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Research Article

PEG-delivered CRISPR-Cas9 Ribonucleoproteins System for Gene-editing Screening of Maize Protoplasts

Rodrigo Ribeiro Arnt Sant'Ana[1], Clarissa Alves Caprestano[1], Rubens Onofre Nodari[1], Sarah Zanon Agapito-Tenfen[2],*

[1] CropScience Department, Federal University of Santa Catarina, Florianópolis, Brazil; rodrigoarnt@hotmail.com, clarissacapre@gmail.com, rubens.nodari@ufsc.br

[2] GenØk – Centre for Biosafety, Siva innovasjonssenter Postboks 6418, 9294 Tromsø, Norway; sarah.agapito@genok.no

*Correspondence: sarah.agapito@genok.no; ORCID 0000-0002-9773-0856

Abstract: CRISPR-Cas9 technology allows the modification of DNA sequences *in vivo* at the location of interest. Although CRISPR-Cas9 can produce genomic changes that do not require DNA vector carriers, the use of transgenesis for stable integration of DNA coding for gene-editing tools into plant genomes is still the most used approach and it can generate unintended transgenic integrations, while Cas9 prolonged expression can increase cleavage at off-target sites. In addition, the selection of genetically modified cells from millions of treated cells, especially plant cells, is still challenging. These downfalls can be avoided with the delivery of preassembled ribonucleoprotein complexes (RNPs) composed of purified recombinant enzyme Cas9 and *in vitro*- transcribed guide RNA (gRNA) molecules in a protoplast system. We therefore aimed to develop the first DNA-free protocol for gene-editing in maize and introduced RNPs into their protoplasts with PEG 4000. We performed effective transformation of maize protoplasts using different gRNAs sequences targeting the inositol phosphate kinase gene and applying two different exposure times to RNPs. Using low-cost Sanger sequencing protocol, we observed an efficiency rate of 0.85 up to 5.85%, which is equivalent to DNA-free protocols used in other plant species. A positive correlation was displayed between exposure time and mutation frequency. Mutation frequency was gRNA sequence- and exposure time-dependent. In summary, we demonstrated the suitability of RNP transfection as an effective screening platform for gene-editing in maize. This efficient and relatively easy assay method for selection of gRNA suitable for editing of gene of interest will be highly useful for genome editing in maize, since genome size and GC-content are large and high in maize genome, respectively. Nevertheless, the large amplitude of mutations at target site requires scrutiny when checking mutations at off-target sites and potential safety concerns.

Keywords: gene editing; mutagenesis; genetically modified; GMO; crop breeding; RNP; genetic screening

1. Introduction

The emergence of technologies related to genetic improvement, such as transgenesis and more recently genome editing, have changed the way humans grown food for thousands of years. Today, the most promising tool to DNA manipulation is CRISPR (*Clustered Regularly Interspaced Short Palindromic Repeats*), a gene-editing technology that has been adapted from bacterial immune system against viral infections (Jinek et al. 2012). CRISPR-Cas9 Ribonucleoproteins (RNPs) consists of an endonuclease enzyme called Cas9 and a guide RNA molecule (gRNA) that contains the target-specific sequence for guiding the enzyme to the target site in the host genome. Cas9 introduces a site-specific double-stranded DNA break (DSB) followed by the cell natural repair of disrupted genome integrity by error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR) (Hsu et al. 2013). Therefore, this tool allows the *in vivo* modification of the DNA at the gene sequence of interest, with unprecedent speed and making it a milestone in manipulating and producing living modified organisms.

Although much is already known about the principles of CRISPR-Cas9 genome editing, the likelihood of different outcomes in terms of resolution, efficiency, accuracy and DNA modification structure has shown to be species-dependent. Various factors including target site choice, guide RNA (gRNA) design, the properties of the endonuclease, the type of DSB introduced, whether or not the DSB is unique, the quantity of endonuclease and gRNA, and the intrinsic differences in DNA repair pathways in different species, tissues and cells will result in differences in the mutation signatures generated in plant species (Bortesi et al. 2016).

While CRISPR technology has already been tested on commercial crops to increase yield, drought tolerance and growth under limited nutrient conditions, improve nutritional properties and develop resistance to plant pathogens (Barrangou et al. 2016); breeding and research of major monocotyledon species, more specifically maize, are still at its infancy. Maize has shown to be an exemption in the plant portfolio for the *in silico* analysis of potential Cas9 target sites as only 29.5% of annotated transcripts matched a specific sgRNA (Xie et al. 2014). Among eight analyzed plant species, maize had the largest genome, the highest GC content and the greatest number of annotated transcripts. Thus, reflecting the abundance of highly repetitive DNA and dispersed repeats which may be challenging to develop unique target sites for the majority of genes in maize.

Despite such challenges, CRISPR technology opens up the possibility for genome changes without foreign introgression of DNA vectors. CRISPR-Cas9 technology can be used as ribonucleoprotein complexes without the introgression and expression of a transgenic cassette in the host genome (Metje-Sprink et al. 2019). Such approach would avoid a number of generations of backcrossing, expression vectors and other invasive methods of cell penetration (e.g. biolistics) that can lead to gene disruption, including large deletions, partial trisomy, genome shattering events and plant mosaicism (Liu et al. 2019). Overall, these side effects can mask or interfere in the target gene functional analysis and further additional biosafety concerns prior to commercial release.

Delivery of preassembled Cas9 protein-gRNA RNPs or plant DNA-free genome-editing technique is not exempt from off-target effects but it represents an approach in which the effects of Cas9 can be isolated from other more invasive techniques (Kanchiswamy, 2016; Agarwal et al. 2018; Metje-Sprink et al. 2019). This approach was first demonstrated in *Arabidopsis thaliana*, tobacco, lettuce and rice protoplasts including regeneration of gene-edited lettuce (Woo et al. 2015). After that, few successful attempts were also accomplished on grapevine and apple (Malnoy et al. 2016), Petunia × hybrida (Subburaj et al. 2016), potato (Andersson et al. 2018); and on soybeans and tobacco using CRISPR/Cpf1 (Clustered Regularly Interspaced Short Palindromic Repeats from *Prevotella* and *Francisella*), recently named Cas12a (for review please read Metje-Sprink et al. 2019). Maize and wheat plants with targeted mutations have been also successfully obtained by delivering gold particles coated with the RNPs into embryo cells (biolistics), followed by post-bombardment culture and plant regeneration (Svitashev et al. 2016). However, the frequency of obtaining genome-edited plants was relatively low, since only 0.3-0.9% of regenerated maize plants possessed bi-allelic mutations (Svitashev et al. 2016).

The few studies published on maize genome editing rely mostly on stable transformation (Liang et al. 2016; Char et al. 2017; Feng et al. 2018; Dong et al. 2018). In the manuscript, we delivered Cas9-gRNA RNP into maize leaf protoplasts via PEG-calcium mediated transfection, and indicated that In/Del mutations occurred with relatively high efficiency of 1-6% among the PEG-calcium treated protoplast. We targeted the inositol phosphate kinase gene (IPK) involved in the phytic acid biosynthetic pathway. To develop a standard protocol for different maize varieties, we designed gRNAs and primers complementary to coding regions in exon 3 that are conserved in the species in order to evaluate the efficiency and spectrum of DNA changes generated by CRISPR-Cas9 technology in maize, and also add relevant information to the safety of gene-edited organisms. This efficient and relatively easy assay method for selection of gRNA suitable for editing of gene of interest will be highly useful for genome editing in maize, since genome size and GC-content are large and high in maize genome, respectively.

2. Material and Methods

2.1 Target Site Selection and in vitro Cleavage Assay

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The *Zea mays* IPK gene data was obtained from NCBI GenBank (accession B73RefGen_v3). *In vitro* test was performed to confirm RNP complex efficiency to cleave target DNA. The target site was amplified using specific primers. The crRNAs were designed for the third exon of the IPK gene in maize using the platform CRISPR-Cas9 guide RNA Design Checker (Integrated DNA technologies Inc, IDT). Commercially available Cas9 protein (160 kDa) was also purchased from IDT (Table 1).

Table 1. List of primers and crRNAs used for amplification and mutation of IPK gene target locus in maize.

Primer	**Sequence (5' – 3')**	**Amplicon size (bp)**
ZmIPK-F	GAAGAAGCAGCAGAGCTTCA	876
ZmIPK-R	CAGAAGAAATCCGTGAGGACAG	
crRNA	**Sequence (5' – 3')**	**Cleaveged fragments (bp)**
crRNA1	AGCTCGACCACGCCGCCGAC	279 | 597
crRNA2*	GGGATCCGTCTCCTTCTCCC	617 | 259
crRNA3*	ATCTTCAAGGTCTACGTCGT	525 | 351
crRNA4*	CAGGAGTTCGTCAACCATGG	498 |378
crRNA5	ACAAGCTCTACGGAGACGAC	141 | 735

Note: *Selected crRNAs used for protoplasts transfection.

gRNA, crRNA (100 nM) and tracrRNA (100 nM) were incubated for 5 min at 95°C, according to manufacturer instructions. Cas9 (100 nM), gRNA and 1×NEB buffer 3 were incubated for 10 min at 25°C to form the RNP complex. Amplified PCR products (300 ng) were then incubated for 60 min at 37°C with the RNP complex. Proteinase K (800U/ml) was added to stop the reaction. The products were visualized using 1% agarose gel electrophoresis (Malnoy et al. 2016).

2.2 Maize Protoplast Isolation and Fluorescent Transfection Assay

Mesophyll protoplasts were isolated from the middle portion of the second leaf following the protocol described by Sheen et al. (1991) with some modifications. Etiolated maize seedlings were grown in vermiculite, after disinfestation of the seeds with 70% alcohol (60 s), NaOCl2% (twice of 15 min) and triple washing with distilled water. Ten-days old deedlings were used (3 days under 16 h light/day and 7 days in darkness). Leaves were cut into thin strips (0.5-1 mm) and immersed in cell-wall digestion enzyme solution (0.3% macerozyme R-10, 1.5% cellulase R-10, 10 mM of MES pH 5.7, 0.6 M mannitol, 10 mM $CaCl_2$, 5 mM ß-mercapto, 0.1% BSA). The material was left in vacuum for 30 min and gentle shaking at 40 rpm in the dark for 4 h. The protoplasts were released thoroughly by shaking at 80 rpm for 5 min. After digestion, the protoplasts were diluted with the same volume of cold W5 solution [2 mM MES (pH 5.7), 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl] and filtered through a double filter (40 µM Nylon mesh). Protoplasts were collected after centrifugation at 100 g for 3 min and washed 2 times in 10 ml of W5 solution. Protoplasts were resuspended in cold MMG solution [0.4 M mannitol, 4 mM MES (pH 5.7), 15 mM $MgCl_2$]. Its viability and concentration were determined using Fluorescein Diacetate (FDA) dye in the hemocytometer. To confirm the internalization of the RNP complex inside cells, an assay was performed using fluorescent labelled tracrRNA molecules (ATTO 550, IDT) (Liu et al. 2018). Microphotographs were taken using an inverted optic microscope ix80 Olympus.

2.3 Maize Protoplast Transformation

Maize protoplasts were gene-edited by introducing CRISPR-Cas9 RNP complex (no integration of exogenous DNA) via PEG-mediated transfection (Figure 1). Protoplast transformation was adapted from Woo et al. (2015) and Malnoy et al. (2016). First, 15 µg the two components of the gRNA (crRNA and tracrRNA) were incubated at 95°C for 5 min. After allowing to cool at room temperature, 45 µg of Cas9 and 1×NEB buffer 3 were added, then mixed and incubated at 25°C for 10 min. Finally, the RNP complex was mixed with 100 µl of protoplasts (1 x 10^5 protoplasts), 250 µl of PEG solution (40% PEG 4000, 0.2 M mannitol, 0.1 M $CaCl_2$) (pH 6.0) and incubated at 25°C in the dark. Two incubation times were tested: T1 = 20 min and T2 = 40 min. W5 solution (950 ul) was added and the tubes were centrifuged at 100 g for 3 min. Protoplasts were resuspended in 1 ml W1 solution [4 mM MES (pH 5.7), 0.5 M mannitol, 20 mM KCl] and then transferred to multi-well plates for 24 h under gentle agitation (40 rpm) in the dark at 25°C.

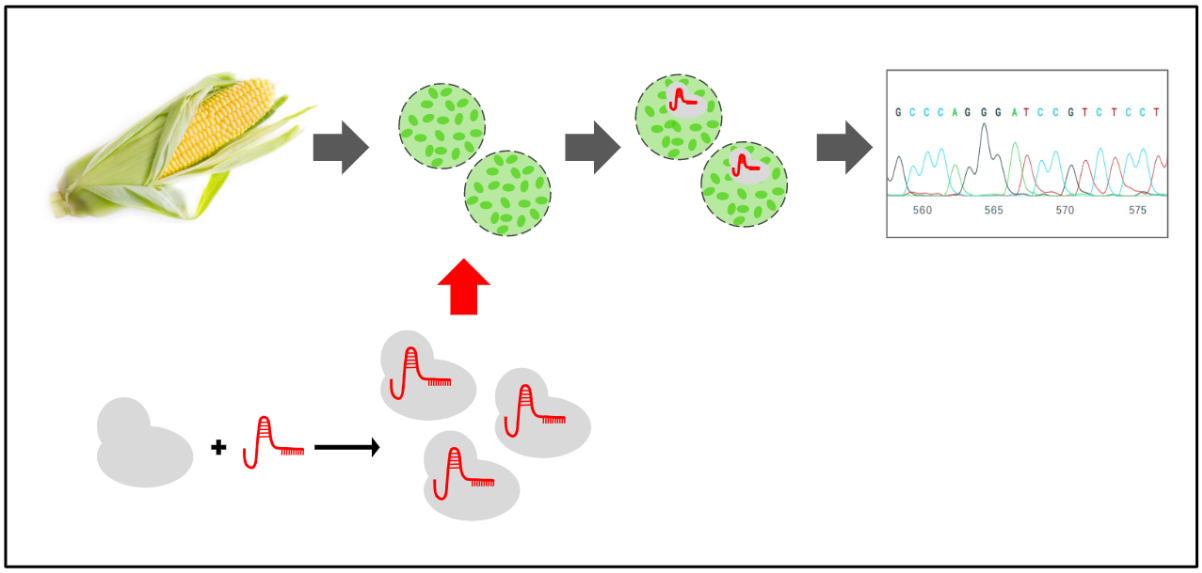

Figure 1. Methodological approach of the project. Corn seeds were germinated *in vitro*, the second leaves of the seedlings were used to obtain the protoplasts. The protoplasts were exposed to the CRISPR-Cas9 ribonucleoproteins complex and after 24 h the DNA was extracted from the samples. PCR fragments were amplified and sequenced.

2.4 Gene-editing Efficiency Analysis by Sanger Sequencing

In order to characterize the spectrum and frequency of DNA changes at the target gene, genomic DNA was isolated using DNeasy Plant Mini Kit (QIAGEN®), followed by amplification of the target region by PCR using Taq Q5 High-Fidelity DNA Polymerase (NEB®) and primers listed in Table 1. PCR samples were purified and sequenced using the BigDye Terminator 3.1v Kit (ThermoFisher Scientific). Samples were resuspended in formamide, denatured at 95°C for 5 min and incubated on ice for 3 min. Sequencing was performed using the Sanger (Sanger et al. 1977) automated sequencer from 3500xL Dx Genetic Analyzer for Sequencing (Applied Biosystems ™).

CRISPR-Cas9 DNA changes were calculated based on the insertions and deletions (indels) around the cleavage site (3 bp upstream of the PAM sequence) using the Inference of CRISPR Editing Software - ICE software. It has been previously shown that ICE software results are comparable to Next Generation Sequencing (NGS) results (Hsiau et al. 2019).

3. Results

3.1 In Vitro Cleavage Assay

Cleavage activity of gRNAs 1 to 5 was tested using 0.5 µg of crRNA and 1.5 µg of Cas9 enzyme to 300 ng of DNA. While all designed gRNAs were able to cleave PCR products of the IPK gene in our study, the different gRNAs sequences varied in their cleavage efficiency (Figure 2). gRNA2, 3 and 4 showed the highest activity and were therefore chosen for subsequent experiments on transfection of maize protoplasts.

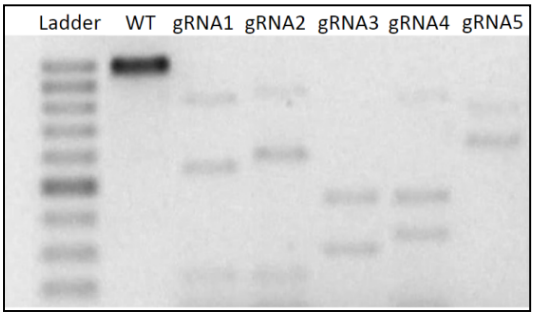

Figure 2. Schematic diagram of the Maize IPK gene locus with the gRNAs target sites. *In vitro* CRISPR assay showing the original and the cleaved fragments of IPK gene in maize that were submitted to the RNP complex with the crRNA1, 2, 3, 4 and 5. Note: WT = Wild Type (control).

3.2 Targeted Mutagenesis in Maize Using CRISPR-Cas9 Ribonucleoproteins

Frequently, results indicating low efficiency of CRISPR-Cas9 editing using RNPs delivery cannot discriminate low transfection rates from poor DNA cleavage and repair activity. In order to overcome this limitation and confirm internalization of Cas9-gRNA RNPs, we have performed a fluorescent microscopy assay. Labeled tracrRNA molecules confirmed internalization of the RNP complex (Figure 3). Although this is not a quantitative method, it can be observed that at least one third of the labelled molecules are internalized.

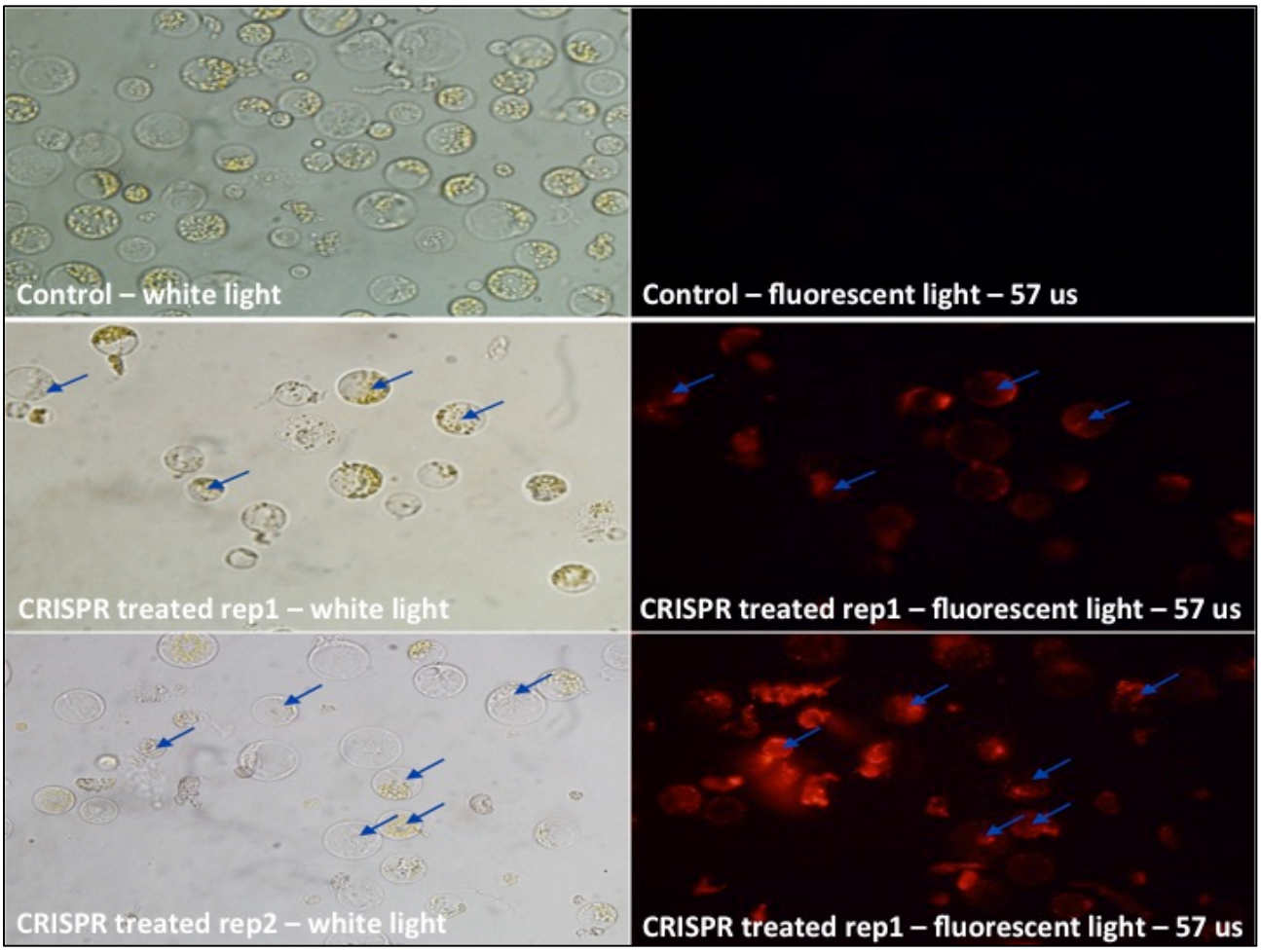

Figure 3. (a)Maize protoplasts submitted to the RNP complex containing tracrRNA labeled (ATTO 550, IDT) and (b) Maize protoplasts control. Image obtained through fluorescence microscopy (IX 80 Olympus).

RNPs containing gRNAs 2, 3 and 4 were transfected into isolated protoplasts with PEG 4000 and the results are displayed as the percentage of indels detected at the cleavage site based on Sanger sequencing and analysis with ICE software. DNA sequences from the universal primer used to amplify the IPK gene in maize (876 bp) from treated samples were compared to the same fragment in negative control (no RNP delivered). Other negative controls (Cas9 or gRNAs delivered alone) were also tested against the first negative control and showed no DNA changes (Table 2).

Different concentrations of Cas9 protein and gRNA have been tested in pilot experiments (data not shown) and the concentration of 45 ug of Cas9 and 15 ug of gRNA to 100 ul protoplasts in a 3:1 ratio resulted in the best cost-efficiency correlation. This is also in agreement with previous reports on RNPs delivery into protoplasts which ranged from 30-60 ug of Cas9 in a 1:1 and 3:1 ratio (Woo et al, 2015; Malnoy et al. 2016; Svitashev et al. 2016). Exposure time was tested for all three gRNA sequences in 20- and 40-minutes exposure. While a longer exposure time to RNP complex led to a higher mutation index for all gRNAs tested, the increase in mutation rate was not consistent among gRNA sequences (approx. 7-fold, 3-fold and 1.3-fold for the gRNAs 2, 3, and 4, respectively) (Figure 4). Deletions were shown more frequent than insertions in this model system. Higher insertion rate was only observed for gRNA 2 at 40 min time exposure. In more detail, we show gene-edited sequences obtained for each gRNA at 40 min exposure time in Figure 5. About six sequence variants were of major contribution in gene-editing efficiency for the three selected gRNA sequences.

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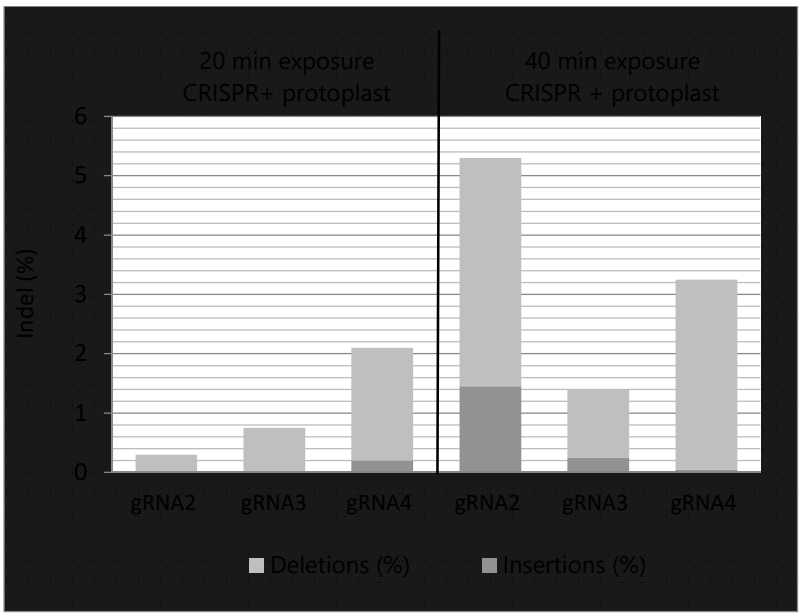

Figure 4. Frequency of mutations in maize generated by gene editing (CRISPR) and measured by ICE software. Different gRNAs and exposure time of the protoplasts to the RNP complex are represented. Percentages of deletions and insertions are represented in green and red, respectively.

gRNA2 – 40 min exposure time

INDEL	Contribution	Sequence
0	93.5%	GATCCGTCTCCTTCT|CCCAGGTCTCTCCAAC
+1	2.7%	GATCCGTCTCCTTCT|NCCCAGGTCTCTCCAA
-1	1.8%	GATCCGTCTCCTTCT|-CCAGGTCTCTCCAAC
-2	1.3%	GATCCGTCTCCTTCT|--CAGGTCTCTCCAAC
-5	0.2%	GATCCGTCTCCTTCT|-----GTCTCTCCAAC
-6	0.1%	GATCCGTCTCCTTCT|------TCTCTCCAAC
-8	>0.1%	GATCCGTCTCCTTT|-------GTCTCCAAC

gRNA3 – 40 min exposure time

INDEL	Contribution	Sequence
0	94.2%	CTTCAAGGTCTAACG|CGTCTGGCGGCCAGC
-19	0.9%	CTTCA----------|----------CCACG
-1	0.5%	CTTCAAGGTCTACG-|CGTCTGGCGGCCACG
+6	0.3%	CTTCAAGGTCTACGT|NNNNNNCGTCGGCG
+8	0.2%	CTTCAAGGTCTACGT|NNNNNNNCGTCGG
-24	0.2%	CTTCA----------|----------
-28	>0.1%	C-------------|----------

gRNA4 – 40 min exposure time

INDEL	Contribution	Sequence
0	95%	GGAGTTCGTCAAACC|ATGGCGCGGCTCT
-2	3.3%	GGAGTTCGTCAAAC-|TGGCGCGGCTCAT
-3	0.8%	GGAGTTCGTCAAA--|TGGCGCGGCTCAT
-1	0.4%	GGAGTTCGTCAAACC|TGGCGCGGCTCAT
-7	0.1%	GGAGTTCGTCAA---|----GGCGGCTCAT
+12	0.1%	GGAGTTCGTCAAACC|NNNNNNNNNNNTG
-1	0.1%	GGAGTTCGTCAAACC|-GGCGGCGGCTCT
-16	>0.1%	GGAGTTCGTCAA---|----------T

Figure 5. Sequence distribution of most efficient mutations identified with ICE software around IPK gene target site in *Zea mays*. Edited sequences were obtained after CRISPR-Cas9 RNP transfection to maize protoplasts. 45 µg of Cas9 preassembled with 15 µg of each gRNA were used in the protoplast transformation. Different exposure times of the RNP complex with the protoplast are presented. Cut sites are represented by black vertical dotted lines, insertions are represented by 'N' and deletions by black horizontal dotted lines.

Results on the percentage of mutated sequences (technology efficiency), the size of the DNA change (number of base pair change), the type of DNA change (deletions or insertions) and a theoretical knockout score (KO score) are

summarized in Table 2. The indel percentage at 20 min exposure was on average 1.63% in contrast to a 4.37% at 40 min exposure time. Overall, gRNA 4 was most efficient and consistent at both exposure time. Intriguingly, gRNA 2 showed the lowest efficient at 20 min (0.85%) but the highest efficiency at 40 min (5.85%). The Knockout Score accounts for reads containing an amino acid frameshift change or 21+ bp indel. Thus, indicating contributing indels that are likely to result in a functional knockout of the targeted gene. In this study, the average KO score was 0.83% for the 20 min and 3.17% for the 40 min exposure treatment, which suggests that the majority of indels were frameshift modifications. In addition, only one gRNA at one time point had a single base pair change as the most frequent mutation (gRNA 2 with a -1 bp). Notably, a deletion of 19 bp was the most abundant DNA change for gRNA 3 (0.9%). Moreover, all other gRNAs and exposure time showed a 2 bp deletion as the most frequent DNA change. Overall, the DNA change ranged from -28 bp to +12 bp change.

Table 2. Mutation rates in *Z. mays* IPK gene target region based on Sanger sequencing and ICE software analysis.

Sample	Incubation time (min)	% of indel	Model fit (R^2)	KO score	Mutation range (bp)	Greater Contribution (bp)
Cas9 only	20	0		0	0	0
gRNA2 only	20	0		0	0	0
gRNA3 only	20	0		0	0	0
gRNA4 only	20	0		0	0	0
Cas9 + gRNA2 rep1	20	0	0.99	0	0	0
Cas9 + gRNA2 rep2	20	1	1	1	-4 to -2	-2
Cas9 + gRNA3 rep1	20	0	1	0	-7 to -1	-2
Cas9 + gRNA3 rep2	20	1	1	1	-7 to -1	-2
Cas9 + gRNA4 rep1	20	3	0.99	2	-7 to +12	-2
Cas9 + gRNA4 rep2	20	1	0.99	1	-2 to +3	-2
Cas9 + gRNA2 rep1	40	4	0.99	4	-7 to +1	-1
Cas9 + gRNA2 rep2	40	6	1	6	-8 to +1	+1
Cas9 + gRNA3 rep1	40	1	1	1	-19 to -1	-1
Cas9 + gRNA3 rep2	40	2	0.96	2	-28 to +8	-19
Cas9 + gRNA4 rep1	40	2	0.99	2	-3 to -2	-2
Cas9 + gRNA4 rep2	40	5	1	4	-16 to +12	-2

Note: KO = Knockout.

4. Discussion

CRISPR-Cas9 technology is a powerful tool for plant breeding and research. While still evolving as a technology to determine the rules for gRNA design and the algorithms to predict target and 'off-target' sequences, CRISPR applications still relies on empirical results to test the performance of new systems (Lin et al. 2018). Notably, gene-editing results outcomes are frequently species-dependent (Bortesi et al. 2015). Therefore, a CRISPR platform for difference species with a rapid and efficient evaluation protocol is needed before commercialization.

Our experiments demonstrated the suitability of the PEG-delivered CRISPR-Cas9 RNPs system for gene-editing screening in maize. We showed that high-efficiency gene-edited maize cells can be obtained using less time-consuming (15 days) and labor-intensive procedures (PCR, agarose gel electrophoresis and Sanger sequencing). In addition, the advantage of our system in relation to the use of vectors is that it prevents the integration and expression of exogenous DNA sequences, isolating the effect of gene-editing modification and avoiding transgene-introgressed side effects. Although non-integrating plasmids could be transfected into plant cells to deliver programmable nucleases; transfected plasmids are degraded in cells by endogenous nucleases, and the resulting small DNA fragments can be inserted at both on-target and off- target sites in host cells (Kim et al. 2014). For example, Braatz et al. (2017) performed whole-genome sequencing after transfection of expression construct CRISPR-Cas9 in *Brassica napus* and found that transformation resulted in at least five independent insertions of vector backbone sequences in the plant genome.

4.1 Ribonucleoprotein delivery in plants

In this report, we show a positive correlation between the time of exposure to RNPs and the efficiency of site-directed mutagenesis in maize, as ascertained with Sanger sequencing. In previous reports referring to the use of RNPs in plant protoplasts (Table 3), the authors used one or more different concentrations of RNPs and Cas9:gRNA ratios, but the effect of exposure time on mutation frequencies was not tested (Sandhya et al. 2020, Wada et al 2020). CRISPR RNPs were delivered to apple, grapevine, brassica sp., lettuce, tobacco and rice plants at less or equal than 20 min exposure time and their efficiencies ranged between 0.1 and 40% (Malnoy et al. 2016; Murovec et al. 2018; Woo et al. 2015). In petunia and

wheat protoplasts exposure for 30 min granted 0.2 up to 45% efficiency (Subburanj et al. 2016; Liang et al. 2017); thus, suggesting that time of exposure might not alone explain indel frequency in different plant systems. In our system, when all other factors are maintained, exposure time consistently increased indel frequency for all three gRNA sequences tested (up to 6.6 fold change increase).

Table 3. Publications with DNA-free gene editing in plants using CRISPR-Cas9 RNPs and other delivery methods for maize.

Reference	Plant species	Plant material	Transfection method	Gene-editing efficiency
RNP delivered in plants				
Woo et al. 2015	*Arabidopsis thaliana, Lactuca sativa, Nicotiana attenuata, Oryza sativa*	Protoplasts	PEG-mediated	5.7 - 40.0%
Malnoy et al. 2016	*Malus domestica, Viti vinifera*	Protoplasts	PEG-mediated	0.1 - 6.9%
Subburaj et al. 2016	Petunia hybrid	Protoplasts	PEG-mediated	2.4 - 21.0%
Liang et al. 2017	*Triticum aestivum*	Protoplasts, immature embryos	PEG-mediated, Biolistics	0.2 - 45.3%
Andersson et al, 2018	*Solanum tuberosum*	Protoplasts	PEG-mediated	1.0 - 25.0%
Murovec et al. 2018	*Brassica oleracea, Brassica rapa*	Protoplasts	PEG-mediated	0.1 - 24.5%
Toda et al. 2019	*Oryza sativa*	Zygotes	PEG-mediated	14.0 – 64.0%
Other delivery methods in maize				
Liang et al. 2014	*Zea mays*	Protoplasts	Vector via PEG-mediated	13.1%
		Immature embryos	*Agrobacterium*-mediated	16.4 - 19.1%
Xing et al. 2014	*Zea mays*	Protoplasts	Vector via PEG-mediated	N.A
Svitashev et al. 2015	*Zea mays*	Immature embryos	RNP via biolistics	1.3 - 4.6%
Liang et al. 2016	*Zea mays*	Protoplasts	Vector via biolistics	80.0 - 90.0%
Qi et al. 2016	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	57.1 - 71.4%
Feng et al. 2016	*Zea mays*	Protoplasts	Vector via PEG-mediated	2.8 - 27.0%
		Immature embryos	*Agrobacterium*-mediated	19.0 - 31.0%
Zhu et al. 2016	*Zea mays*	Protoplasts	Vector via PEG-mediated	4.0 - 11.9%
		Immature embryos	*Agrobacterium*-mediated	65.8 - 86.9%
Svitashev et al. 2016	*Zea mays*	Immature embryos	RNP via Biolistics	0.01 - 0.7%
Char et al. 2017	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	12.0 - 74.0%
Shi et al. 2017	*Zea mays*	Immature embryos	Vector via Biolistics	60.0 - 98.0%
Chen et al. 2018	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	N.A
Feng et al. 2018	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	5.0 - 100%
Dong et al. 2019	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	N.A
Lee et al. 2019	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	90.0 -100%
Kelliher et al. 2019	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	N.A
Doll et al. 2019	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	N.A
Wu et al. 2020	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	N.A
Liu et al. 2020	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	N.A
Gao et al. 2020a	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	N.A
Gao et al. 2020b	*Zea mays*	Immature embryos	Vector via biolistics	N.A
Barone et al. 2020	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	25.0 - 100%
Zhang et al. 2020	*Zea mays*	Immature embryos	*Agrobacterium*-mediated	N.A
This study	*Zeamays*	Protoplasts	RNA via PEG-mediated	0.85 - 5.85%

Note: * N.A is 'not applicable' because transgenic plants were either selected using antibiotic marker genes or the analysis was not performed.

The Cas9:gRNA ratio also influences target efficiency in a species-specific manner. Three different Cas9:gRNA ratios were tested in apple and grapevine protoplasts (Malnoy et al. 2016). While the 1:1, 1:3 and 3:1 ratios did not differ in mutation frequency for grapevine (0.1%), the 1:1 and 3:1 ratios increased indel frequency in two (6.6 and 2.6 fold change

respectively) out of three gRNA sequences in apple. Overall, the results obtained for the 3:1 ratio are equivalent to our results applying the same ratio (from 3.3 up to 6.7% efficiency). Cas9 concentration has shown to be of major factor influencing the delivery of RNPs to plant cells. Woo et al. (2015) tested 20 and 60 ug of Cas9 to Arabidopsis protoplasts and found that the editing efficiency was not directly related to Cas9 concentration but also dependent to the time course of analysis. At 24 h after delivery, more efficiency was observed when applying 20 instead of 60ug of Cas9 (71 in contrast to 54%). Opposite results were obtained at 72 h after delivery. On the other hand, increasing the amount of Cas9 (7.5, 15. 30 and 60 ug) was consistent with a crescent indel frequency in brassica sp. protoplasts (Murovec et al. 2018). The efficiency results obtained in our study was similar to those obtained applying approximately 60-90 ug of Cas9 thus, indicating that a lower amount of Cas9 (45 ug) but a higher exposure time (40 min) might have similar cleavage levels. Overall, it is clear that the limited amount of studies investigating RNP delivery into plant cells is insufficient to draw definitive conclusions for increasing gene-editing efficiency using this system.

4.2 CRISPR delivery methods in maize

Other delivery methods have been tested for maize as screening methods or gene-editing breeding methods and these include: PEG-mediated vector transfection, agrobacterium-mediated and biolistics (Table 3). The vast majority of studies still rely on vector-based transformation delivery of CRISPR. None of the listed studies have provided a cell-based screening method without the insertion of foreign vector-based DNA. On average, PEG-mediated vector transfection reached an average of about 12% efficiency, whereas agrobacterium and biolistics reached 44 and 83%, respectively. In addition, most of these methods used embryogenic callus as explant material. Callus-based methods harbor chimeric tissues thus, requiring subsequent genetic fixation to allow stable inheritance of the edited traits. Therefore, these are not suitable material for genetic screening of successful gene-edited plants. Currently, many protocols are available for regeneration of whole plants from protoplasts. These include lettuce, tobacco and rice, petunia, wheat, apple and soybean. It is also suggested as a future choice for gene-edited maize and the list seems to expand because of the capabilities of the RNP technology (Svitashev et al. 2016, Park and Choe 2019). The trade-off in efficiency percentage plays in return of avoiding unintended DNA integration and potential undesirable biosafety risks.

4.3 Analytical platforms for gene-editing detection

Different analytical platforms for the detection and identification of CRISPR outcomes reflects in frequency results as they show different analytical sensitivity. Woo et al. (2015) showed a 40% transformation efficiency in tobacco when samples were analyzed by Illumina sequencing platform. In contrast, the same samples showed a much lower efficiency rate (17%) when analyzed by T7 cleavage assay. While T7 cleavage assays are nowadays being limited to a qualitative rather than quantitative detection method, high throughput sequencing platforms are time-consuming and costly option for screening protocols. Usually, such platforms are available in other labs or through service providers which requires a long processing time and high costs for samples and assays that are still at screening stage. In order to overcome such problems, we have proposed a model that analyzes gene-edited cell pools using data from common Sanger sequencing analysis. More specifically, we use the ICE: *Inference of CRISPR Edits* software, which unable the analysis of mixed populations and strongly correlates with next-generation sequencing of amplicons using Sanger sequencing data (Hsiau et al. 2019).

However, the proposed model is focused on providing a simple, cost-efficient analysis of gene-editing outcomes at a screening stage. The model is currently limited to detect mutations with more than 30 bp deletions or more than 14 bp additions. The analysis also does not account very small mutant populations (<0.1%) neither present mutations with substitution of bp (Hsiau et al. 2019). Although our platform does not apply exogenous DNA generating potential integration, it cannot be ruled out that microhomologies with gRNA sequences produce spurious cleavage or larges genomic rearrangements (Vu et al. 2017). Therefore, long run high through put sequencing analysis are recommended for follow up breeding programs and safety tests (Agapito-Tenfen et al. 2018).

5. Conclusions

We have shown that RNPs can be used for targeted CRISPR-Cas9 via PEG delivery as a model system to screen for gene-editing outcomes in maize. Target insertion and deletion DNA changes (indels) were induced using 45 ug of Cas9 and gRNA used at a 3:1 ratio, and a positive correlation between exposure time (20 and 40 min) and indel frequency was observed. By targeting preserved coding regions, we can anticipate that the model can be applied to several maize varieties by validation using IPK gene. However, *in vivo* indel frequencies differed among gRNA sequences. In addition, the proposed method for sequencing analysis is also restricted to a window of 30 bp deletions and 14 bp addition. Further studies will be focused on CRISPR-Cas9 off-target activity and on the regeneration of edited protoplasts.

Authors Contributions: R.R.A.S. and S.Z.A. interpreted the data and drafted the manuscript. R.R.A.S. performed the statistical analysis. R.R.A.S. and C.A.C. conducted the CRISPR experiments. R.O.N. assisted with data interpretation. S.Z.A., R.O.N., C.A.C. and R.R.A.S. conceived the study. S.Z.A. coordinated the investigation. All authors reviewed the manuscript.

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FIGURE LEGENDS

Figure 1. Methodological approach of the project. Corn seeds were germinated *in vitro*, the second leaves of the seedlings were used to obtain the protoplasts. The protoplasts were exposed to the CRISPR-Cas9 ribonucleoproteins complex and after 24 h the DNA was extracted from the samples. PCR fragments were amplified and sequenced.

Figure 2. Schematic diagram of the Maize IPK gene locus with the gRNAs target sites. *In vitro* CRISPR assay showing the original and the cleaved fragments of IPK gene in maize that were submitted to the RNP complex with the crRNA1, 2, 3, 4 and 5. Note: WT = Wild Type (control).

Figure 3. (a)Maize protoplasts submitted to the RNP complex containing tracrRNA labeled (ATTO 550, IDT) and (b) Maize protoplasts control. Image obtained through fluorescence microscopy (IX 80 Olympus).

Figure 4. Frequency of mutations in maize generated by gene editing (CRISPR) and measured by ICE software. Different gRNAs and exposure time of the protoplasts to the RNP complex are represented. Percentages of deletions and insertions are represented in green and red, respectively.

Figure 5. Sequence distribution of most efficient mutations identified with ICE software around IPK gene target site in *Zea mays*. Edited sequences were obtained after CRISPR-Cas9 RNP transfection to maize protoplasts. 45 µg of Cas9 preassembled with 15 µg of each gRNA were used in the protoplast transformation. Different exposure times of the RNP complex with the protoplast are presented. Cut sites are represented by black vertical dotted lines, insertions are represented by 'N' and deletions by black horizontal dotted lines.

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