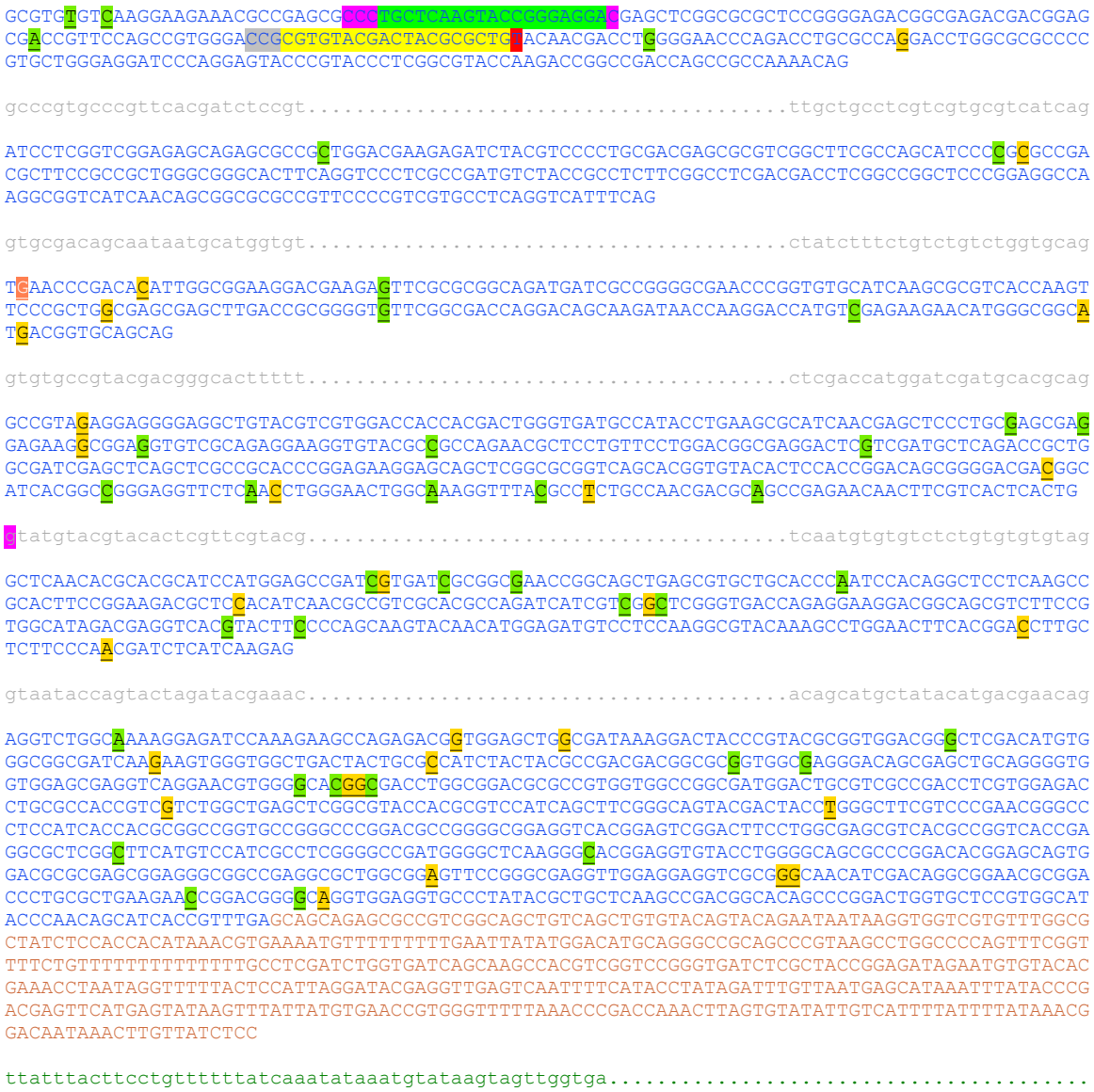
.....agcagcgtggtggacatattattgtttcctctccgtccatcacccacccc

GGTCGGTCTCAACTCAAGCTCTCAGCCACACACGCCGCCATCAGGCACGTATGCGACCTGACCTCGTGTTACATGCTACTACGGCCGTGT
ATATAACAGCGATCAATCTACCGTGACCACCCCCATCCTCTCTAACCCACGGAAGCAAGACAAAAGCTCGTGCTGGACGACATCTCCCCT
GTCGATCCACGACCATGATGCAGCAGCTCCGTCACAGCCAGCCGAGCCCGTGCCTCTGCGGCCTGCGGGCGGCACGGCCTATGCTCGCCC
TCGGCGCAGCAGCATCCCGTTCGCGGCCCGCCGGAAAACTGCAACCGAGCGTCTGCCTCGGCCTCGGCCATGTAGCCCCAGCCGCGGCGA
GAGGACAGCCCCGTCCCCGTGCCGTTGCCGACTCGGCGCTGGGAGCATCGCCTACGAGCGTGCATGTCGGAGGCAAGCTGCTGCTGCAGA
ACTTCGCCGCCGACAGCCAGCAGCGGCTCAAGCTCTCCATCCAGCTTGTCAGCGCCACCGTGGCCG

gtacgcgcgcgaactcggccatatg.....cattttttcttacgtacgcgtgcag

ATCCCGACGGGCGCGGGGTGAAGGCGGAGGCGTCGGTGCTGGACGCCGTCGTGGGCAGCGGGGACAGCGAGCTCGACGTGGACCTGATCT
GGGACGAGGCGCTGGGCGCGCCCGGCGCGGTGGTGGTGAAGAACCACTCCGACTTCCCCGTGTACCTGAGGCTGCTGAGCGTGCCGGCCG
GCGTCGGCGGCGCCGACGACGAGGCCGCCGCCGTCCACTTCGCCTGCAACGGATGGGTGTACCCCGTCGACAAGCACCCGTACCGCCTCT
TCTTCACCAACGAC

gtgcgtgcgtgcgcagctagctaac.....cgtacgctgttcccttcctctgcag



The following results corresponds to candidate guides in exon 1 on the CRISPR-P website. In green and pink are shown the sequence used as target for sgRNA.

Guide-9	92	GCCGAGGGTACGGGTACTCCTGG	CCAGGAGTACCCGTACCCTCGGC	0 SNP
Guide-10	92	GTCCTCCCGGTACTTGAGCAGGG	CCCTGCTCAAGTACCGGGAGGAC	0 SNP
Guide-11	92	GGAGCGACCGTTCCAGCCGTGGG		1 SNP
Guide-12	91	ACAGCGCGTAGTCGTACACGCGG	CCGCGTGTACGACTACGCGCTGT	0 SNP
Guide-13	91	ACGCGGTCCCACGGCTGGAACGG		
Guide-17	88	GAGCGCCCTGCTCAAGTACCGGG		
Guide-25	83	ACGCGCTGTACAACGACCTGGGG		
Guide-27	77	GACCTGGCGCGCCCCGTGCTGGG		
Guide-28	77	GGACCTGGCGCGCCCCGTGCTGG		

Guide-29	75	GGCCGGTCTTGGTACGCCGAGGG		

3. Oligos used for LOX6 CRISPR Cloning:

LOX6-G12-F 5'-GCACAGCGCGTAGTCGTACACG-3'

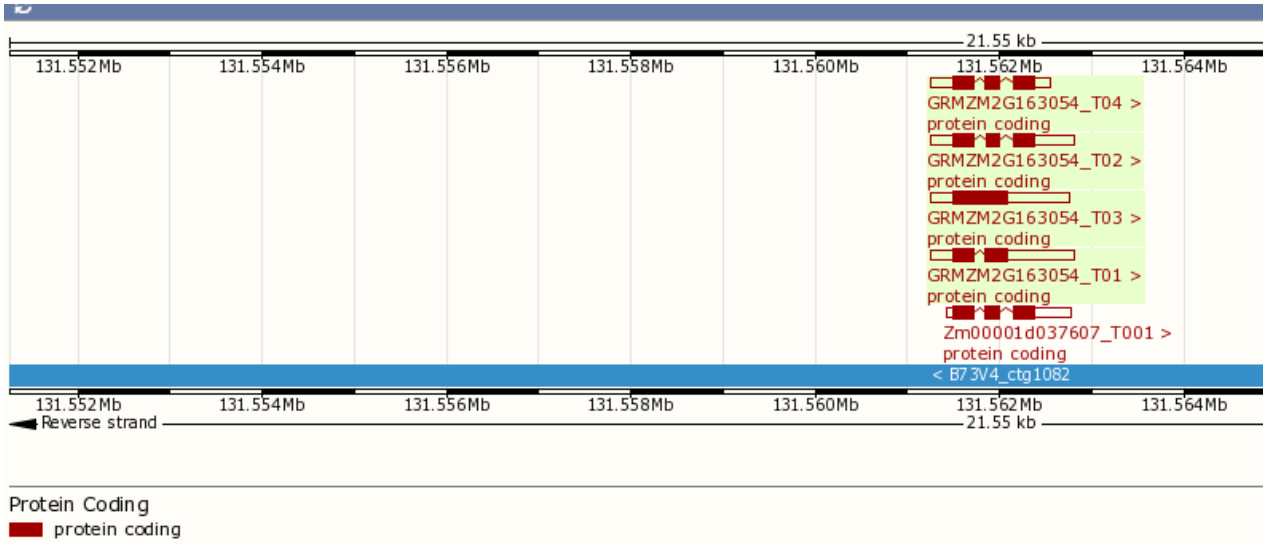
LOX6-G12-R 5'-AACCGTGTACGACTACGCGCTG-3'

LOX6-G10-F 5'-TTGTCCTCCCGGTACTTGAGCA-3'

LOX6-G10-R 5'-AACTGCTCAAGTACCGGGAGGA-3'

sgRNA Design for WRKY125 CRISPR (GRMZM2G163054)

1. Choice of the mRNA between the different splicing sequences for ZmWRKY125. The mRNA corresponds to Zm00001d037607_T04. sgRNA were found after CRISPR-P analysis.



2. SNPs study to avoid differences in sequence between the inbred used for the transformation and the template used for sgRNA. Highlighted are SNPs in ZmWRKY125.

GRMZM2G163054_T04

.....atatacacacaagcagcagcacacatttgtggaattgaaatgtgacatat
CTCAGCAGCAGCACAAAAAGAAGAATTGACATGTGACGAATCTCAGAACTTTCGGATCAG
CTAGCTTTTGAGAAACCTCCACGAAGGCTTGCAGAGGTCGCAGGCCGGATGTATAAATTG
CAGAGCCCGGCCGGCACGCCAAAAATATTATCCAGACAGACAGGCACATCAACGGACAGA
CACACACAAGCAAGGCGGCTAGCGGTGCAAGTAGTGCGAAGCTAGCTAGGTGCTGGTGCA
TGCAATGGCGGCTTCGCTGGGTCTGAACCCTGAAGCTGTCTTCACTTCCTACACCTCCTC
GCCGCCGTTCATGTCGGACTACGTGGCGGCGAGCTTCCTGCCGCCGGCCGTCGTCGACTC
CACGGACTTCTCTGCAGAGCTCGATGATCTTCACCACCACTTGGATTACTCATCGCCGGC
GCCGACCTTGGCCGGGGCTCGGAGCGATCGCAGCGAGAAGCAGATGAT
gtatgtactacctatgcacagcaaa.....acggcgatttcatttcatccatcag
CAGGTGGTGTGAGGGTGGTGGTGGCGAGAAGAGACTCGGTAGGATCGGGTTCAGAACGAG
ATCAGAGGTGGAGATCTTGGACGATGGATTCAAATGGAGGAAGTATGGCAAGAAGGCTGT
CAAGAGTAGCCCAAATCCAAG
gtacacccatatgttcacctggaat.....tgcatgcacacgtacgtacgtgcag
GAACTACTACCGCTGCTCGTCGGAGGGCTGCGGCGTGAAGAAGCGGGTGGAGAGGGACCG
CGACGACCCCCGCTACGTCATCACCACCTACGACGGCGTCCACAACCACGCAAGCCCCGC

AGCCGCCGCCATCATCCAGTACGGCGGCGGCGGCGGATTCTATAGCCCGCCGCACAGCGG
CTCGCCGTCGGCCGCCTCCTACTCGGGCTCCTTCGTCCTCTGACTTCTCCGGGCCTTGAC
CCAGAGTGATCTGATCTACCTGCTTTATCCCAGTCGTAGGATGAGAAGAGTGTTCAGACT
TCAGAGACATCAGTTCAGCGGCCGGCTAAATTCGGACCTGATTGTAGATATTTACACCTC
AATTATTGGTTACCTCTTGTGACGACTGACGAGGCATCATAATC
gtcactaggataattgatcacgcatgtttttcgcttaattagtttctgtg…….

The following results corresponds to candidate guides in exon 1 on the CRISPR-P website. In green and pink are shown the sequence used as target for sgRNA.

			reverse complement
Guide-4	91	ACGTAGTCCGACATGAACGGCGG	CCGCCGTTCATGTCGGACTACGT
Guide-8	88	ATCGCTCCGAGCCCCGGCCAAGG	CCTTGGCCGGGGCTCGGAGCGAT
Guide-10	84	AGGAAGTGAAGACAGCTTCAGGG	CCCTGAAGCTGTCTTCACTTCCT

Guide-2	94	GCCACGTAGTCCGACATGAACGG
Guide-7	89	GTTCATGTCGGACTACGTGGCGG
Guide-9	87	GAGCTCTGCAGAGAAGTCCGTGG
Guide-12	79	GAAGTCCGTGGAGTCGACGACGG
Guide-13	77	GCGATGAGTAATCCAAGTGGTGG
Guide-15	70	GCCGGCCGTCGTCGACTCCACGG
Guide-16	67	GCCGTTCATGTCGGACTACGTGG
Guide-17	67	GGCGGCGAGCTTCCTGCCGCCGG

Oligos used for WRKY125 CRISPR Cloning:

ZmWRKY125_Guide-7_F: TTGTTCATGTCGGACTACGTGGCGG

ZmWRKY125_Guide-7_R: AACCCGCCACGTAGTCCGACATGAA

ZmWRKY125_Guide-8_F: GCATCGCTCCGAGCCCCGGCCAAGG

ZmWRKY125_Guide-8_R: AACCCTTGGCCGGGGCTCGGAGCGA

ZmLOX6 and ZmWRKY125 CRISPR-Cas9 cloning

Cloning of ZmLOX6 and ZmWRKY125 was performed according to the method reported hereafter.

sgRNA A- L1609 and sgRNA-L1611 cloning were confirmed by mini preps digestion using:

- pstI for L1609;
- EcoRV for L1611:

The digestion patterns are described in Table 1 and Figure 7:

Table 1. L1609 and L1611 pstI and EcoRV digestion

	empty vector fragments bp	**positive ligation fragment bp**
sgRNA A- L1609: pstI digestion	6860 5069 3264 442	11924 3264 442
sgRNA-L1611: EcoRV digestion	2160 378	2533

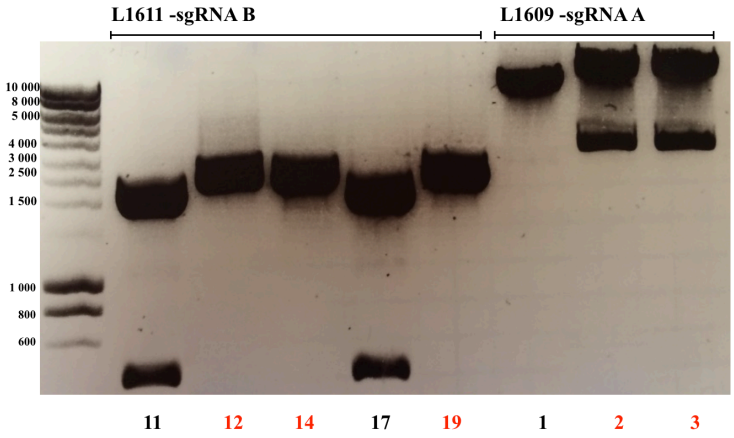


Figure 7. L1609-sgRNA A and L1611-sgRNA B for ZmLOX6 and ZmWRKY125 Cloning. Faint bands of 442 for L1609 are not detectable. In red positive digestion, in black negative digestion (empty vector). At the bottom miniprep numbers.

L1611-sgRNA B was moved to L1609. The ligation ratio between insert and vector was 3:1. Both the vectors are digested with EcoRV and I-CeuI (Figure 2), then transformed in DH5α competent cells by heat shock as performed before. To avoid continuous cutting of restriction enzyme, the ligation product was inactivated at 80°C for 20 minutes (I-CeuI denaturation conditions).

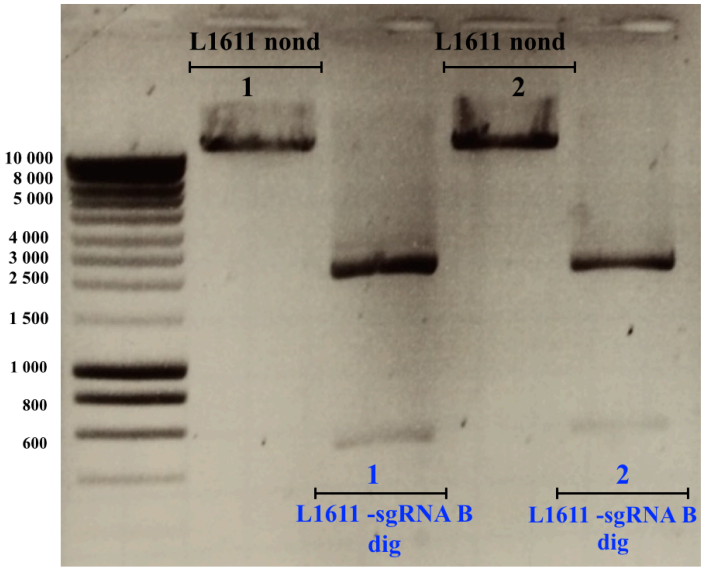


Figure 8. L1611-sgRNA B for ZmLOX6 and ZmWRKY125 Cloning. In blue vector digestion, in black negative digestion (empty vector). bands bp: 2046+492 belongs to digested (dig) L1611-sgRNA B; over 10000 bp: non-digested (nond) plasmid used as control sample.

Transformation of *A. tumefaciens* with *ZmLOX6 and ZmWRKY125*

The ligation obtained have been used for *A.tumefaciens* transformation by following mobilization protocol. This transformation procedure consists of petri dishes single growth of three components, as described in Material and Methods and in Figure 9:

1. plasmid of interest containing sgRNA A + sgRNA B and Cas9 cassette;
2. pHelper pSB1 which allows the integration of the plasmid of interest in *A.tumefaciens;*
3. *A.tumefaciens* strain LBA4404.

After the growth of each component all of them will be put together in order to complete the transformation step as illustrated in Material and Methods.

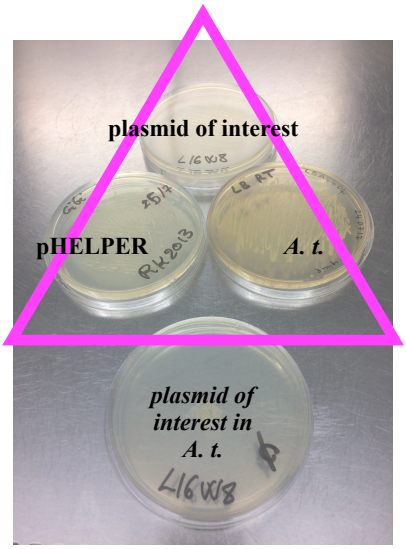


Figure 9. Representation of *A. tumefaciens* transformation by following the mobilization protocol. The plasmid of interest, the pHELPER and *A. tumefaciens* are mate together in a final petri dish. This case represent the transformation of ZmWRKY125 (L16W8).

Editing of genes involved in *Fv* resistance by single and multiple gene knockout by CRISPR-Cas9 in maize.

CRISPR maize design and results obtained for ZmLOX6 editing in this chapter are part of the paper "Single and multiple gene knockout by CRISPR-Cas9 in maize" by Doll et al. The study examined CRISPR-Cas9-mediated targeted mutagenesis in maize during routine use for functional genetics studies by single or multiple gene mutagenesis via stably transformed maize plants. Both systems allow the expression of multiple guide RNAs and different strategies have been employed to knock-out either independent or paralogous genes. A total of 12 plasmids, representing 28 different single guide RNAs (sgRNAs), were generated in order to target 20 genes. At least one mutant allele was obtained for 18 genes, whereas two genes were recalcitrant to gene editing. Among all mutated plants, 19% (16/83) showed biallelic mutations. Small insertion or deletions of less than 10 nucleotides were the type of mutation mostly observed, no matter whether the gene was targeted by one or more sgRNAs. Deletion of a defined region located between the target sites of two guide RNAs were also reported although the exact size of the deletion size was slightly variable. Three types of mutations were observed in the 93 mutant maize plants analyzed: indels, larger deletions and local chromosome rearrangements. More importantly, the mutation efficiency was very variable at different levels. Firstly, two of the 20 genes could not be mutated at all despite the use of two guide RNAs per gene and the generation of 8 and two transformation events, respectively. Secondly, among the 18 genes successfully mutated, not all transformation events caused mutations. For example, in the case of GRMZM2G352274 only one of the 16 transformation events yielded a mutation. And thirdly, in transformation events carrying novel mutations, not all guide RNAs present in the same plant provoked mutations. The reasons for failure are likely linked either to the sgRNA design or to the accessibility of the target sequence. The two recalcitrant genes GRMZM2G035701 and GRMZM2G040095 (LIPOXYGENASE 6) are located in gene-rich regions on the long arm of chromosome 8 and close to the end of chromosome 2, respectively, which do not present any obvious feature explaining failure; LOX6 regenerated plants are shown in figure 10. No sequence confirmation about off-targets effects have been performed, so we can't exclude that the Cas9 has cut a redundant gene close to the target site. The ease of multiplexing is frequently cited as one of the major advantages of CRISPR-Cas9 technology over other site-directed nucleases such as meganucleases, zinc finger nucleases or TALENs and CRISPR-Cas9 constructs harbouring 14 guide RNAs have been used successfully in Arabidopsis (Peterson et al. 2016). Three double mutants in gene family members residing on different chromosomes, two double mutants in paralogs distant by 53 kb or 75 kb and a triple mutant in paralogs distant by 13 kb or 29 kb were

generated. These examples underline the power of CRISPR-Cas9 technology since the production of double or triple knockout mutants in tightly linked genes would have been nearly impossible to achieve by crossing of single mutants and would have required the analysis of thousands of recombinants. Multiplexing is of particular interest in maize, which is an ancient tetraploid known to contain numerous functionally redundant paralogs hampering functional analysis, and the production of multiple mutants by CRISPR-Cas9 will certainly become a prime tool in functional genomics in this species.

Figure 10**.** Regenerated plants of LOX6-CRISPR-Cas9.

Table 2a. Summary of transformation events according LOX6-CRISPR and LOX4OE transformations. The transformation event corresponds to one callus growth led to one ore more regenerated plants. (V, confirmed; X, absent)

LOX6-CRISPR T0 PLANTS				
transformation event	PLANT code	DNA EXTRACTION	*LOX6* SEQ ANALYSIS	Cas9
1	X361-1	V	V	X
1	X361-2	V	V	X
2	X362-1	V	V	V
3	X363-1	V	V	V
4	X364-1	V	V	V
4	X364-2	V	V	V
5	X365-1	V	V	V
5	X365-2	V	V	V
5	X365-3	V	V	V
6	X366-1	V	V	V
6	X366-2	V	V	V
7	X367-1	V	V	V
8	X368-1	V	V	V

Table 2b. Summary of transformation events according WRKY125 -CRISPR transformation. The transformation event corresponds to one callus growth led to one ore more regenerated plants. (V, confirmed; X, absent)

WRKY125-CRISPR T0 PLANTS				
transformation event	PLANT code	DNA EXTRACTION	*WRKY125* SEQ ANALYSIS	Cas9 CONFIRM
1	X471-1	V	V	V
1	X471-1	V	V	V
2	X472-1	V	V	V
3	X473-1	V	V	V
4	X474-1	V	V	V
5	X475-1	V	V	V
6	X476-1	V	V	V
7	X477-1	V	V	V
8	X478-1	V	V	V
9	X479-1	V	V	V
10	X480-1	V	V	V
11	X481-1	V	V	V
12	X482-1	V	V	V
13	X483-1	V	V	V
14	X484-1	V	V	V

Design of *LOX4* over expression vector

In order to identify *ZmLOX4* CDS, a multiple alignment of LOX4 protein was performed between *Zea mays, Hordeum vulgare, Oryza indica, Oryza sativa, Setaria italica, Sorghum bicolor*. The software used was CLUSTAL O (1.2.4) multiple sequence alignment and the multiple alignment obtained for LOX4 protein is reported in supplemental data of this chapter. The most conserved ZmLOX4 protein corresponding to GRMZM2G109056_T01 was used.

To test if LOX4 was conserved in A188 as in B73 we have sequenced the gene in one of the highest conserved region which corresponds to exon1; the sequencing started from intron1 to intron2. We have noticed that LOX4 in A188 has 2 indels of 70 bp in intron1 and 5 point mutations in exon1. We reported 4 transversion and 1 transition, from the 5' region the point mutations are: C in T, C in G, G in A, C in T and T in G. The alignment are shown in supplemental data (Figure SD.3) while the PCR product are presented in Figure 11.

The cDNA of LOX4 was added by attB1 and attB2 in order to perform LR Gateway reactions with plasmid L1781 in over-expression promoter for kernel development, LOX4 cDNA sequence is shown in supplemental data SD.1 and SD.2. The L1781 have been used to maize transformation at the same conditions adopted for CRISPR-Cas9 in ZmLOX6 and ZmWRKY125 transformations. The results obtained are reported in Table 3. For LOX4OE 1,015 embryos have been transformed, 6 transformation events and a total number of 11 plants were obtained and are still under investigation.

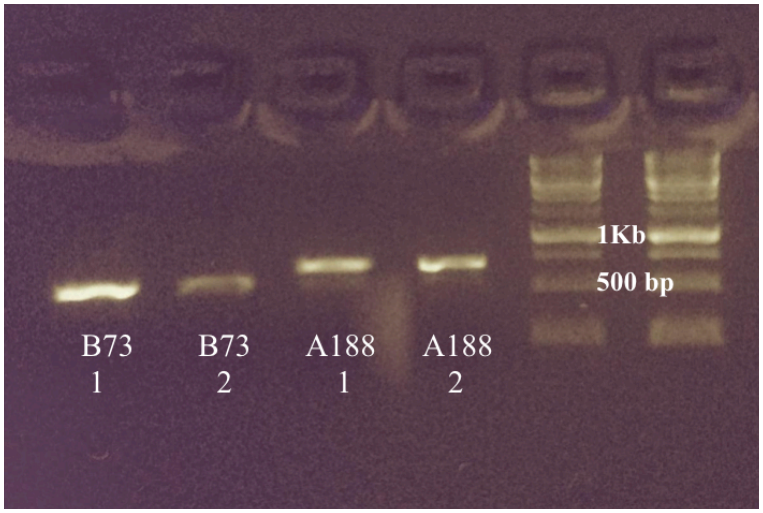


Figure 11. PCR product ZmLOX4 for 5'UTR. primers used
forL: gttttgtgtggtagggcttgcgtt
revT: tcctgagtaagaatactcacgtcg
B73 expected bands: bp 539. A188 shows slightly longer bands becouse of 2 indels of 70 bp. B73 and A188 PCR product have been sequenced, results shown in supplemental data.

LOX4OE-T0 PLANTS			
transformation event	PLANT NAME	DNA EXTRACTION	*LOX4*SEQ ANALYSIS
1	X451-1	V	V
1	X451-2	V	V
2	X452-1	V	V
3	X453-1	V	V
3	X453-2	V	V
3	X453-3	V	V
4	X454-1	V	V
4	X454-2	V	V
5	X455-1	V	V
6	X456-1	V	V
6	X456-2	V	V

Table 3. Summary of transformation events according LOX4OE. The transformation event corresponds to one callus growth led to one ore more regenerated plants.

Material and Methods

Cloning of the sgRNA A and sgRNA B in plasmid L1609 and L1611.

sgRNA A cloning started with sgRNA A oligos annealing. 1µM of oligo is used in a final volume of 20µL.

The annealing follows this program:

-94°C: 2 minutes;

-94°C: 30 seconds;

-(-1°C: 30 seconds up to 37 °C)

After the oligos annealing, the plasmids were digested with SapI for 1 hour at 37 °C.

50 ng ofL1609 SapI digestion and 2µL of sgRNA A (after annealing) were used for the ligation mix. The ligase used is active at 25°C for 5-10 minutes. The ligation mix is used to transform DH5α E.Coli competent cells by heat shock. The ligation is added to DH5α on ice for 10 minutes, then the ligation product is moved to 42°C for 2 minutes, finally it turns on ice for 2 minutes. A recovery step is required by adding 200µL of LB and 45 minutes at 37°C in agitation.

Agrobacterium transformation LBA4404: mobilization protocol

Transfer of the plasmid from E.coli to A.tumefaciens using a plasmid helper that takes the plasmid and passes it into the A. tumefaciens.

Day1: Plating LBA4404 from glycerol stocks. note: prepare agro plate with YT + rif + tetracycline. incubate at 29 ° C for 48h. if you have not transformed the plasmid to be cloned, turn it today and plunge it.

Day2: plate the HELPER on LB + KANA. HELPER code: RK2013 (RDP laboratory). Re-enter the plasmid of interest to have fresh for day 3. note: both HELPER and the plasmid of interest must be abundant. pick up a small amount of bacteria and plate on the loop on a new one, take the shape # add HELPER and plasmid of interest at 37 ° C

Day3: prepare a LB plate without antibiotic. Put the HELPER and the plasmid of interest in equal parts.incubate at 29 ° C for 48h.

Day5: plate the result of the day on two plates: YT + rif + antibiotic (SPECT) incubate at 29 ° C for 48h

Day7: island only 6 COLONIES and plate with YT + Rif + antibiotic (SPECT)

Day8: PCR of gene control: VIR, BAR, better also on plasmid of interest. incubate at 29 ° C for 24h (not 48h). Choose 1 or 2 colonies for growth of inoculum ON and then glycerol stock.

Maize transformation and calli cultivation

Preparation of immature embryos TIMING 35 min for handling, 90 days for growing plants

1.Grow maize plants in individual 270 mm pots in a greenhouse. Maintain daytime temperature between 30 and 35 1C and night time temperature between 20 and 25 1C. Ideally, the light intensity should be stronger than 60,000 lx and the photoperiod should be more than 12 h.

CRITICAL STEP The quality of the immature embryos is one of the most import factors for achieving highly efficient maize transformation. Good embryos are obtained only from vigorous plants grown in a well-conditioned greenhouse. Air-conditioning and supplemental lights are needed to ensure a supply of good embryos year-round. Usually, more than 150 kernels can be collected from a single ear of A188. Production of a much lower number of kernels on a single cob implies that the growth conditions are not optimal. If transformation efficiency is poor, the greenhouse conditions should be optimized before investigating other aspects of the protocol, such as types of vectors and strains, and media compositions.

2. Between 8 and 15 days after pollination (DAP), harvest an ear that contains immature embryos at the right developmental stage. CRITICAL STEP: The use of immature embryos at the right developmental stage is a critical factor, and the size of the embryos is a very good indicator of the stage. Immature embryos that are between 1.0 and 1.2 mm in length along the axis are optimal for transformation. Time (DAP) required for embryos to reach the best stage differs depending on the genotypes and the season. So carefully examine the sizes of embryos and determine the time of collection. A typical pattern of the growth of embryos is shown in Figure 2. For example, the time of collection for A188 was 8 DAP in August, 10 DAP in June and 15 DAP in January at our facility. As long as the sizes of embryos are in the above range, the frequency of transformation is reproducible year-round.

3. Husk the ear and detach kernels from the cob by cutting the base of the kernel with a scalpel. Insert a scalpel into the detached kernels and remove the immature embryos. If plants are grown in a greenhouse free from disease and pest, cobs in husks are clean and immature embryos can be removed without surface sterilization of the cobs.

4. Immerse the embryos in 2.0 ml of LS-inf medium in a 2.0 ml microcentrifuge tube at room temperature until the remaining embryos have been collected. Finish the collection of immature embryos within 1 h. More than 200 immature embryos can be collected in 1 h by a single, skilled technician.

6. Add 2.0 ml of LS-inf medium and vortex as in Step 5. Pre-treatment with heat and centrifuging TIMING 30 min 7| Incubate the embryos in the microcentrifuge tube in a water bath at 46 1C for 3

min. CRITICAL STEP The optimal conditions for this heat treatment will differ depending on genotype. The conditions described here work well for genotypes A188, H99 and A634. Since immature embryos of W117 were more sensitive to heat than these genotypes, lower temperature and/or shorter treatment may be suitable for successful transformation in W117. Optimization will be necessary to find the best conditions for other strains and can be assessed by co-culturing with a. In addition, it is also important to assess callus induction from heat-treated immature embryos.

8. Cool the microcentrifuge tube on ice for 1 min.

9. Remove the medium and add 2.0 ml of LS-inf medium.

10. Centrifuge the microcentrifuge tube with a fixed-angle rotor with a maximum radius of 83 mm at 20,000g at 4 1C for 10 min. CRITICAL STEP : the optimal centrifugation conditions will differ depending on genotype. The conditions described here work well for genotypes A188, A634, W117 and H99. Optimization will be necessary to find the best conditions for other strains and can be assessed by co-culturing with a strain of *A. tumefaciens* carrying an intron-GUS gene (Steps 11–13) and GUS staining at Step 18.

Preparation of inoculum TIMING 5 min for handling, 2 days for cultivation

11. Culture *A. tumefaciens* strain on a YP plate that contains appropriate antibiotics in the dark at 28 1C for 2 days.

12. Collect the bacteria with a loop and suspend in 1.0 ml of LS-inf-AS medium at a density of 1 10^9 colony-forming units per ml (OD 1⁄4 1.0 at 660 nm). Inoculum should be prepared fresh. Growth of *Agrobacterium* in liquid culture before transformation is not necessary.

Inoculation and co-cultivation TIMING 30 min for handling, 7 days for cultivation

13. Remove the medium from the microcentrifuge tube in Step 10 and add 1.0 ml of bacterial suspension from Step 12.

14. Vortex the microcentrifuge tube at 2,700 r.p.m. for 30 s. 15| Incubate for 5 min at room temperature.

16. Transfer the suspension of the embryos and bacteria to an empty Petri dish (60 15 mm).

17. Remove and discard 0.7 ml of the liquid from the suspension.

18. Transfer the embryos onto fresh LS-AS solid medium with the scutellum face up and seal the Petri dishes with Parafilm. Up to 200 embryos may be placed on a single plate. If the procedure is being optimized using strains that contain the intron-gus gene, transient expression of GUS can be analyzed at this point, as detailed.

19. Incubate in the dark at 25 1C for 7 days; this is the co-cultivation step.

Selection of transformed calli TIMING 140 min for handling, 52 days for cultivation

20. Transfer the embryos to LSD1.5A medium and seal the Petri dishes with surgical tape. Up to 25 embryos may be placed on a single plate. CRITICAL STEP Do not rinse the embryos. Rinsing with

an antibiotic solution tends to result in poor growth of cells. The surgical tape, which allows aeration, is much better for the growth of plant cells than air-tight tapes.

21. Incubate in the dark at 25 1C for 10 days; this is the first selection.

22. Transfer the embryos to LSD1.5B medium, and seal the Petri dishes with surgical tape. Up to 25 embryos may be placed on a single plate.

23. Incubate in the dark at 25 1C for 21 days; this is the second selection.

24. Cut type I calli proliferated from the scutellum into pieces of between 3 and 5 mm in diameter under a stereoscopic microscope (Fig. 4), transfer to LSD1.5B medium and seal the Petri dishes with the surgical tape. Up to 25 pieces may be placed on a single plate.

25. Incubate in the dark at 25 1C for 21 days; this is the third selection. Proliferated type I calli are transgenic.

Regeneration of transformed plants TIMING 240 min for handling, 28 days for cultivation, 100 days for growing plants

26. Cut the further proliferated type I calli from Step 25 into pieces of between 2 and 3 mm in diameter under a stereoscopic microscope, transfer to LSZ medium and seal the Petri dishes with Parafilm. Up to 25 pieces may be placed on a single plate.

27. Incubate under continuous illumination (5,000 lx) at 25 1C for 14 days.

28. Transfer a regenerated shoot to a tube of LSF medium, and cover with a polypropylene cap.

29. Incubate under continuous illumination (5,000 lx) at 25 1C for 14 days.

30|. Transfer each plant to a 230 mm pot containing appropriately supplemented soil (see REAGENT SETUP).

31. Grow transgenic plants in a greenhouse as detailed in Step 1 for 3–4 months and harvest progeny seeds. Plants in the following generations may be grown as detailed in Step 1.

Examination of progeny for expression of a selection marker gene TIMING 10 days for growing plants, 30 min for handling, 2–4 days for incubation, 10 min for examination

32. This examination is performed by the modified method of Wang and Waterhouse[36]. Sow individual progeny seeds of transgenic plants in soil in 40 mm 40 mm plastic pots and grow as detailed in Step 1. It is preferable that more than 30 seeds are sown and examined.

33. Excise a leaf segment of 10 mm in length from a 10-day-old seedling, insert 3 mm of the tip of the segment in ELA medium, and seal the Petri dish with Parafilm.

34. Incubate the plates under constant illumination (5,000 lx) at 25 1C.

35. Examine the segment for changes in the color 2, 3 and 4 days after the start of incubation. A resistant segment, which expresses the transgene, stays green whereas a sensitive segment (non-transgenic) turns yellow.

REAGENT SETUP

- 10X LS major salts Dissolve 19.0g KNO_3 , 16.5 g NH_4NO_3, 4.4 g $CaCl_2\ 2H_2O$, 3.7 g $MgSO_4\ 7H_2O$ and 1.7 g KH_2PO_4 in 900 ml distilled water and make up the volume to 1,000 ml (see ref. 35). Store at 4 1C. The final concentrations of components in this solution are 188 mM KNO_3, 206 mM NH_4NO_3, 30 mM $CaCl_2\ 2H_2O$, 15 mM $MgSO_4\ 7H_2O$ and 12.5 mM KH_2PO_4

- 100X FeEDTA Dissolve 2.78 g $FeSO_4\ 7H_2O$ in 900 ml of hot distilled water and add 3.73 g ethylenediamine-N,N,N¢,N¢-tetraaceticacid, disodium salt (Na_2EDTA). Cool and make up the volume to 1,000 ml. Store at 4 1C. The final concentrations of components in this solution are 10 mM $FeSO_4\ 7H_2O$ and 10 mM Na_2EDTA.

- 100X LS minor salts Dissolve 2.23 g $MnSO_4\ 5H_2O$, 1.06 g $ZnSO_4\ 7H_2O$, 620 mg H_3BO_3, 83 mg KI, 25.0 mg $Na_2MoO_4\ 2H_2O$, 2.5 mg $CuSO_4\ 5H_2O$ and 2.5 mg $CoCl_2\ 6H_2O$ in 900 ml of distilled water and make up the volume to 1,000 ml. Store at 4 1C. The final concentrations of components in this solution are 9.3 mM $MnSO_4\ 5H_2O$, 3.7 mM $ZnSO_4\ 7H_2O$, 10 mM H_3BO_3, 0.5 mM KI, 0.1 mM $Na_2MoO_4\ 2H_2O$, 0.01 mM $CuSO_4\ 5H_2O$ and 0.01 mM $CoCl_2\ 6H_2O$.

- 100X modified LS vitamins Dissolve 10 g myoinositol, 100 mg thiamine hydrochloride, 50 mg pyridoxine hydrochloride and 50 mg nicotinic acid in 900 ml of distilled water and make up the volume to 1,000 ml. Store at 4 1C. The final concentrations of components in this solution are 55.5 mM myoinositol, 0.30 mM thiamine hydrochloride, 0.24 mM pyridoxine hydrochloride and 0.41 mM nicotinic acid. 100 mg liter ^{1} 2,4-D Add 1 N NaOH drop-wise to 100 mg 2,4-D until completely dissolved. Make up to 1,000 ml with distilled water. Store at 4 1C. The final concentration of 2,4-D in this solution is 0.45 mM.

- 100 mg liter ^{1} 2,4-D Add 1 N NaOH drop-wise to 100 mg 2,4-D until completely dissolved. Make up to 1,000 ml with distilled water. Store at 4 1C. The final concentration of 2,4-D in this solution is 0.45 mM.

- 100 mg liter zeatin Add 1 N NaOH drop-wise to 100 mg zeatin until completely dissolved. Make up to 1,000 ml with distilled water. Store at 4 1C. The final concentration of zeatin in this solution is 0.46 mM.

- 100 mg liter ^{1} IBA Add 1 N NaOH drop-wise to 100 mg IBA until completely dissolved. Make up

to 1,000 ml with distilled water. Store at 4 1C. The final concentration of IBA in this solution is 0.49 mM.

- 100 mg liter ^{1} 6BA Add 1 N NaOH drop-wise to 100 mg 6BA until completely dissolved. Make up to 1,000 ml with distilled water. Store at 4 1C. The final concentration of 6BA in this solution is

0.44 mM.

- 100 mM acetosyringone Dissolve 392.4 mg acetosyringone in 10 ml of dimethyl sulfoxide and dilute with 10 ml distilled water. Filter-sterilize and store in the dark at 41°C.

- 100 mM X-gluc Dissolve 52 mg X-gluc (Sigma B6650) in 1 ml of ethylene glycol monomethyl ether (Sigma E5378). Store in the dark at 20 °C. Ethylene glycol monomethyl ether is toxic. Wear suitable protective clothing, gloves and eye/face protection.

- 50 mM Na_2HPO_4 Dissolve 17.91 g Na_2HPO_4 $12H_2O$ in 900 ml of distilled water and make up to 1,000 ml.

- 50 mM NaH_2PO_4 Dissolve 7.8 g NaH_2PO_4 $2H_2O$ in 900 ml of distilled water and make up to 1,000 ml.

- YP plate (for A. tumefaciens) Dissolve 5 g yeast extract, 10 g peptone and 5 g sodium chloride in 900 ml of distilled water and adjust pH to 6.8 with NaOH. Make up to 1,000 ml and add 15 g agar (Difco). Autoclave at 121 1C for 15 min. Cool the medium to 50 1C, add appropriate antibiotics, which depend on the type of plasmid(s) in the strain, and pour 20 ml aliquots into Petri dishes (90 15 mm). LS-inf medium (for preparation of immature embryos) Add 100 ml of 10 LS major salts, 10 ml of 100 FeEDTA, 10 ml of 100 LS minor salts, 10 ml of 100 modified LS vitamins and 15 ml of 100 mg liter ^{1} 2,4-D (final concentration is 1.5 mg liter ^{1}) to 700 ml of distilled water. Dissolve 68.46 g sucrose, 36.04 g glucose and 1.0 g Casamino acids in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.2 and sterilize with a 0.22 mm cellulose-acetate filter.

- LS-inf-AS medium (for infection) Add 1 ml of 100 mM acetosyringone to 1 ml of LS-inf medium. LS-AS medium (for co-cultivation) Add 100 ml of 10 LS major salts, 10 ml of 100 FeEDTA, 10 ml of 100 LS minor salts, 10 ml of 100 modified LS vitamins, 15 ml of 100 mg liter ^{1} 2,4-D (final concentration is 1.5 mg liter ^{1}) and 0.05 ml of 100 mM $CuSO_4$ to 700 ml of distilled water. Dissolve 20 g sucrose, 10 g glucose, 0.7 g proline and 0.5 g MES in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agarose. Autoclave and cool to 50 1C, and add 1 ml of 100 mM acetosyringone and 0.05 ml of 100 mM $AgNO_3$ and pour 30 ml aliquots into Petri dishes (90 20 mm). Store in the dark at room temperature (20–25 1C).

- LSD1.5A medium (for first selection of transformed cells) Add 100 ml of 10 LS major salts, 10 ml of 100 FeEDTA, 10 ml of 100 LS minor salts, 10 ml of 100 modified LS vitamins and 15 ml of 100 mg liter ^{1} 2,4-D (final concentration is 1.5 mg liter ^{1}) to 700 ml of distilled water. Dissolve 20 g sucrose, 0.7 g proline and 0.5 g MES in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agar (Sigma, A6013-500G). Autoclave at 121 1C for 15 min. Cool to 50 1C and add 1 ml of 250 g liter ^{1} carbenicillin (final concentration is 250 mg liter ^{1}), 0.4 ml of 250 g liter ^{1} cefotaxime (final concentration is 100 mg liter ^{1}), 0.1 ml of 100 mM $AgNO_3$ and either 0.25 ml of

20 g liter [1] phosphinothricin(final concentration is 5 mg liter [1]) for bar selection or 0.3 ml of 50 g liter [1] hygromycin (final concentration is 15 mg liter [1]) for hpt selection. Pour 30 ml aliquots into Petri dishes (90 20 mm) and store in the dark at room temperature.

- LSD1.5B medium (for second and third selection of transformed cells) This is identical to LSD1.5A medium except for the amount of selective agent. Add 0.5 ml of 20 g liter [1] phosphinothricin (final concentration is 10 mg liter [1]) instead of 0.25 ml or 0.6 ml of 50 g liter [1] hygromycin (final concentration is 30 mg liter [1]) instead of 0.3 ml in this case. LSZ medium (for regeneration of transformed plants) Add 100 ml of 10 LS major salts, 10 ml of 100 FeEDTA, 10 ml of 100 LS minor salts, 10 ml of 100 modified LS vitamins, 50 ml of 100 mg liter [1] zeatin (final concentration is 5 mg liter [1]) and 0.1 ml of 100 mM $CuSO_4$ to 700 ml of distilled water. Dissolve 20 g sucrose and 0.5 g MES in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agar (Sigma, A6013-500G). Autoclave at 121 1C for 15 min. Cool to 50 1C, and add 1 ml of 250 g liter [1] carbenicillin (final concentration is 250 mg liter [1]), 0.4 ml of 250 g liter [1] cefotaxime (final concentration is 100 mg liter [1]) and either 0.25 ml of 20 g liter [1] phosphinothricin (final concentration is 5 mg liter [1]) for bar selection or 0.6 ml of 50 g liter [1] hygromycin (final concentration is 30 mg liter [1]) for hpt selection. Pour 30 ml aliquots into Petri dishes (90 20 mm) and store in the dark at room temperature.

- LSF medium (for rooting of transformed plants) Add 100 ml of 10 LS major salts, 10 ml of 100 FeEDTA, 10 ml of 100 LS minor salts, 10 ml of 100 modified LS vitamins and 2 ml of 100 mg liter [1] IBA (final concentration in 0.2 mg liter [1] to 700 ml of distilled water. Dissolve 15 g sucrose and 0.5 g MES in the mixture and make up the volume to 1,000 ml and adjust pH to 5.8. Add 3 g gellan gum and warm to 90 1C to dissolve. Pour 10 ml aliquots to glass test tubes (25 mm in diameter 100 mm in height). Cover the tubes with polypropylene caps and autoclave at 121 1C for 15 min. Store at room temperature.

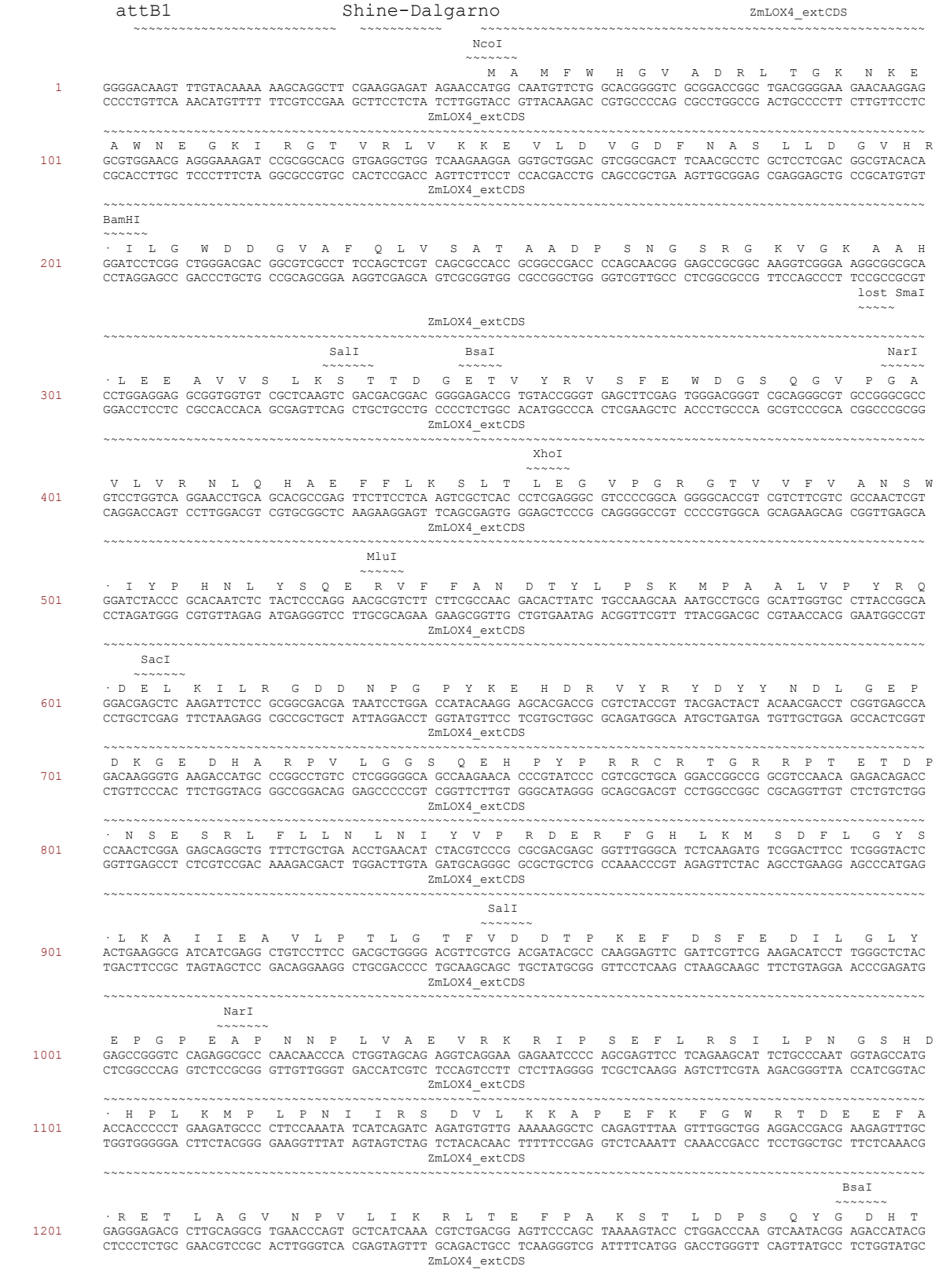
- ELA medium (for detached leaf analysis) Add 100 ml of 10 LS major salts, 10 ml of 100 FeEDTA, 10 ml of 100 LS minor salts and 5 ml of 100 mg liter [1] 6BA (final concentration is 0.5 mg liter [1]) to 700 ml of distilled water. Dissolve 0.5 g MES in the mixture and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agar (Sigma, A6013-500G). Autoclave at 121 1C for 15 min. Cool to 50 1C and add either 0.1 ml of Basta (Bayer Crop Science) for analysis of expression of bar gene or 2 ml of 50 mg ml [1] (final concentration is 100 mg liter [1]) hygromycin for analysis of expression of hpt gene. Pour 30 ml aliquots into Petri dishes (90 20 mm) and store in the dark at room temperature.

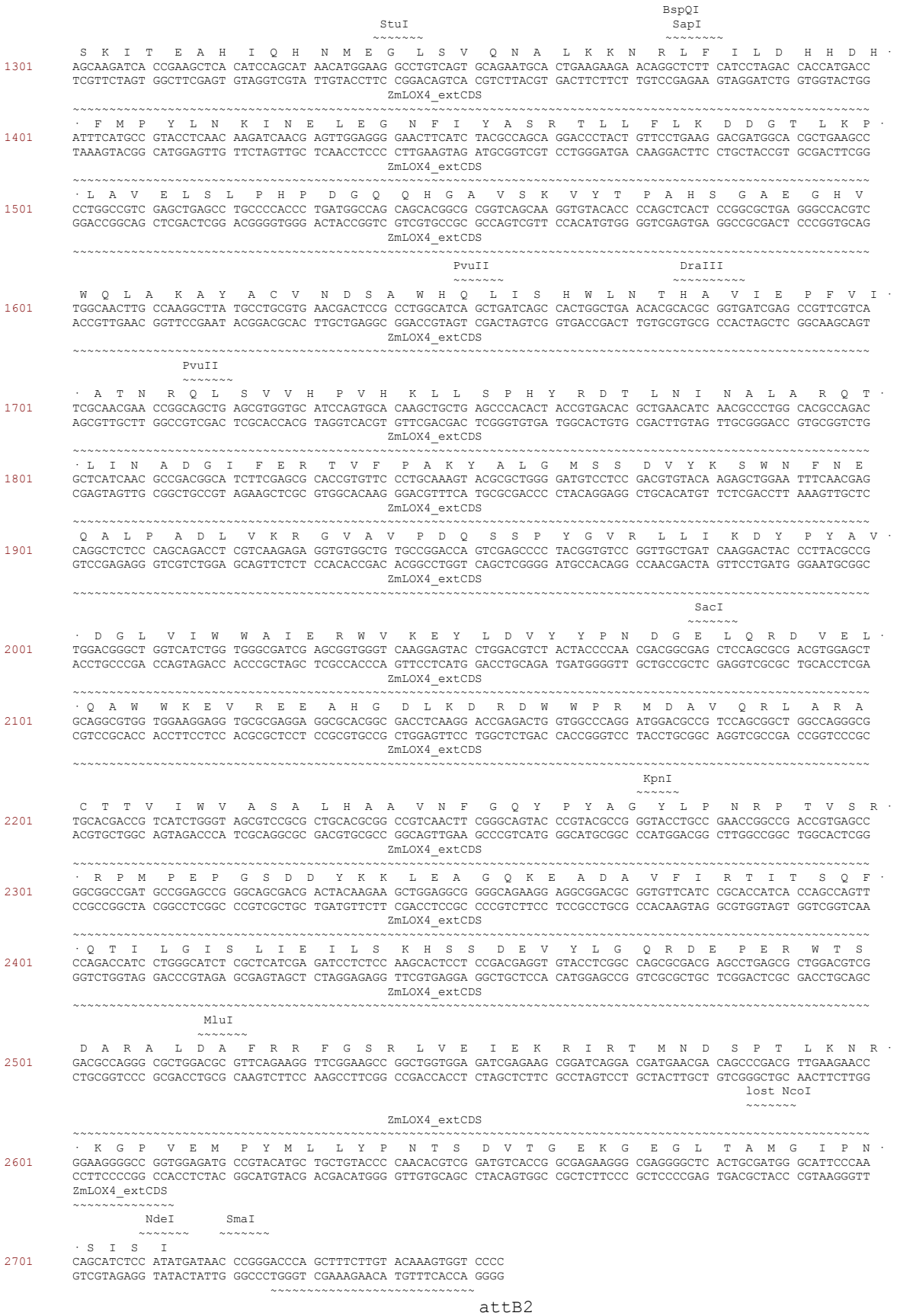
- Soil for pots A soil mixture for horticultural use that is commercially available and well drained is usually good. Adjust the major nutrients to 0.4 g N per liter, 0.4 g P per liter and 0.4 g K per liter by adding a commercial fertilizer.

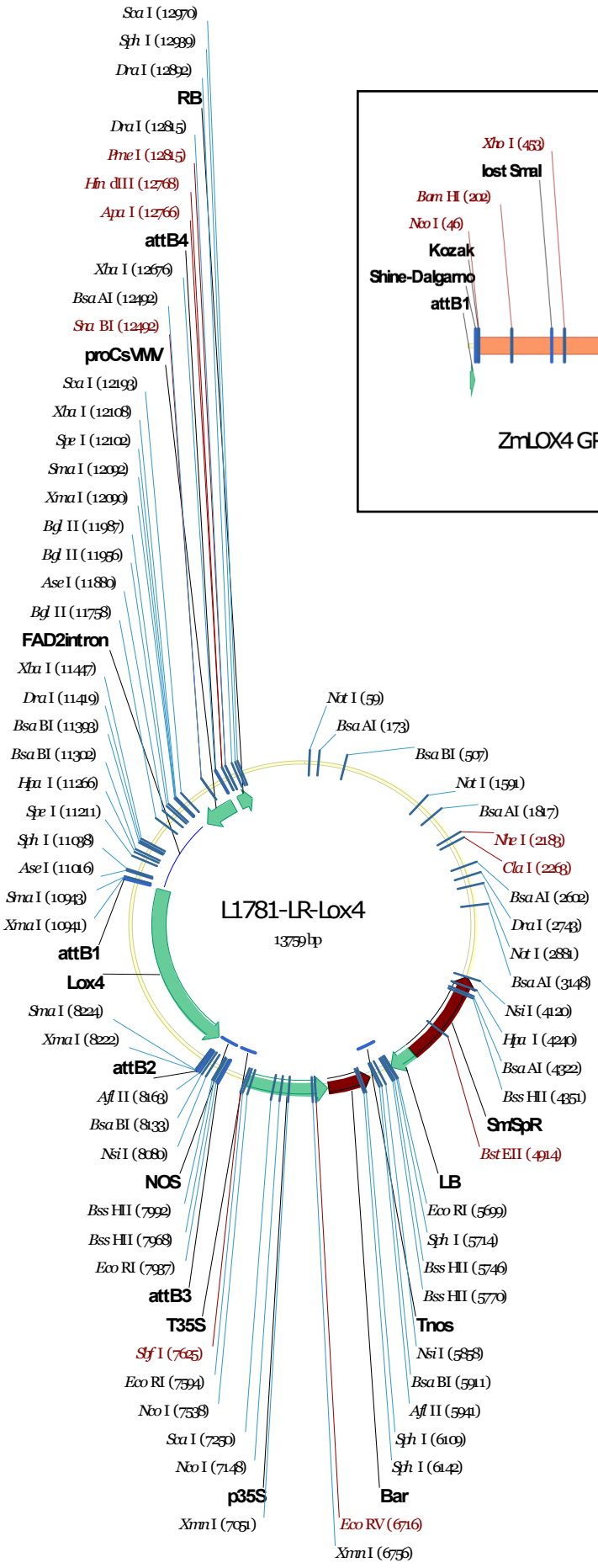
- Buffer P Add 50 mM Na_2HPO_4 (about 500 ml) to 1,000 ml of 50 mM NaH_2PO_4 until the pH reaches 6.8. Sterilize using a 0.22 mm cellulose-acetate filter and store at room temperature. Mix 9.9 ml of this buffer and 0.1 ml of Triton X-100 before use.
- Buffer X Mix 8 ml of buffer P, 0.1 ml of 100 mM X-gluc and 2 ml methanol just before use.

Supplemental data

SD.1 Sequence of LOX4 cDNA:




BspQI
StuI
SapI
PvuII
DraIII
PvuII
SacI
KpnI
MluI
lost NcoI
ZmLOX4_extCDS
NdeI
SmaI
attB2



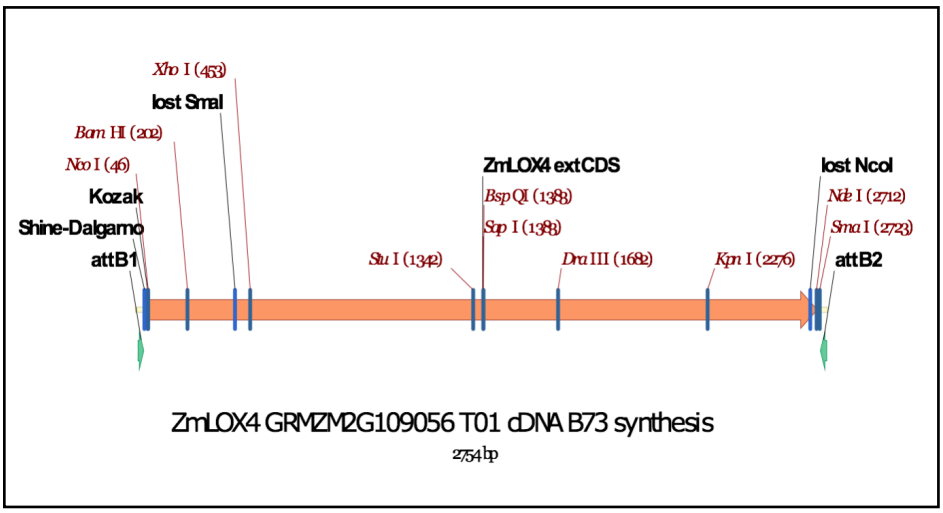


Figure SD.2. A: L1781-LR-LOX4 plasmid map details. B. LOX4 cDNA map with modified features as the following restriction sites loss: NcoI, SmaI. attB1 and attB2 were added in order to perform LR Gateway reactions with plasmid L1781.

Figure SD.3.1 LOX4 in B73, B73 and A188 alignments, indel and point mutations. ZmLOX4 of B73 genome. Blue: exon 1 and exon 2; red: ATG; orange and green: primer sequences. The black square corresponds to the pcr products sequenced for LOX4 analysis in A188.

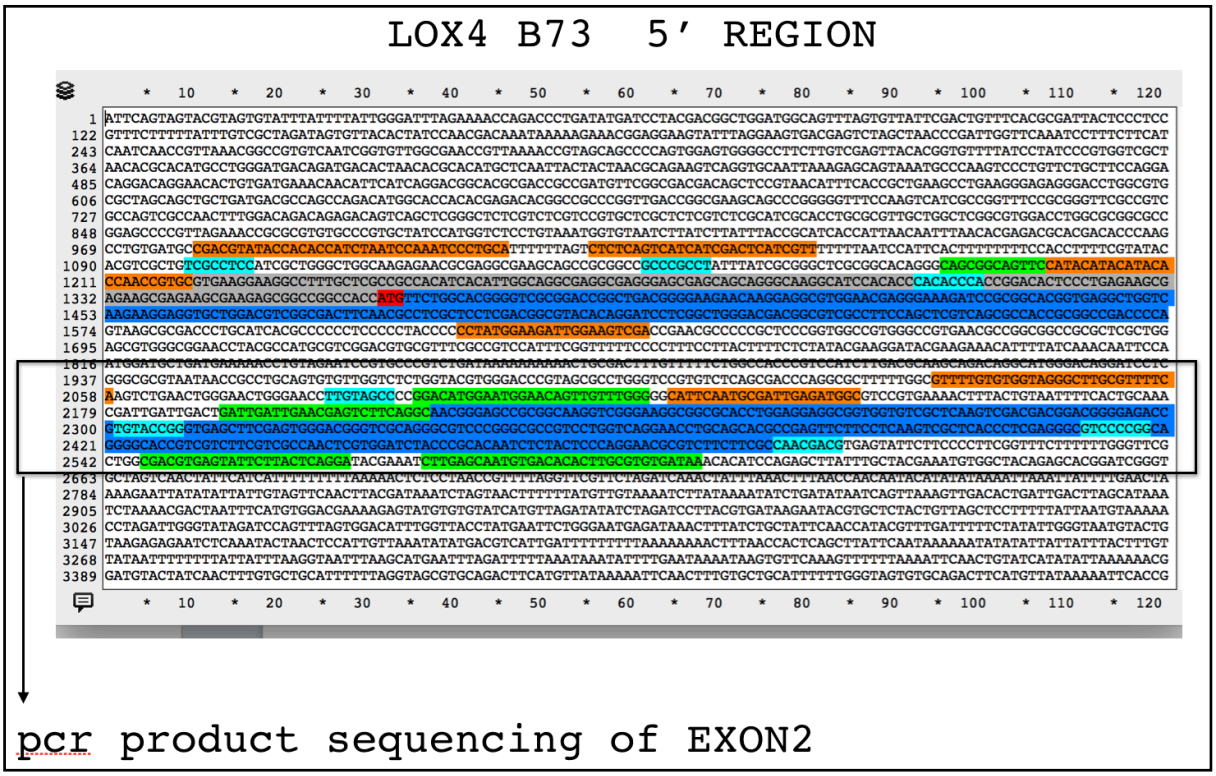
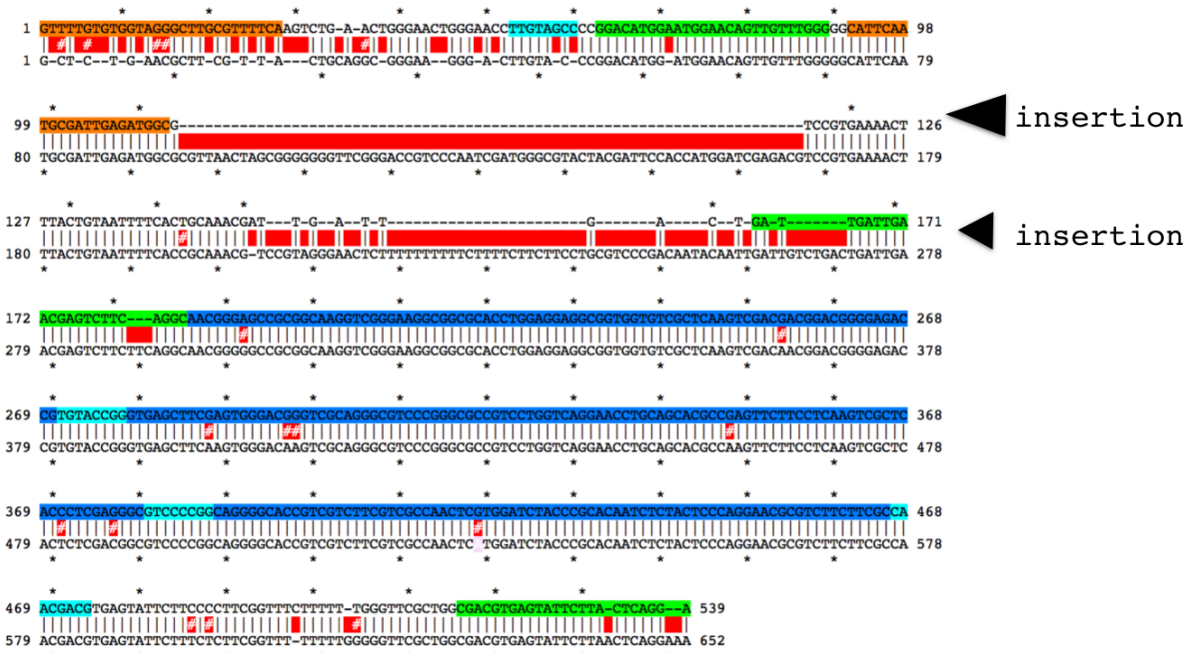


Figure SD.3.2 *ZmLOX4* blast between B73 (top) and A188 genome (bottom). blue: exon 1 and exon 2; red: ATG; orange and green: primer sequences. Two intel of 70bp are evidenced in the first intron and in the second exon, mismatches are reported in red square. Two indels (black triangles) sequenced in A188.



SD.4 LOX4 sequence in A188 from intron 1 to intron 2.

TGGTCTTTCGTGCTACTCATCACTCGGTATATCCGCCTGCAGTGTGTTGGTCGCGTGGTA
CGTGGGACCCGCAGCGCCTAGGTCCGTGTCTCAGCGACCCAGGAGCTTTTTGGCGTTTT
GCGTGGCAGGCTTGCGTTTTCAAGTCTGAACTGGGAACCGGGAACTGGGAACCTTGTA
GCCCCGGACATGGAATGGAACAGTTGTTTGGGGGCATTCAATGCGATTGAGATGGCGCG
TTAACTAGCGGGGGGGTTCGGGACCGTCCCAATCGATGGGCGTACTACGATTCCACCAT
GGATAGAGACGTCCGTGAAAACTTTACTGTAATTTTCACCGCAAACGTCCGTAGGGAAC
TCTTTTTTTTTTCTTTTCTTCTTCCTGCGTCCCGACAATACGATTGATTGTCTGACTGATTG
AACGAGTCTTCTTCAGGCAACGGGGGCCGCGGCAAGGTCGGGAAGGCGGCGCACCTG
GAGGAGGCGGTGGTGTCGCTCAAGTCGACGACGGACGGGGAGACCGTGTACCGGGTG
AGCTTCGAGTGGGACGAGTCGCAGGGCGTCCCGGGCGCCGTCCTGGTCAGGAACCTGC
AGCACGCCGAGTTCTTCCTCAAGTCGCTCACTCTCGACGGCGTCCCCGGCAGGGGCAC
CGTCGTCTTCGTCGCCAACTCATGGATCTACCCGCACAATCTCTACTCCCAGGAACGCGT
CTTCTTCGCCAACGACGTGAGTATTCTTTCTCTTCGGTTTTTTTTGGGGGTTCGCTGGCG
ACGTGAGTATTCTTACTCAGTATACGAAGTCTTGAGCAGTGTGACACACTTGCGTGTGAT
AAACACATCCATAGCTTATTTGCTACGAAATGTGGCTACAGACACGACGGGGGGTATTTT
AAAATTTTTTTAAAGGGGAAAAAAAAAAAAAAAAAAAAAAGGGG

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Single and multiple gene knockout by CRISPR-Cas9 in maize

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Abstract:	CRISPR-Cas9 technology is a simple and efficient tool for targeted mutagenesis of the genome. It has been implemented in many plant species, including crops such as maize. Here we report single and multiple gene mutagenesis via stably transformed maize plants using both our newly developed CRISPR-Cas9 vector and a previously published gateway-based system. Both systems allow the expression of multiple guide RNAs and different strategies were employed to knock out either independent or paralogous genes. A total of 12 plasmids, representing 28 different single guide RNAs (sgRNAs), were generated in order to target 20 genes. For 18 of these genes, at least one mutant allele was obtained, while two genes were recalcitrant to sequence editing. 19% (16/83) of mutant plants showed biallelic mutations. Small insertions or deletions of less than 10 nucleotides were most frequently observed, regardless of whether the gene was targeted by one or more sgRNAs. Deletions of defined regions located between the target sites of two guide RNAs were also reported although the exact deletion size was variable. Lastly, double and triple mutants were created in a single step, which is especially valuable for functional analysis of genes with strong genetic linkage.
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Author Comments:	Dear Plant Cell Reports editors, Please find attached our manuscript entitled “Single and multiple gene knockout by CRISPR-Cas9 in maize" by Nicolas M. Doll, Laurine M Gilles, Marie-France Gérentes, Christelle Richard, Jeremy Just, Virginia M. G. Borrelli, Ghislaine Gendrot, Gwyneth C. Ingram, Peter M. Rogowsky and Thomas Widiez. We would like to submit this manuscript to the special issue of the journal Plant Cell Reports on "Precision genetic engineering tools for next generation plant breeding" that was commissioned by Laurence Tomlinson and Fabien Nogué earlier this year. In this manuscript we detail our approach for multiplexed CRISPR-Cas9 genome editing in maize. Firstly we introduced our cloning system. We then demonstrate the success of this strategy by the editing of 18 genes out of 20 targeted and present the different cases of the 93 edited alleles we obtained. The mutagenesis efficiency, the type of mutations obtained, the simultaneous knockout of tightly genetically linked genes and the rate of transmission to the next generation are addressed. Finally, we position our results in the context of our current knowledge on plant gene editing and discuss their limitations and advantages. My collaborators and I are looking forward to hearing back from you. Yours sincerely, Thomas Widiez
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Single and multiple gene knockout by CRISPR-Cas9 in maize

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KEY MESSAGE

The analysis of 93 mutant alleles in 18 genes demonstrated that CRISPR-Cas9 is a robust tool for targeted mutagenesis in maize, permitting efficient generation of single and multiple knockouts.

ABSTRACT

CRISPR-Cas9 technology is a simple and efficient tool for targeted mutagenesis of the genome. It has been implemented in many plant species, including crops such as maize. Here we report single and multiple gene mutagenesis via stably transformed maize plants using both our newly developed CRISPR-Cas9 vector and a previously published gateway-based system. Both systems allow the expression of multiple guide RNAs and different strategies were employed to knock out either independent or paralogous genes. A total of 12 plasmids, representing 28 different single guide RNAs (sgRNAs), were generated in order to target 20 genes. For 18 of these genes, at least one mutant allele was obtained, while two genes were recalcitrant to sequence editing. 19% (16/83) of mutant plants showed biallelic mutations. Small insertions or deletions of less than 10 nucleotides were most frequently observed, regardless of whether the gene was targeted by one or more sgRNAs. Deletions of defined regions located between the target sites of two guide RNAs were also reported although the exact deletion size was variable. Lastly, double and triple mutants were created in a single step, which is especially valuable for functional analysis of genes with strong genetic linkage.

KEYWORDS (4 to 6 keywords)

CRISPR, gene editing, maize, SDN1, mutagenesis, *Zea mays*

Abbreviations

bp: Base pairs

Cas9: CRISPR associated protein 9

CRISPR: Clustered regularly interspaced short palindromic repeats

DSB: Double strand break

EMS: Ethyl methanesulfonate

ESR: Embryo surrounding region

HR: Homologous recombination

MMEJ: Microhomology mediated end joining

NHEJ: Non homologous end joining

PAM: Protospacer adjacent motif

SDN1: Site directed nuclease 1

sgRNA: Single guide RNA

shRNA: Short hairpin RNA, also referring to scaffold RNA

T-DNA: Transfer DNA

Introduction

Maize plant has a dual role, being a major crop species and a model species in genetics. Genome edited *waxy* maize characterized by modified starch composed entirely of amylopectin was one of the first crops edited using CRISPR-Cas9 technology that obtained clearance to be cultivated and sold without GM-type oversight by the US Department of Agriculture (Waltz 2016). This example illustrates the intense interest in the potential of CRISPR-Cas9 technology for both applied and fundamental research. The starch industry has appreciated *waxy* maize for decades, because the absence of amylose makes starch easier to process. Although the *waxy* trait is not novel, CRISPR-Cas9 technology allowed the direct creation of *waxy* deletions in elite lines over one or two generations, avoiding time consuming backcrosses and genetic drag experienced with conventional introgression (Cigan et al. 2017).

In fundamental research, understanding the contribution of genes to phenotypic traits in maize has been a challenge for many decades. By comparing a standard (wild-type) to a mutant, the contribution of a genetic sequence to a biological process can be assessed. Although a large number of natural maize mutants exist, increasing their diversity through mutagenesis has been a long term goal (Candela and Hake 2008). For example, the original *waxy* mutation discovered in 1909 (Collins 1909) has been joined over time by hundreds of additional alleles reflecting emerging mutagenesis tools. In the sixties, mutagenesis induced by the chemical agent ethyl methanesulfonate (EMS) was popular and allowed the generation of an allelic series at the *Waxy* locus with different levels of residual amylose (Briggs et al. 1965). A few years later irradiation mutagenesis, which preferentially creates deletions rather than point mutations helped to generate true loss-of-function mutants that are more informative for functional genetics (Amano 1968). With the advent of molecular biology, transposon mutagenesis was developed, since transposon insertions could be easily localized in the genome. The cloning of the *Waxy* gene by transposon tagging was a prime example for the success of this strategy (Shure et al. 1983). Lately, over the past few years CRISPR-Cas9 technology has emerged as an appreciated alternative to sequenced indexed mutant collections (Settles et al. 2007; Vollbrecht et al. 2010), mainly because these collections do not saturate the maize genome, and because CRISPR-Cas9 technology can be targeted.

In contrast to random mutagenesis tools, which require the molecular screening of large mutagenized populations to find a mutation in a given gene, targeted mutagenesis of a gene gives ready access to a specific mutant and its phenotype, but has been a major challenge. It has been made possible by the development of techniques inducing double strand breaks (DSB) of genomic DNA at a predetermined site (Puchta and Fauser 2014). DSB are then repaired by one of several cellular repair mechanisms that can be non-conservative and can

therefore lead to mutations at the desired location. The most frequent repair process in plants is Non Homologous End Joining (NHEJ), during which a DNA ligase joins the damaged strands, and which can be classified as either classical NHEJ or alternative NHEJ, also known as Microhomology Mediated End Joining (MMEJ) (Lieber 2010). Classical NHEJ primarily induces the insertion or deletion of a low number of nucleotides, whereas MMEJ generally leads to larger deletions (McVey and Lee 2008; Puchta and Fauser 2014). DSB can also be repaired by homologous recombination (HR), which can also be classified into two classes: conservative and non-conservative. Non-conservative HR, called single strand annealing, occurs if a repeated sequence of more than 30 nucleotides is present upstream and downstream of the DSB (McVey and Lee 2008). The presence of these repeated sequences renders single strand annealing very efficient for mutagenesis, as it repairs up to 1/3 of the DSB and can generate large deletions (Siebert and Puchta 2002; Steinert et al. 2016). Conservative HR is of particular interest because it can be used for the replacement or the insertion of a sequence of interest, present on an extra chromosome, at the desired genomic locus. However, although the presence of DSB increases the efficiency of conservative HR, this remains two orders of magnitude lower than that of NHEJ (Steinert et al. 2016).

Inducing a DSB at a predetermined site in the genome requires both the recognition of the target sequence and the cleavage of the DNA, hitherto achieved using endonucleases. Several technologies have been developed to direct endonucleases to sequences of interest, either by engineering the DNA binding domains of naturally occurring meganucleases (Choulika et al. 1994) or by linking modular DNA-binding domains such as zinc finger (Bibikova et al. 2003; Porteus and Carroll 2005; Shukla et al. 2009) or Transcription Activator-Like Effector (TALE) domains (Christian et al. 2010) to endonuclease domains such as FokI. All three technologies have been successfully implemented in maize (Bibikova et al. 2003; Porteus and Carroll 2005; Shukla et al. 2009; Gao et al. 2010; Liang et al. 2014; Char et al. 2015). In 2013, the adaptation of the bacterial immune system CRISPR-Cas9 of *Streptococcus pyogenes* offered a novel type of technology in which the recognition of the DNA was not due to a protein domain but to a short guide RNA (sgRNA) that forms an active complex with the Cas9 protein (Jinek et al. 2012; Cong et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). The sgRNA is composed of 20 nucleotides which are homologous to the genomic region targeted, followed by a short hairpin RNA (*shRNA*), also referring to scaffold RNA. Within the genome the 20 targeted nucleotides should be followed by a protospacer adjacent motif (PAM) composed of the nucleotides NGG. DSB induced by Cas9 are generally located three base pairs upstream of the PAM site. The ease of design and low cost explain the rapid success of this user friendly and efficient technology in a wide range of organisms including plants.

In the last five years, CRISPR-Cas9 technology has been successfully adapted to maize. For the introduction of the CRISPR-Cas9 machinery, direct DNA transfer to protoplasts (Liang et al. 2014; Xing et al. 2014), particle bombardment of immature embryos (Xing et al. 2014; Svitashev et al. 2015; Feng et al. 2016; Zhu et al. 2016) and *Agrobacterium*-mediated transformation of immature embryos (Xing et al. 2014; Svitashev et al. 2015; Feng et al. 2016; Zhu et al. 2016; Char et al. 2017) have been used. Protoplast experiments serve mainly for the evaluation of the efficiency of different sgRNA designs, since there is presently no protocol for the regeneration of maize plants from protoplasts. Biolistics avoid the use of *Agrobacterium*, which is regulated in certain countries since it is a plant pathogen. *Agrobacterium*-based stable transformation and subsequent elimination of the CRISPR-Cas9 casette by backcross nonetheless remains the most widely used method. The transfer is almost exclusively based on DNA molecules encoding *Cas9* and the sgRNA but the bombardment of Cas9 expressing plants with sgRNA (Xing et al. 2014; Svitashev et al. 2015; Feng et al. 2016; Zhu et al. 2016) and of wild-type plants with pre-assembled Cas9–sgRNA ribonucleoproteins (RNP), have also been reported (Svitashev et al. 2016). Multiplexing with more than one guide RNA in a single construct is of particular interest in maize due to the lengthy and not very efficient transformation protocol. Two techniques have been developed: one based on a multi-guide RNA activated by a single promoter and processed by tRNA motif-mediated self-cleavage into several sgRNAs, and another based on tandem repeats of different U3 and U6 promoters each controlling one guide RNA (Qi et al. 2016; Char et al. 2017). As expected, the mutations resulting from targeted mutagenesis were mainly deletions or insertions of a few nucleotides probably due to classical NHEJ. Larger deletions of more than 10 bases, potentially resulting from an MMEJ repair, have also been reported but are less frequent (Xing et al. 2014; Svitashev et al. 2015; Feng et al. 2016; Zhu et al. 2016). Furthermore, true genome editing, *i.e.* the predetermined modification of an allele based on a repair matrix carrying the desired mutation by HR (Xing et al. 2014; Svitashev et al. 2015; Feng et al. 2016; Zhu et al. 2016) and the replacement of a promoter has also been achieved in maize (Svitashev et al. 2015; Shi et al. 2017).

Here we describe the CRISPR-Cas9-based mutagenesis of 20 maize genes selected for their putative implication in maize kernel development. The mutagenesis efficiency, the type of mutations obtained, the simultaneous knockout of tightly genetically linked genes and the rate of transmission to the next generation will be addressed.

Results

Multi-sgRNA plasmids for single and multiple gene editing

In order to carry out single or multiple gene mutagenesis using CRISPR-Cas9 technology in maize, two types of vectors were used. The first type was designed in-house and will be named RDP vectors hereafter (**Fig. 1**). The final construct typically contains two guide RNAs and is built by combining derivatives of the initial plasmids L1609 and L1611 (**Fig. 1**) by restriction and ligation. L1609 is a binary vector containing a T-DNA suitable for *Agrobacterium*-mediated maize transformation, which encompasses a plant selection marker conferring resistance to the Basta® herbicide and a *Cas9* coding sequence driven by the maize ubiquitin promoter, which is active in most plant tissues (Christensen and Quail 1996). The specific 20-nt sequence that will hybridize with the target site in the genome and thus guide the Cas9 complex to the gene(s) of interest, is inserted between the *Oryza sativa* U3 (*OsU3*) promoter and the *shRNA* (**Fig. 1**). The other initial plasmid L1611 allows the cloning of a second 20-nt targeting sequence between the *TaU6* (*Triticum aestivum U6*) promoter and a *shRNA*. The sub-cloning of this *TaU6::sgRNA* cassette into the modified L1609 plasmid leads to the generation of the final RDP vector with two sgRNAs (**Fig. 1**). The second type of CRISPR-Cas9 vector used was derived from the Gateway® compatible plasmid pGW-CAS9 developed by Iowa State University (Char et al. 2017) and will be referred to as Iowa vectors hereafter. Two to four sgRNA cassettes flanked by attL sites were entirely synthetized prior to recombination into pGW-CAS9.

Both the RDP and Iowa vectors used in this study contain multi guide RNAs, allowing the targeting of several genes with a single construct. We also designed multi-target 20 nt sequences, targeting up to 10 loci in the genome with one sgRNA, allowing for example to target paralogous genes (**Online Resource 1**). In our functional genetics approaches, we targeted the coding sequence to increase the likelihood of generating loss-of-function mutations. Four main strategies for sgRNA design were employed to achieve different types of gene knock-out(s) (KO) (**Fig. 2**): (1) targeting two unique, non-related genes with a single guide RNA each, (2) targeting a unique gene with two guide RNAs, (3) targeting paralogs with a single or multiple guide RNAs and (4) targeting a unique gene with four guide RNAs (**Fig. 2**).

Different types of mutations are created using multi guide RNAs strategies

A total of 20 genes were targeted with different RDP or Iowa vectors (**Table 1**). After stable transformation of maize immature embryos, DNA was extracted from young leaves of transgenic T0 plants to assess the type and frequency of the mutations generated. Based on

PCR amplification of the target site, and subsequent Sanger sequencing, at least one mutant allele was obtained for 18 of the 20 genes. All edited alleles are summarized in **Table 1**.

For genes targeted by a single guide RNA (strategies 1 and 3 in **Fig. 2**), a total of 56 mutations were generated in 13 genes (top section of **Table 1**). With the exception of one guide RNA targeting GRMZM2G352274, all other guide RNAs gave rise to new alleles, ranging from one to 12 different alleles (in the case of GRMZM2G089517). In this context it should be noted that the number of alleles does not reflect mutation efficiency, since transformation rates varied over time and not all transformation events were carried to the plantlet stage, and also because identical mutations could be generated independently in different plants. The mutations generated were predominantly (82%, 46/56) small indels, defined as short (<10 bp) insertions or deletions or mixtures of both (**Table 1**). As expected the vast majority of these indels occurred 3 bp upstream of the PAM sequence, the position where the Cas9 nuclease cleaves double stranded DNA (Zuo and Liu 2016). Less frequently (14%, 8/56), larger deletions (>10 bp) were observed, the largest one observed reaching 136 bp (**Table 1**). Interestingly, the majority of these larger deletions concerned a single gene, GRMZM2G089517, in which 6 of the 8 larger deletions were found. In addition, two substantial insertions (10 bp and 11 bp) of unrelated DNA occurred in this gene, both accompanied by the deletion of a few nucleotides, as well as three classical indels (**Table 1**). This atypical example suggests that a specific gene context may influence the type of mutations generated, possibly by favouring a particular repair mechanism. However, in the case of GRMZM2G089517 it was not possible to implicate a specific mechanism with certainty, since the start and end points were not shared between the large deletions and since a search for repeated nucleotides did not detect obvious micro-homologies in proximity to the cutting site. Lastly, two other types of mutations were observed only once. The first, which again concerns the atypical GRMZM2G089517 gene, consists of a substitution of two nucleotides on either side of the PAM site, for which it is difficult to provide a mechanistic explanation (**Table 1**). The second atypical mutation was found in GRMZM2G046086, in which 35 bp next to the putative cutting site were substituted by an insertion of 62 bp (**Fig. 3 and Table 1**). This insertion comprises an adenine nucleotide plus 61 nucleotides corresponding to a stretch of intergenic DNA region found 602 bp downstream of the putative cutting site (**Fig. 3**). Interestingly, this 61 bp intergenic sequence is still present at the original location in the two alleles of the T0 plant, indicating that it was duplicated to create this atypical mutation.

We next analysed the 6 genes that had been targeted by two guide RNAs concomitantly (strategy 2 in **Fig. 2**). The rationale behind this strategy was to increase the probability of success with a single construct, since a mutation at either target site would be sufficient for loss-of-function. In the ideal case the two guide RNAs, spaced between 40 bp and 100 bp apart, would induce deletions of a predictable size that could be easily detected by

simple PCR in agarose gels and avoid the Sanger sequencing step to detect and follow the mutant allele. A total of 27 mutations were generated in 4 of the 6 genes, whereas neither deletions between the two cleavage sites nor other mutations were obtained for GRMZM2G040095 and GRMZM2G035701. There were no obvious reasons for the two failures, since the sgRNA design followed the same rules as for the 4 successful constructs. More intriguingly, the two sgRNA for GRMZM2G035701 (failure) were actually present on the same construct and in the same plants as the two sgRNA for GRMZM2G149940 (success). The large majority of the mutations identified (78%, 21/27) did not involve a deletion between the two guide RNA targets, but were caused by indels or larger deletions at one (74%, 20/27) or both target sites (4%, 1/27) (**Table 1**). Clear preferences for one of the two target sites were noted in all four cases and likely reflect differences in mutation efficiency or target site accessibility. Only 22% (6/27) of mutations harboured deletions of the region located between the two target sites (**Table 1**). In only one case (GRMZM2G049141) the 100 bp deletion corresponded exactly to the zone between the two putative cleavage sites. Regarding the other five deletions, small indels at one or both target sites either caused deletions that were slightly smaller (GRMZM2G039538 and GRMZM2G363552) or slightly larger (GRMZM2G049141) than the expected size (**Table 1**). In summary, it was possible to generate deletions in regions between two guide RNAs. However, the exact size of the deletion was variable and deletions between two target sites were less frequent than indels generated by the action of an individual guide RNA.

Lastly, a vector with four guide RNAs was designed to target a unique gene (strategy 4 in **Fig. 2**). Three guide RNAs gave rise to mutations in GRMZM2G471240, which were all of the indel type (**Table 1**). No deletions between the four target sites were observed.

Mutation efficiency

In total 28 guide RNAs were expressed in plants, 20 using RDP vectors, and 8 using Iowa vectors. Three targeted two genes in conserved regions. Among them, 22 resulted in at least one mutation and 6 did not induce any sequence change in the analysed plants (**Table 2**). For RDP vectors, 17 guide RNAs induced at least one mutation and three did not generate a mutation. For Iowa vectors, the proportion of unsuccessful guides was higher (3/8) but this result should be interpreted with caution because considerably fewer transformation events were obtained when using Iowa vectors in our conditions. This was certainly due to the non-optimal combination of our *Agrobacterium* strain LBA4404 (pSB1) and the binary vector, and more precisely an incompatibility between the origins of replication of pSB1 and pGW-Cas9 (Char et al. 2017).

Bi-allelic mutations, meaning that alleles on both the maternal and paternal chromosomes carried mutations, were detected in 19% (16/83) of the mutated plants, and more precisely in 18% (13/74) of the mutants obtained with RDP vectors and in 33% (3/9) of the mutants generated with Iowa vectors (**Table 2**).

Mutation efficiency was calculated as the number of transformation events harbouring at least one mutation as a proportion of all transformation events obtained for a given guide RNA (**Table 2**). Although this number may be somewhat influenced by differences in the accessibility of certain targets, for example due to chromatin differences between centromeric and telomeric chromosome regions, or by competition between guide RNAs in the plants that produced more than one guide, it was clear that mutation efficiency was very variable despite similar rules for guide RNA design (**Table 2)**. Concerning the promoter used to drive guide RNA expression in the RDP vectors, mutations were obtained using both the *OsU3* and the *TaU6* promoter. Averaging the percentages for each promoter, a higher overall mutation efficiency was observed with the *TaU6* promoter (65%) as compared to the *OsU3* promoter (39%) (**Table 2**). Using the same approach, a slightly higher efficiency was noted when the 20-nt target and the NGG were chosen on the coding (+) strand (58%) compared to the non-coding (-) strand (48%) (**Table 2**). Finally, the mutation efficiency was not strongly correlated to the overall GC content of the 20 nt targeted sequence (r=0.31, **Table 2**).

Although the sample number (3 cases) in which 1 guide RNA was used to target two paralogous genes precluded a quantitative analysis, the mutation efficiency seemed to be in the same range for both target genes with 63%/88% for the first (GRMZM2G039538/GRMZM2G363552), 25%/25% for the second (GRMZM2G039538/GRMZM2G363552) and 75%/50% for the third guide RNA GRMZM2G140302/GRMZM2G046086) (**Table 2**). Our results on the first two case suggests that the difference in mutagenesis efficiency between two guide RNAs targeting the same gene was more important than the difference between the mutagenesis efficiency for a single guide RNA targeted the two paralogs.

Transmission of edited genes to the next generation

Mutations must be present in germline cells in order to be passed on to the next generation. We therefore tested whether mutations detected in leaf material of T0 plants fulfill this criterion. During the detection by PCR amplification and Sanger sequencing of leaf material of T0 transformation events, two kinds of chromatograms indicative of editing were observed (**Online Resource 2)**: (1) the most common case was a switch from a homogenous chromatogram to two overlapping sequences with similar peak height (**Online Resource 2a)**,

indicating two alleles present in approximately the same proportion in the extracted DNA; (2) less frequently, sequencing chromatograms showed a main signal and a very weak overlapping signal (**Online Resource 2b)**, suggesting that the proportion of mutated DNA is very low compared to wild-type DNA. In the first case, we systematically observed transmission of the mutation to the T1 generation, suggesting that the edited alleles were fixed and present in all leaf cells and that the mutations had probably occurred early on in the maize transformation process, likely during the callus formation step. It should be noted that all alleles presented in **Table 1** were of this type. In the second case, we never observed any transmission of the mutations to the next generation suggesting that the mutations were present only in few leaf cells and that the mutations had probably occurred during leaf development. These data indicate that although chimeras may exist in maize but that fully edited T0 plants are predominant and that the distinction between chimeric and fully edited T0 plants can be made on the basis of the Sanger chromatograms.

To avoid additional mutations in targeted genes and to minimize mutations at off target sites in T1 plants, the T-DNA was routinely segregated away based on a negative PCR assay for the *BAR* gene (**Online Resource 3)**. Only transgene-free mutant T1 plants were used for subsequent analysis.

Creation of multiple mutants

One of the advantages of the CRISPR-Cas9 technology is that it allows the creation of multiple mutants in a single step, thereby avoiding time consuming crosses and/or backcrosses. With regard to unlinked genes located on different chromosomes, three double mutants were produced in the T0 generation using a construct with two guide RNAs, one for each gene (strategy 1 **in Fig. 2**). They concerned members of the same gene family in the case of GRMZM2G157313/GRMZM2G014499 (two double mutants in four transformation events, **Table 2**) and GRMZM2G059165/GRMZM2G120085 (1/3) and true paralogs in the case of GRMZM2G039538/GRMZM2G363552 (5/8). More importantly, multiple mutants were also obtained in genes that were tightly linked on the same chromosome, and for which the production of a double knockout mutant would have been difficult to achieve. Double mutants were identified for GRMZM2G089517/GRMZM2G352274 (separated by 75 kb on chromosome 5 (1 mutant found out of 14 transformation evens) and GRMZM2G145466/GRMZM2G573952 (located within 53 kb on chromosome 7 (1 out of 2). Finally, we successfully managed to knock out three small (<600 bp) paralogous genes that are situated in the same region of chromosome 1. These genetically strongly linked genes are *ZmEsr1*, *ZmEsr2* and *ZmEsr3* (GRMZM2G046086, GRMZM2G315601, GRMZM2G140302) (Opsahl-Ferstad et al. 1997). Since *ZmEsr2* and *ZmEsr1* are separated by only 29 kb, and *ZmEsr1* and *ZmEsr3* by only 13 kb, the production of a triple knockout mutant underlines the

power of CRISPR-Cas9 technology. Using the CRISPR-Cas9 strategy 3 illustrated in **Fig. 2**, a plant with a frame-shift mutation in each of the three *ZmEsr* genes was obtained. By a simple self-pollination, we have been able to generate T1 plants homozygous for the three mutated *ZmEsr* genes that are now available for functional analysis. However, no large deletions between the cleavage sites in the linked genes were found, despite specific PCR reactions designed to detect them.

Discussion

The present study examined CRISPR-Cas9-mediated targeted mutagenesis in maize aimed at routine use for functional genetics studies. Analysing mutations in 20 genes in genome edited maize plants, it was conducted at a larger scale than previous studies in maize, which either simply demonstrated the feasibility for a single gene or addressed a maximum of 5 genes (Liang et al. 2014; Xing et al. 2014). It also focused on regenerated plants rather than protoplasts or calli, systematically analysed offspring and is the first study to use the inbred line A188. The results indicate that CRISPR-Cas9 is a robust technology for gene knockout in maize, and can be used to generate various types of mutations with a high frequency of success. Furthermore it allows the production of double and triple mutants in tightly linked genes.

Three types of mutations were observed in the 93 mutant maize plants analysed: indels, larger deletions and local chromosome rearrangements. The occurrence of larger chromosome rearrangements, such as those reported recently for mouse embryonic stem cells (Kosicki et al. 2018), cannot be excluded but would not be detected with our method. Indeed the detection method, based on PCR amplification and subsequent Sanger sequencing, can only detect mutations in which the two primer binding sites on either side of the putative cleavage site are conserved in head to head orientation and remain at a distance allowing standard PCR amplification. Small indels as produced in the case of classical NHEJ repair (Ma et al. 2016; Bortesi et al. 2016) were, as expected, the most frequent outcome (80%, 74/93) and were documented for each of the 18 genes that were successfully mutagenized. They were generally located at, or close to, the putative cleavage site 3 bp upstream of the PAM. Larger deletions (>10 bp) ranging from 11 bp to 136 bp were considerably less frequent (11%, 10/93) and concerned only 4/18 genes. Thought to be generated by the MMEJ repair mechanism, short (2 bp to 4 bp) microhomologies were indeed present on both sides of the putative cleavage site in the wild-type sequence of GRMZM2G120085 (GC), GRMZM2G149940 (CCG) and GRMZM2G049141 (GACT) and the large deletions tended to correspond more or less precisely to recombination products between these direct repeats. In

contrast, for GRMZM2G089517 larger deletions were more frequent than indels, the start and end points of the deletions were not conserved between events, and two other atypical mutations were obtained: a combination of a 7 bp deletion with an 11 bp insertion, and two point mutations flanking the PAM. The mechanism generating these atypical mutations remains unclear, although it is known that strand resection and random DNA synthesis can lead to unpredictable outcomes during MMEJ repair (Wang and Xu 2017; Sinha et al. 2017).

An unexpected allele was also detected for GRMZM2G046086 alongside 5 other classical indels. This allele consists of a 35 bp deletion accompanied by the insertion of a 61 bp DNA fragment copied from the intergenic region downstream of the gene (**Fig. 3**).

Importantly, defined deletions (6%, 6/93) of predetermined size and position were successfully provoked by the simultaneous action of two guide RNAs on target sites separated by between 44 bp and 102 bp in a given gene. The precision of these deletion events was not perfect, since only one deletion was precisely of the expected size, whereas the other five contained indels of 1 bp or 2 bp at least at one end of the deletion. In addition, this approach worked only for 3/7 targeted genes and in the three successful cases indels at only one of the target sites were more frequent than the deletion. Since variations in target accessibility over such short distances in coding regions are unlikely, this suggests that very similar efficiency of the two guide RNAs is crucial for the successful generation of defined deletions. Differences between guide RNA efficiency may also partially explain why larger deletions involving target sites distant between 13 kb and 75 kb in genetically linked paralogs were not detected, despite the fact that CRISPR-Cas9-mediated deletions of up to 120 kb have been documented in plants (Gantner et al. 2018).

The overall mutation efficiency (averaging the percentages for each guide RNA) of 53% was in the global range (2% to 100%) of previous reports on targeted mutagenesis in maize, as was the 19% rate of biallelic mutations obtained (Liang et al. 2014; Xing et al. 2014; Lee et al. 2018). Since higher rates have been achieved in maize with the same basic elements (maize ubiquitin promoter, codon optimized Cas9, cereal U3 or U6 promoters), the specific choices made during vector design, such as the choice of different versions of the *ZmUbi* promoter, the choice of the terminator, the position of promoter-*Cas9* and *Cas9*-terminator junctions, as well as the presence of an NLS domain, of tags for immuno-detection or of introns in the *Cas9* coding sequence, are possible parameters for optimisation. However, this suboptimal rate of biallelic mutations also has advantages in the context of functional genetics studies of genes involved in maize kernel development, since mutations could be lethal for the embryo and/or seedling in the homozygous state (Neuffer and Sheridan 1980; Doll et al. 2017).

It is therefore preferable to generate heterozygous plants and to assess the (lethal) phenotype after self-pollination in segregating ears.

More importantly, the mutation efficiency was very variable at different levels. Firstly, two of the 20 genes could not be mutated at all, despite the use of two guide RNAs per gene and the generation of 8 and two transformation events, respectively. Secondly, among the 18 genes successfully mutated, not all transformation events caused mutations. For example, in the case of GRMZM2G352274 only one of the 16 transformation events yielded a mutation. Thirdly, in transformation events carrying novel mutations, not all guide RNAs present in the same plant induced mutations. The reasons for failure are likely linked either to the intrinsic quality of the sgRNA design or to the accessibility of the target sequence. Although the design of all sgRNAs followed the same rationale, the online and in house tools used only ensure a relatively high minimum quality standard, but they do not exclude quality differences between the possible designs. The GC content of the binding site (Ren et al. 2014; Labuhn et al. 2018), the secondary structure of the sgRNA, and its capacity not only to guide but also to activate the nuclease activity of Cas9 are known to be important parameters (Liu et al. 2016). In this context it is noteworthy that the GC content of both target sites in GRMZM2G035701 (failure) was relatively low (45%), whereas the GC content of the two sites in GRMZM2G149940 (targeted with success by the same construct in the same plants) was considerably higher (60% and 65%). The criteria for target site accessibility are less clear. Although Cas9 cleavage activity is not thought to be strongly affected by DNA CpG methylation (Hsu et al. 2013), it is generally accepted that the chromatin status of the target region influences the efficiency of CRISPR-Cas9 approaches, that DNase I hypersensitivity (DHS) is a good indicator for Cas9 binding (Wu et al. 2014) and that heterochromatin may be less accessible (Jensen et al. 2017). On the other hand, the accessibility of genes located in globally heterochromatic, centromeric regions of maize chromosomes to Cas9-mediated targeted mutagenesis has been demonstrated in protoplasts (Feng et al. 2016). In our study, the two recalcitrant genes GRMZM2G035701 and GRMZM2G040095 are located in gene-rich regions on the long arm of chromosome 8 and close to the end of chromosome 2, respectively. These regions do not present any obvious features explaining failure.

Differences in mutation efficiency between transformation events are expected, since the genomic environment is known to influence the expression level of transgenes, in the present case of the *Cas9* and *sgRNA* genes. However, very low success rates, such as the single edit for GRMZM2G352274 in 16 transformation events, are difficult to explain by insufficient expression, in particular since the second guide RNA present in the same plants caused mutations in 14/16 events. In this as in other cases, the competition of guide RNAs of unequal quality, or differences in target gene accessibility, are more likely explanations for

differences in successful mutagenesis than positional effects on transgene expression. Our study suggests that other parameters with a minor impact on mutation efficiency were the choice of the type III promoter with a preference for the *TaU6* over the *OsU3* promoter, and the choice of the DNA strand with mutagenesis improved by binding of the sgRNA to the template rather than non-template strand. This last observation is likely caused by a quicker release of the Cas9 from the template strand due to displacement by RNA polymerase II and faster repair of the DSB by the cellular machinery (Clarke et al. 2018). Overall, these results can be translated into 5 recommendations for gene-knockout in maize: 1) The use at least two guide RNAs per gene. 2) The generation of at least 5 transformation events. 3) The retargeting of recalcitrant genes with constructs targeting a single gene. 4) The use of maize or wheat *U6* promoters. 5) The preferential use of target sequences on the coding strand.

Chimerism is an important issue in CRISPR-Cas9-mediated mutagenesis, since in stably transformed plants constitutively expressing *Cas9* and *sgRNA* genes, genome editing can occur at any time and in any number of cells during the life cycle of the plant, raising the question of whether the mutations detected in the leaves or other organs of primary transformants will be present in the germline and thus transmitted to the offspring. Our results indicate that chimerism does occur, but that in the majority of events detected in leaf material are fully edited and that sequencing chromatograms with overlapping sequencing peaks of equal height are predictive for transmission to the next generation. This is in agreement with earlier reports in maize (Liang et al. 2014; Xing et al. 2014) and seems to indicate that the majority of editing events occur very early on during the transformation of immature maize embryos, likely at the callus stage.

The ease of multiplexing is frequently cited as one of the major advantages of CRISPR-Cas9 technology over the use of other site-directed nucleases such as meganucleases, zinc finger nucleases or TALENs, and CRISPR-Cas9 constructs harbouring as many as 14 guide RNAs have been used successfully in Arabidopsis (Peterson et al. 2016). Three double mutants in gene family members residing on different chromosomes, two double mutants in paralogues separated by 53 kb or 75 kb, and a triple mutant in paralogues separated by 13 kb or 29 kb were generated in our study. These examples underline the power of CRISPR-Cas9 technology since the production of double or triple knockout mutants in tightly linked genes would have been nearly impossible to achieve by crossing of single mutants, and would have required the analysis of thousands of recombinants. Multiplexing is of particular interest in maize, which is an ancient tetraploid known to contain numerous functionally redundant paralogues, hampering functional analysis. As a result the production of multiple mutants by CRISPR-Cas9 will almost certainly become a prime tool for functional genomics studies in this species.

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Author contribution statement

NMD, PMR and TW conceived and designed research; NMD, LMG, MFG, CR, VMG, GG, and TW conducted experiments. MFG, CR and GG performed maize transformation. NMD prepared tables and figures. JJ performed bioinformatics analyses to produce **Online Resource 1**. NMD, GI, PMR and TW wrote the manuscript. PMR and TW were involved in project management and obtained funding.

Data availability statement

The two plasmids generated during the current study are available at GeneBank under the accession number MH662439 for L1609 and MH662440 for L1611.

Conflict of Interest

LMG is employed by Limagrain Europe. PMR is part of the GIS-BV (“Groupement d’Intérêt Scientifique Biotechnologies Vertes”).

Material and Methods

Plant Material and Growth Conditions

The maize (*Zea mays*) inbred line A188 (Gerdes and Tracy 1993) and derived transgenic or edited plants were grown in growth chambers that fulfill the French S2 safety standards for the culture of transgenic plants. In the 15 m^2 growth chambers the plants were illuminated by a mixture of 10 LED spots of 500 W (Neptune LED, Ste Anne sur Gervonde) set at 60% intensity and 8 high-pressure sodium lamps of 400 W, resulting in the spectrum presented in **Online Resource 4** and a photosynthetic photon flux density (PPF) of about 300-400 µmol.s-1 at plant height. The photoperiod consisted of 16 h light and 8 h darkness in a 24 h diurnal cycle. Temperature was set to 24°C/17°C (day/night) during the first 84 days after sowing (DAS) and then to 26°C/28°C for the remaining 30 days of the life cycle. The relative humidity was controlled at 55% (day) and 65% (night). Seeds were germinated in 0.2 L of Favorit MP Godets substrate (Eriterre, Saint-André-de-Corcy) and were transferred at between 12 and 20 DAS to 8 L of Favorit Argile TM + 20% perlite substrate (Eriterre, Saint-André-de-Corcy) supplemented with 50 ml of Osmocote Exact Hi.End 5-6M (15-9-12+2MgO+TE) fertilizer (Scotts, Ecully). All plants were propagated by hand pollination.

Vector cloning

The integrative plasmid L1609 (**Fig. 1**) is based on the backbone of pSB11 (Ishida et al., 1996), from which a SapI site was removed. It contains between the T-DNA borders a rice codon optimized *Cas9* (Miao et al., 2013) driven by a synthetic maize ubiquitin promoter lacking several restriction sites, a rice U3 promoter separated from a sgRNA scaffold (Shan et al., 2013) by two adjacent but otherwise unique SapI sites, unique EcoRV and I-CeuI sites and a Basta® resistance cassette. The small plasmid L1611 (**Fig. 1**) contains a wheat U6 promoter followed by two adjacent SapI sites and a shRNA (Shan et al., 2013), the entire cassette being flanked by unique EcoRV and I-CeuI sites. Annealed oligonucleotides with SapI compatible overhangs and corresponding to 20 nt targeted sequences containing at their 5' end an A in the case of the U3 promoter or a G in the case of the U6 promoter were cloned in L1609 and L1611 respectively. The U6 driven target cassette present in L1611 was subsequently excised with EcoRV and I-CeuI and cloned into the L1609 derivative downstream of the U3 driven target cassette. The resulting plasmid was transferred to *Agrobacterium tumefaciens* strain LBA4404 (pSB1) and used for maize transformation. Alternatively, Gateway compatible assemblies of two to four cassettes consisting each of a long or short maize U6 promoter, followed by a 20 nt target site starting with a G and a shRNA (Char et al. 2017) were entirely synthesized (GENEWIZ, New Jersey) and recombined into plasmid pGW-Cas9 (Char et al. 2017) containing between T-DNA borders a maize codon optimized Cas9 driven by maize

ubiquitin promoter and a Basta® resistance cassette conferring glufosinate-ammonium herbicide resistance.

20-nt target sequence choice

For the design of sgRNAs targeting specifically a single gene in the maize genome, the online tools CRISPR-P (http://crispr.hzau.edu.cn/CRISPR/) (Lei et al. 2014) and CRISPOR (http://crispor.tefor.net/) (Haeussler et al. 2016) were interrogated and targets at convenient positions with high scores in both tools were chosen. Since these tools are not readily suited to target several members of a gene family with a single sgRNA, we wrote custom Perl scripts to design sgRNAs directed against up to 10 genes each. All candidate CRISPR-Cas9 targets were identified in the B73 maize reference genome sequence v3.26 (Schnable et al. 2009) using the following criteria: 23-mers ending with NGG, not containing more than 4 Ts in a row, and with no variant of the last 15 nt ending in NAG existing in the genome. Using Jellyfish v2.2.0 (Marçais and Kingsford 2011), we counted the number of occurrences in the genome of the last 15 nt of each candidate, and kept only those occurring at most 10 times. The resulting database (**Online Resource 1)** contained 18,422,860 20 nt sequences, targeting 22,541,809 loci. We queried it to identify targets in the genes we wanted to edit. In both cases the design was only retained, if the sequence of the reference genome of genotype B73 v3.26 (Schnable et al. 2009) available in the design tools did not show any polymorphism in the 20-nt target sequence and the PAM with the sequence of genotype A188 used for transformation.

Maize transformation and screen for Cas9-free edited plants

Immature embryos of maize inbred line A188 were transformed with *A. tumefaciens* strain LBA4404 harboring pSB1 and the construct of interest according to a standard protocol (Ishida et al. 1996, 2007). T-DNA integrity was checked as described elsewhere (Gilles et al. 2017). Genome editing was evaluated on leaves of T0 plants, individually for each targeted gene by specific PCR amplification of the targeted region (see **Online Resource 3** for primer sequences) followed by Sanger sequencing. Segregation of T-DNA in T1 plants was evaluated by PCR amplification on the *Bar* gene, checking the presence and quality of genomic DNA by PCR amplification of the GRMZM2G136559 control gene (see **Online Resource 3** for primer sequences).

Figure legends

Fig. 1 CRISPR-Cas9 cloning vectors.

Cloning strategy for RDP vectors. The final RDP plasmids contain two small guide RNAs (*sgRNA1* and *sgRNA2*) and are generated by assembly of the two initial plasmids L1609 and L1611. First the 20 nt corresponding to the recognition sequences are synthesized as oligonucleotides with SapI compatible ends and inserted between the U3 or U6 promoter and the scaffold RNA (*shRNA*) after SapI digestion in both plasmids, forming *sgRNA1* and *sgRNA2*. Then the *TaU6::sgRNA2* cassette is transferred by EcoRV/CeuI digestion into the plasmid already containing the *OsU3::sgRNA1* cassette. *BAR* = Basta® resistance gene, *Cas9* = rice codon optimized *Cas9* gene, LB = T-DNA left border, *OsU3* = rice U3 promoter, *pActUbi* = maize ubiquitin promoter, *pOsAct* = rice actin promoter, RB = T-DNA right border, *shRNA* = short hairpin RNA, *sgRNA* = small guide RNA, *TaU6* = wheat (*Triticum aestivum*) U6 promoter, 20 nt = recognition sequence of 20 nucleotides inserted before the *shRNA*

Fig 2 Different approaches to generate single and multiple gene knockout in maize.

Scheme illustrating different types of strategies for single or multiple gene knockout. The first strategy consists of targeting two distinct genes with specific guide RNAs for each gene, the second of targeting a single gene with two guide RNAs, the third of targeting several paralogous genes with one or several guide RNAs, and the fourth of targeting a single gene with four guide RNAs

Fig 3 Scheme of an atypical mutant allele of *ZmEsr1* (GRMZM2G046086). The intronless *ZmEsr1* gene is represented by a square box with the open reading frame in blue and the UTRs in red. Numbering starts at the first nucleotide of ATG start codon. The duplicated intergenic sequence is depicted in yellow. The 35 bp segment deleted in the mutant allele is indicated in dark blue

Table 1 CRISPR-Cas9 alleles generated in 20 maize genes.

a: Sequence of the coding strand of the targeted genes around the recognition site (underlined) and the PAM (in blue)

b: Position of the mutation relative to the putative cleavage site (3 bp upstream of the PAM)

c: Number of independent transformation events with the same mutation

Table 2 Guide RNAs used and relationship with plant transformation events.

a: Mutation efficiency is defined as percentage of transformation events leading to at least one mutation within the targeted gene(s)

b: Chromosome carrying the targeted gene

c: DNA strand targeted by sgRNA, relative to gene orientation. "-" refers to the non-coding strand and "+" to the coding strand

Electronic Supplementary Material

Online Resource 1 List of CRISPR-Cas9 targets identified in maize B73 genome (v3.26), presented as 23-mers ending with NGG

Online Resource 2 Detection of genome editing in T0 plants

Online Resource 3 List of the primers used for amplifying the edited region for each gene as well as for selecting T-DNA negative segregant plants

Online Resource 4 Emission spectrum of the light used for maize culture in growth chambers

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Table 1

ID in maize genome V3	Allele	Sequence[a]	Position from the putative cutting site[b]	Occurrence [c]	mutation type
Gene targeted by one guide with RDP vectors					
GRMZM2G157313	WT A188	GACCGGAACGCGACATG-GTACGGAGCACA			
	ins G	GACCGGAACGCGACATGGGTACGGAGCACA	0	X2	indel
GRMZM2G014499	WT A188	ACAGGTCTACATAGTGT-ACCTGGGCGAGC			
	del TGTACCT	ACAGGTCTACATAG---------GGCGAGC	-3		indel
	ins T	ACAGGTCTACATAGTGTTACCTGGGCGAGC	0	X2	indel
GRMZM2G059165	WT A188	AGCATTCTGCACCAGGGTATCGGACCCAT			
	del T	AGCATTCTGCACCAGGG-ATCGGACCCAT	1		indel
GRMZM2G120085	WT A188	CGCACTCCTCGC-AAGCCCCTCGCTTCCCC			
	del CTCCTCGCAAG	CGCA----------CCCCTCGCTTCCCC	-8		larger deletion
	del AA	CGCACTCCTCGC--GCCCCTCGCTTCCCC	0		indel
	del A	CGCACTCCTCGC--AGCCCCTCGCTTCCCC	0	X2	indel
	ins A	CGCACTCCTCGCAAAGCCCCTCGCTTCCCC	0		indel
GRMZM2G145466	WT A188	GGAGAAGCACACAAAGC-GCGTGGACGCAC			
	del CG	GGAGAAGCACACAAAG--CGTGGACGCAC	0 or 1		indel
	ins A	GGAGAAGCACACAAAGCAGCGTGGACGCAC	0		indel
GRMZM2G573952	WT A188	AAAAGGTGCTACTGCTG-CGTTGGGGGTAG			
	ins A	AAAAGGTGCTACTGCTGACGTTGGGGGTAG	0		indel
	ins G	AAAAGGTGCTACTGCTGGCGTTGGGGGTAG	0		indel
GRMZM2G046086	WT A188	TATTGGCCATCG--TCCAAGACTTGCATCTT			
	del 35pb ins 62pb	see Figure 3	-6		other
	del TCCA ins G	TATTGGCCATCG-G----AGACTTGCATCTT	0		indel
	del CG ins A	TATTGGCCAT---ATCCAAGACTTGCATCTT	-2		indel
	ins C	TATTGGCCATCG-CTCCAAGACTTGCATCTT	0		indel
	ins A	TATTGGCCATCG-ATCCAAGACTTGCATCTT	0		indel
	ins TT	TATTGGCCATCGTTTCCAAGACTTGCATCTT	0		indel
GRMZM2G140302	WT A188	TATTGGCCATCG-TCCAAGACTTGCATCTT			
	del TCCAAG	TATTGGCCATCG-------ACTTGCATCTT	0		indel
	del T	TATTGGCCATCG--CCAAGACTTGCATCTT	0	x2	indel
	ins C	TATTGGCCATCGCTCCAAGACTTGCATCTT	0		indel
	ins A	TATTGGCCATCGATCCAAGACTTGCATCTT	0		indel
GRMZM2G315601	WT A188	AAAAGACCTGTG-CCTAGCGGGCCAGACCC			
	del CCT	AAAAGACCTGTG----AGCGGGCCAGACCC	0		indel
	del C	AAAAGACCTGTG--CTAGCGGGCCAGACCC	0	X3	indel
	ins T	AAAAGACCTGTGTCCTAGCGGGCCAGACCC	0		indel
	ins A	AAAAGACCTGTGACCTAGCGGGCCAGACCC	0		indel
GRMZM2G134341	WT A188	TCCTCCCCAGGT-TGCCGGGTGCGACCTGT			
	del CCGGG	TCCTCCCCAGGT-TG-----TGCGACCTGT	2		indel
	del TG	TCCTCCCCAGGT--CCGGGTGCGACCTGT	0		indel
	ins A	TCCTCCCCAGGTATGCCGGGTGCGACCTGT	0		indel

ID in maize genome V3	Allele	Sequence[a]	Position from the putative cutting site[b]	Occurrence [c]	mutation type
AC208201.3_FG003	WT A188	GCAGACGTGCGACCTGT-ACCGGGGCAGCT			
	ins T	GCAGACGTGCGACCTGTTACCGGGGCAGCT	0	X2	indel
	del ACCGGG	GCAGACGTGCGACCTGT-------GCAGCT	0		indel
	del A	GCAGACGTGCGACCTGT--CCGGGGCAGCT	1		indel
	del GT	GCAGACGTGCGACCT---ACCGGGGCAGCT	-2		indel
	ins A	GCAGACGTGCGACCTGTAACCGGGGCAGCT	0	X2	indel
GRMZM2G089517	WT A188	CTTGAAGTGAGGACTGCAAGAA----GGCCGGCTCTCGCCGACATCTCTG			
	del 136pb	CTTGAAG----------	-15		larger deletion
	del 43pb	----------	-31		larger deletion
	del 32pb	----------CCGGC	-30		larger deletion
	del 17pb	CTTGAAGTGAGGACTGCAA----------	-3		larger deletion
	del 8pb	CTTGAAGTGAGGACTGCAAGAA----------	0		indel
	del 28pb, ins GT	----------GTC	-21		larger deletion
	del 16pb, ins 10pb	CTTGAAGTGAGGACT----------ACCAGAGA	-7		larger deletion
	del 7pb ins 11pb	CTTGAAGTGAGGACTGCAAGATGCTCTTTGGTC	-1		????
	del AAG	CTTGAAGTGAGGACTGCAAG-------GCCGGC	-2		indel
	ins T	CTTGAAGTGAGGACTGCAAGAAT---GGCCGGC	0		indel
	ins A	CTTGAAGTGAGGACTGCAAGAAA---GGCCGGC	0		indel
	2 mutations	CTTGAAGTGAGGACTGCAAGAA----GGTCGGA	3 and 7		other
GRMZM2G089517	WT A188	GAATGGTGCTGTCAAGC-GGCCGGCTCGGC			
	ins T	GAATGGTGCTGTCAAGCTGGCCGGCTCGGC	0		indel
GRMZM2G352274	WT A188	CGG-CAACACATCCAATCGAATGAAGATTCTTCAC			
	ins T	CGGTCAACACATCCAATCGAATGAAGATTCTTC	14		indel
GRMZM2G352274	WT A188	AACGGACTGCTCCTTGCAGGTGGCTCCAT			
Gene targeted by two guides with RDP vectors					
GRMZM2G039538	WT A188	CCTCTTCCACTC-GGGCGGCGAGCTCCAGCAGCATCCTGTACACCTACGACACCGTCAT-GCACGGCTTC			
	del 45pb	CCTCTTCCACTC----------	0 and -1		deletion betwee
	del 44pb	CCTCTTCCACTC-G----------	1 and -1		deletion betwee
	del GGGCG ins C ; ins T	CCTCTTCCACTC-C----GCGAGCTCCAGCAGC	0 and 0		indel
	del GGGC	CCTCTTCCACTC-----GGCGAGCTCCAGCAGC	0		indel
	del G	CCTCTTCCACTC--GGCGGCGAGCTCCAGCAGC	0	x2	indel
	del CTCGGGC ins T	CCTCTTCCA----T---GGCGAGCTCCAGCAGC	-3		indel
	ins T	CCTCTTCCACTCTGGGCGGCGAGCTCCAGCAGC	0	X2	indel
GRMZM2G363552	WT A188	CCTCTTCCACTC-GGGCGGCGAGCTCCAGCAGCATCCTGTACACCTACGACACCGTCAT-GCACGGCTTC			
	del 44pb	CCTCTTCCACTC-G----------	1 and -1	X2	deletion betwee
	del GGG ins TT	CCTCTTCCACTC-TT-CGGCGAGCTCCAGCAGC	0		indel
	del GG	CCTCTTCCACTC--GCGGCGAGCTCCAGCAGC	0		indel
	del G	CCTCTTCCACTC--GGCGGCGAGCTCCAGCAGC	0		indel
	ins G	CCTCTTCCACTCGGGGCGGCGAGCTCCAGCAGC	0		indel
	ins T	CCTCTTCCACTCTGGGCGGCGAGCTCCAGCAGC	0		indel
	ins A	CCTCTTCCACTCAGGGCGGCGAGCTCCAGCAGC	0		indel

ID in maize genome V3	Allele	Sequence[a]	Position from the putative cutting site[b]	Occurrence [c]	mutation type
GRMZM2G049141	WT A188	ACAGTGCCGACGATGGCAGTATATCGTCCAGGCCAGC-(60nt)-GGCCCCAGCCCGGAACCCTGAATCT			
	del 100pb	ACAGTGCCGACG----------	0 and 0		deletion betwee
	del 102pb	ACAGTGCCGACG----------	0 and 2		deletion betwee
	del TGCCGACG	ACAG--------ATGGCAGTATATCGTCCAGGC	-8		indel
	del ATG	ACAGTGCCGACG---GCAGTATATCGTCCAGGC	0		indel
	del 17 pb	A----------GTATATCGTCCAGGC	-11		larger deletion
	del 15 pb	A----------CAGTATATCGTCCAGGC	-11		larger deletion
	del CCTG	ACAGTGCCGACGATGGCAGTATATCGTCCAGGC	1		indel
GRMZM2G040095	WT A188	CGAGCGCCCTGCTCAAGTACCGGGAGGACGAGCTCGGCG-(40nt)-GCCGTGGGACCGCGTGTACGACT			
Gene targeted by 2 guides with Iowa vectors					
GRMZM2G149940	WT A188	CTTCCACCAATA-CCCCGCCGGCTTGATCCCAGCGCCGGTGGCACTGCCGGTTCACGCACCGGTGTCGTC			
	del 54pb	CTTCCACCAATA-CCCCGCCGGCTTGATCCCAG	-1		larger deletion
	del G	CTTCCACCAATA-CCCCGCCGGCTTGATCCCAG	-1		indel
	ins A	CTTCCACCAATAACCCCGCCGGCTTGATCCCAG	0		indel
GRMZM2G035701	WT A188	GAATACAGCTGCTCTTGATCCGGATCATT-(30nt)-GGAGCTCCGAAATAGCGATGTAAGCCAGC			
Gene targeted by 4 guides with Iowa vectors					
GRMZM2G471240	WT A188	GCAATACCTGTAGCACGAAGGCGATGGCC			
GRMZM2G471240	WT A188	GCGGCCTCTCTACGCTG-CCAAGGACATCA			
	del G	GCGGCCTCTCTACGCT--CCAAGGACATCA	0		indel
	ins G	GCGGCCTCTCTACGCTGGCCAAGGACATCA	0		indel
	ins T	GCGGCCTCTCTACGCTGTCCAAGGACATCA	0		indel
GRMZM2G471240	WT A188	GAGGGTGTCCAGGGTCAACGTGGAGACAG			
	del CAA	GAGGGTGTCCAGGGT---CGTGGAGACAG	-2		indel
	del A	GAGGGTGTCCAGGGTC-ACGTGGAGACAG	0		indel
GRMZM2G471240	WT A188	GGAGACAGGGAGGTACG-AACCGGTGACTG			
	del GA	GGAGACAGGGAGGTAC---ACCGGTGACTG	0		indel
	del GA ins C	GGAGACAGGGAGGTAC-C-ACCGGTGACTG	0		indel
	del G	GGAGACAGGGAGGTAC--AACCGGTGACTG	0		indel
	ins T	GGAGACAGGGAGGTACGTAACCGGTGACTG	0		indel
	ins A	GGAGACAGGGAGGTACGAAACCGGTGACTG	0		indel

Table 2

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20nt target (+NGG)	Gene targeted (ID Maize Genome V3)	Number of plant transformation events	Number events with at least one mutation	% successful events (mutation efficiency) [a]	Number of transformation event with bi-allelic mutation	Promoter driving the sgRNA	%GC witin the 20nt targeted	chromosome [b]	DNA strand [c]
RDP vectors									
GCTGGAGCTCGCCGCCCGAGTGG	GRMZM2G039538	8	5	63%	0	TaU6	80	2	-
	GRMZM2G363552		7	88%	0	TaU6	80	7	-
ACCTACGACACCGTCATGCACGG	GRMZM2G039538		2	25%	0	OsU3	55	2	+
	GRMZM2G363552		2	25%	0	OsU3	55	7	+
GACCGGAACGCGACATGGTACGG	GRMZM2G157313	4	3	75%	0	TaU6	60	10	+
ACAGGTCTACATAGTGTACCTGG	GRMZM2G014499		2	50%	0	OsU3	45	3	+
AGCATTCTGCACCAGGGTATCGG	GRMZM2G059165	3	1	33%	0	OsU3	50	7	+
GGGGAAGCGAGGGGCTTGCGAGG	GRMZM2G120085		3	100%	1	TaU6	75	1	-
GGAGAAGCACACAAAGCGCGTGG	GRMZM2G145466	2	2	100%	0	TaU6	60	7	+
AAAAGGTGCTACTGCTGCGTTGG	GRMZM2G573952		1	50%	0	OsU3	50	7	+
ACAGGTCGCACCCGGCAACCTGG	GRMZM2G134341	3	1	33%	1	OsU3	70	7	-
GCAGACGTGCGACCTGTACCGGG	AC208201.3_FG003		2	67%	2	TaU6	65	1	+
AGTGAGGACTGCAAGAAGGCCGG	GRMZM2G089517	16	14	88%	0	OsU3	55	5	+
GTGAAGAATCTTCATTCGATTGG	GRMZM2G352274		1	6%	0	TaU6	35	5	-
GAATGGTGCTGTCAAGCGGCCGG	GRMZM2G089517	5	3	60%	0	TaU6	60	5	+
AACGGACTGCTCCTTGCAGGTGG	GRMZM2G352274		0	0%	0	OsU3	60	5	+
ATGATGAAGATTCAGGGTTCGGG	GRMZM2G049141	7	3	43%	1	OsU3	40	2	-
GGACGATATACTGCCATCGTCGG	GRMZM2G049141		6	86%	1	TaU6	50	2	-
AAGATGCAAGTCTTGGACGATGG	GRMZM2G140302	8	6	75%	2	OsU3	45	1	-
	GRMZM2G046086		4	50%	3	OsU3	45	1	-
GGGTCTGGCCCGCTAGGCACAGG	GRMZM2G315601		6	75%	2	TaU6	75	1	-
ACAGCGCGTAGTCGTACACGCGG	GRMZM2G040095	8	0	0%	0	OsU3	65	2	-
GTCCTCCCGGTACTTGAGCAGGG	GRMZM2G040095		0	0%	0	TaU6	65	2	-
Iowa vectors									
GAATACAGCTGCTCTTGATCCGG	GRMZM2G035701	2	0	0%	0	ZmU6	45	8	+
GCTGGCTTACATCGCTATTTCGG	GRMZM2G035701		0	0%	0	ZmU6	45	8	-
GGATCAAGCCGGCGGGGTATTGG	GRMZM2G149940		1	50%	0	ZmU6	65	2	-
GGAGACGTCTGTGACGACACCGG	GRMZM2G149940		2	100%	2	ZmU6	60	2	-
GGCCATCGCCTTCGTGCTACAGG	GRMZM2G471240	2	0	0%	0	ZmU6	65	1	-
GCGGCCTCTCTACGCTGCCAAGG	GRMZM2G471240		2	100%	0	ZmU6	70	1	+
GAGGGTGTCCAGGGTCAACGTGG	GRMZM2G471240		2	100%	0	ZmU6	65	1	+
GGAGACAGGGAGGTACGAACCGG	GRMZM2G471240		2	100%	1	ZmU6	60	1	+

Table 2 Guide RNAs used and relationship with plant transformation events.

a: Mutation efficiency is defined as percentage of transformation events leading to at least one mutation within the targeted gene(s)
b: Chromosome carrying the targeted gene
c: DNA strand targeted by sgRNA, relative to gene orientation. "-" refers to the non-coding strand and "+" to the coding strand

Figure 1

Fig. 1

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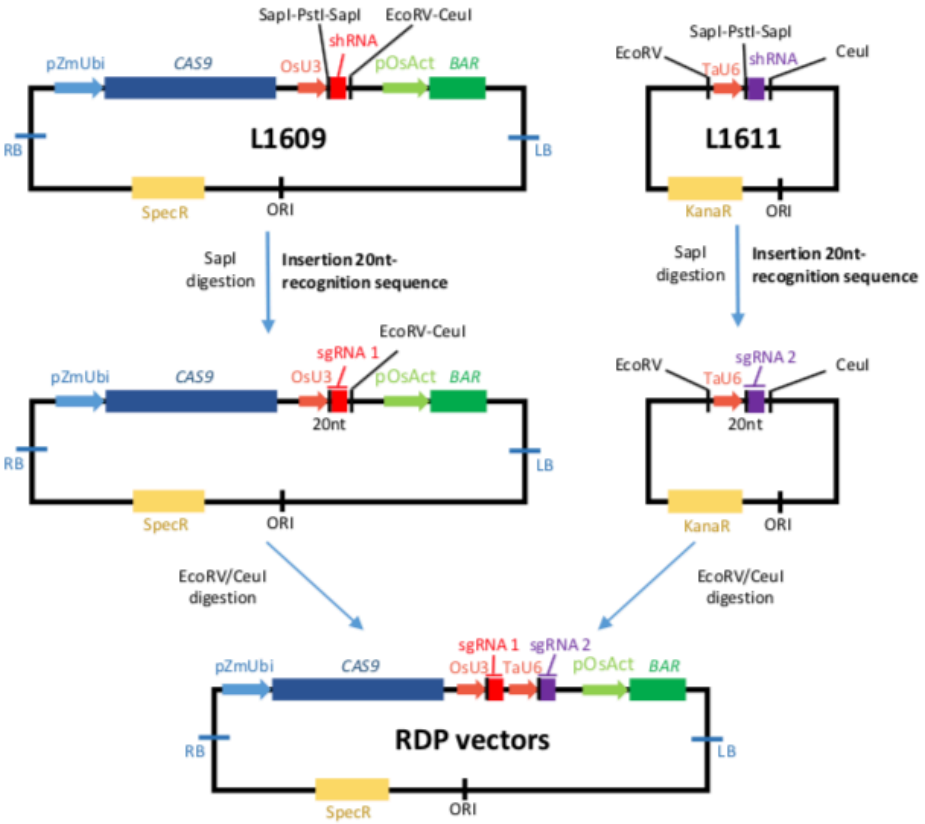

Figure 2

Fig. 2

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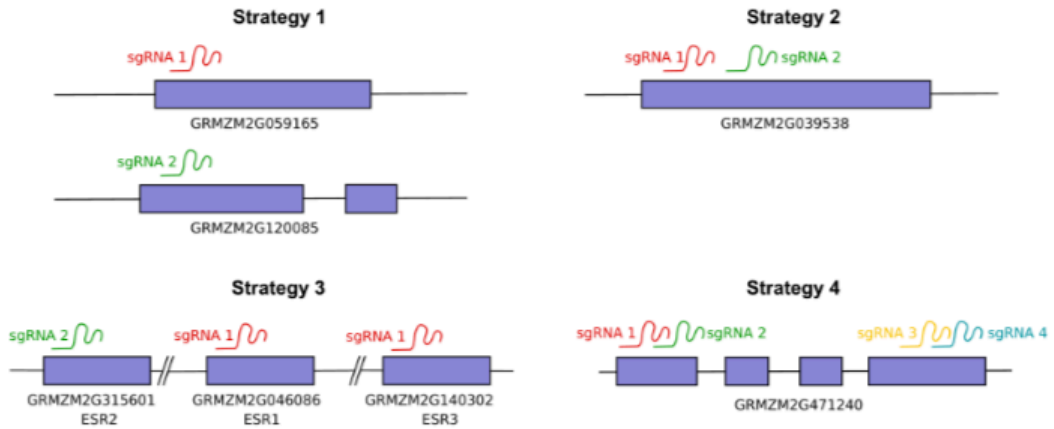

Figure 3

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Fig. 3