Project Final Report

TASK 1: Design and synthesis of different single-stranded DNA oligonucleotide (SDO) molecules with novel chemical modifications for the enhancement of targeted nucleotide exchange

Researchers: Györgyi Ferenc, Zoltán Kupihár, Ferhan Ayaydin
Technician: Katalin László, Tamás Kukli

What have we promised?

Workplan for TASK 1

***1. year**: Design, chemical synthesis, and structural characterization of the SDOs to be used for the introduction of nucleotide exchange in the mutant GFP gene (mGFP) to restore its function (SDO^{GFP}), induction of mutation in maize acetolactate synthase (ALS) gene (SDO^{ALS}) and in anthranilate synthase (AS) maize genes (SDO^{AS}). These SDO molecules will be synthesized for different target sequences with variable lengths and phosphorothioate end-protection. Synthesis of SDOs with lipophilic moieties at 5´-end (cholesterol or palmityl). Synthesis of fluorescence-labeled SDOs for uptake studies.*

***2. Year:** Continuation of supply of synthesized SDOs. Further improvement of OTNE efficiency: design and synthesis of SDO^{GFP}, SDO^{ALS} and SDO^{AS} using 2´-fluoro-arabino nucleotide (FANA) or Locked Nucleic Acid (LNA) units, N-alkinyl nucleotides. Structural characterization of these new molecules. Design and synthesis of SDOs for their combinatory use with CRISPR/Cas9 system in Task 3.*

***3. Year:** Continuous supply of synthetic SDOs with sequence, length and modifications optimized in the 1st and 2nd years.*

Single-stranded DNA oligonucleotide (SDO) molecules serve as templates for both Oligonucleotide Targeted Nucleotide Exchange (OTNE) and CRISPR/Cas9 methods. They are designed to be complementary in sequence to the targeted DNA and to carry the desired mutation, which can be nucleotide replacement, deletion or insertion. We aimed to develop and synthesize novel forms of SDO molecules having new structural elements to enhance the frequency of the targeted nucleotide exchange in plants.

1.1. Fluorescent labeling of the SDOs to track their delivery by fluorescence microscope

For testing the efficiency of the delivery method, oligonucleotides were fluorescently labeled by FAM (green) or Cy3 (red) dyes. The red fluorescent-modified oligonucleotides were designed to target the non-functional, mutant Green Fluorescent Protein (mGFP) gene and at the same time aid the monitoring of cellular uptake efficiency of the SDOs by confocal microscopy under different treatment conditions. This dual-color detection approach proved to be very advantageous during fine-tuning of conditions for biolistic delivery experiments using dedifferentiated callus tissues as well as during optimizations of the cationic polymer delivery method involving protoplasts (e.g., see section 2.4).

1.2. Effect of SDO length, end-protection and target sequence on the frequency of OTNE

For an efficient OTNE, the length of the homology arm and the lifetime of the SDO inside the plant cells are crucial. Therefore, first, we have designed, synthesized and characterized a set of SDO molecules

of different lengths and modifications aiming for the introduction of targeted nucleotide exchange into the mutant GFP gene (SDO^{GFP}). In our experiments, SDOs with different lengths (30, 38, 40, 41, 72, 90 and 140-mer), structure (native, end-protected by phosphorothioate (PTO) or 2'-OMe bonds) and sequence (complementary to either sense or antisense DNA strand) were used. In case of 38-mer SDOs, there was a significant difference in the frequency of OTNE events, where a 3-fold more GFP-positive cells were detected when using "sense" PTO-modified form as compared to its native, unmodified form. The 38-mer PTO SDO^{GFP}, complementary to the antisense strand and carrying the targeted mutation at the center of the sequence, proved to be the most efficient. Therefore, this structure was chosen as a reference structure for comparison in case of correction of the mGFP gene and the other genes (Acetolactate synthase SDO^{ALS}, anthranilate synthase SDO^{AS} and phytoene desaturase SDO^{PSD}) targeted during our OTNE experiments. Based on OTNE-CRISPR efficiency comparison results, in our CRISPR-based experiments, longer SDOs (72 to 140-mer) served as templates. (See section 2.3, 3.3)

1.3. SDOs with incorporated modified nucleotides

Our next step was the design and synthesis of the 38-mer SDO^{GFP} molecules with different chemical modifications, such as "locked nucleic acid" (LNA), ˝ethylene-bridged nucleic acid˝ (ENA), "2′-fluoroarabino nucleic acid" (FANA) and 5MedC chemical modifications to enhance the number of edited cells. These modified nucleotides were incorporated in different amounts and positions into the 38-mer SDOs. For end-protection, beside PTO bonds, inverted deoxycytosine (dC) was also incorporated in combination with a fluorescent dye. In the present treatment conditions, these structures have led to targeted nucleotide exchange frequencies similar to that of 38-mer PTO SDOs.

1.4. Synthesis of SDO-lipid conjugates for efficient delivery

We aimed to enhance the efficiency of gene editing by improving the delivery of the SDO molecules into plant cells. One of our approaches was the synthesis of oligonucleotide-lipid conjugates by coupling cholesterol or palmityl moieties to the 5´-end of oligonucleotides for enhancing the efficiency of their uptake by protoplasts. As a first step, we synthesized either a long alkyl chain (palmityl)-containing phosphoramidite monomer or a cholesterol-based phosphoramidite monomer. To improve the coupling yields for the cholesteryl-containing conjugates, we modified their structure by extending it with a triethylene glycol unit. After the characterization of these phosphoramidites by mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques, they were successfully coupled to the 5'-ends of oligonucleotides, applying solid-phase oligonucleotide synthesis. Using glycerol branching unit, two and four palmityl-containing oligomers were also synthesized and used along with single palmityl- and cholesteryl-containing ones. The modified oligonucleotides were characterized by ESI-mass spectrometry and their lipophilic properties were investigated by reverse phase HPLC analysis.

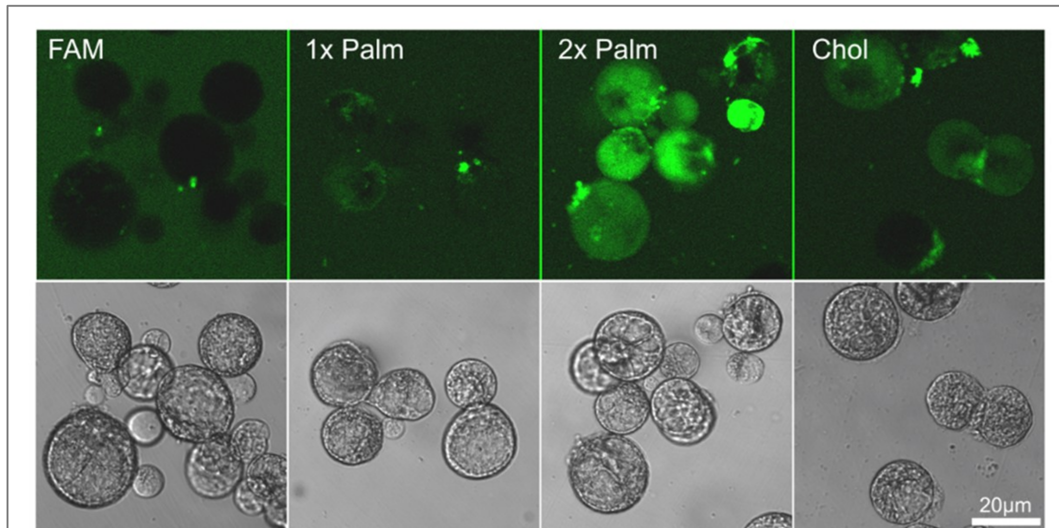


Fig. 1 Delivery of monopalmityl (1x Palm), dipalmityl (2x Palm) and cholestryl (Chol) conjugated green fluorescent (FAM) oligonucleotides to maize protoplasts.

After the optimization of the protocol, we synthesized lipid-modified SDO molecules in large-scale by coupling cholesterol or palmityl moieties to oligonucleotides and used them for delivery experiments. The mono-, di-lipid (palmityl and cholesteryl) derivatives of fluorescently labeled oligonucleotides were tested for cellular uptake efficiency on maize protoplasts using confocal microscopy. Intensive fluorescence was seen inside isolated maize cells after 24 h treatment with dipalmityl and cholesteryl conjugates (**Fig. 1**). The intensity of the dipalmityl derivative was higher than that of the cholesterol-containing one, indicating a more efficient uptake. To increase the efficiency of targeting lipid-modified SDO molecules into the nuclei of maize cells, lipids were coupled to oligonucleotides with an intracellularly cleavable disulfide bond. A manuscript is in preparation regarding the effect of SDO structures on gene editing frequency (Ms. in preparation #1).

Expected results vs. achieved results of TASK 1

- By using novel oligonucleotide constructs, we expected to develop an efficient and optimized technology for targeted nucleotide exchange in higher plants. We have achieved this by optimizing oligomer length, types of chemical modifications, position and number of modified nucleotide units in target sequences for efficient gene editing in maize.
- 38-mer PTO SDO^{GFP}, complementary to the antisense strand with the targeted mutation at the center of the sequence proved to be the most efficient for OTNE experiments. Longer SDOs (e.g.: 72-140mer PTO), however, scored better in CRISPR experiments

Dissemination of results related to TASK 1: Tiricz et al. (2017), Ferenc and Dudits (2017), Rádi et al. (2018, submitted), posters #1, 2, 4, 5, 8, oral presentations #1 ,3

TASK 2: Testing the effects on the OTNE frequency and co-induction of OTNE in genes encoding selectable and non-selectable traits

Researchers: Bettina Nagy, Hilda Tiricz, Elfrieda Fodor, Zoltán Zombori, Ferhan Ayaydin,
Technicians: Zsuzsanna Kószó, Katalin Török, Ildikó Kelemen-Válkony

Workplan for TASK 2

***1. Year**: Bombardment of SDO^{GFP} and SDO^{ALS} molecules with different structures (see Task1) into transgenic maize cells carrying the non-functional mutant GFP gene. In parallel, the SDO molecules will be packaged by cationic liposomes with different compositions and introduced into maize protoplasts. Comparison of the two uptake methods will be based on either the number of GFP positive cells, quantified by flow cytometry, or the chlorsulfuron-resistant micro-colonies. For checking nucleotide exchanges, the targeted genes will be amplified from the extracted genomic DNA of chlorsulfuron-resistant or GFP- positive clones and sequenced.*

***2. Year**: Using parameters optimized for both uptake protocols in the 1st year the SDO^{GFP} and SDO^{ALS} molecules will be co-delivered into the transgenic maize cells. After selection of chlorsulfuron-resistant clones we will search for the GFP-positive ones among them. Stable cell lines will be established from the various genotypes.*

***3. Year**: Molecular characterization of selected clones by sequencing the target genes and confirmation of nucleotide changes. Comparison of the OTNE frequencies resulted from the different chemistry of SDO molecules and from bombardment or liposome uptake system. Furthermore assessing the potentials for targeting two genes into the same cell and inducing simultaneous mutations in a selectable, marker ALS gene and in a gene with a product (GFP) that cannot be exposed to selective pressure in cultured cells*

2.1. Optimization of long-term deep-freezing of stable transgenic maize cultures

In recognition of widespread bacterial contamination in several of our stable transgenic mGFP maize cultures, we focused on long-term deep-freezing of healthy plant cultures to have backup stocks in case such contamination occurs in the future. We used a combination of dimethylsulfoxide (DMSO) and sorbitol as cryoprotection medium and tested different thawing conditions. Using slow replacement of cryoprotection medium with temperature adaptation, we successfully revived long-term deep frozen GFP-transgenic maize cultures which were highly viable (**Fig. 2**). To our knowledge, this is the first record of successful cryoprotection of maize cultures. We have shown that this method is applicable not only for maize but also for tobacco, rice, Arabidopsis and alfalfa cultures (Poster #9).

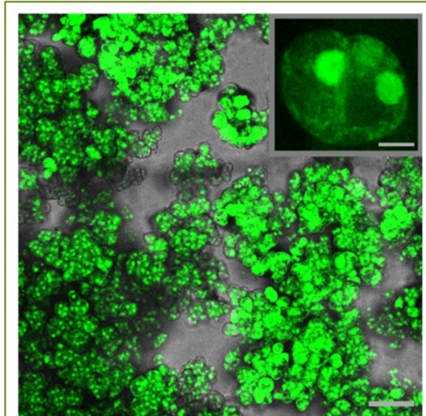


Fig. 2 High viability of liquid nitrogen frozen GFP-transgenic maize cultures after thawing. Inset shows dividing cells after 2 days. Scale bars 100µm and 20µm (inset)

2.2. Relaxed chromatin induced by histone deacetylase inhibitors improves the oligonucleotide-directed gene editing in plant cells

Improving the efficiency of oligonucleotide-directed mutagenesis (ODM) is a prerequisite for a broad application of this gene-editing approach in plant science and breeding. Here we have tested histone

deacetylase inhibitor treatments for induction of relaxed chromatin and for increasing the efficiency of ODM in cultured maize cells. For phenotypic assay we produced transgenic maize cell lines expressing the non-functional Green Fluorescent Protein (mGFP) gene carrying a TAG stop codon. These transgenic cells were bombarded with corrective oligonucleotide as editing reagent to recover GFP expression. Repair of green fluorescent protein function was monitored by confocal fluorescence microscopy and flow cytometry was used for quantification of correction events. Sequencing PCR fragments of the GFP gene from corrected cells indicated a nucleotide exchange in the stop codon (TAG) from T to G nucleotide that resulted in the restoration of GFP function. We show that pretreatment of maize cells with sodium butyrate (5-10 mM) and nicotinamide (1-5 mM) as known inhibitors of histone deacetylases can cause elevated chromatin sensitivity to DNase I that was visualized in agarose gels and was confirmed by the reduced presence of intact PCR template for the inserted exogenous mGFP gene. Maize cells with more relaxed chromatin could serve as an improved recipient for targeted nucleotide exchange as indicated by an average of 2.67–3.62-fold increase in GFP-positive cells (**Fig. 3**). Our results stimulate further studies on the role of the condition of the recipient cells in ODM and testing the application of chromatin-modifying agents in other programmable nuclease-based genome-editing techniques in higher plants (see Tiricz *et al*. 2017).

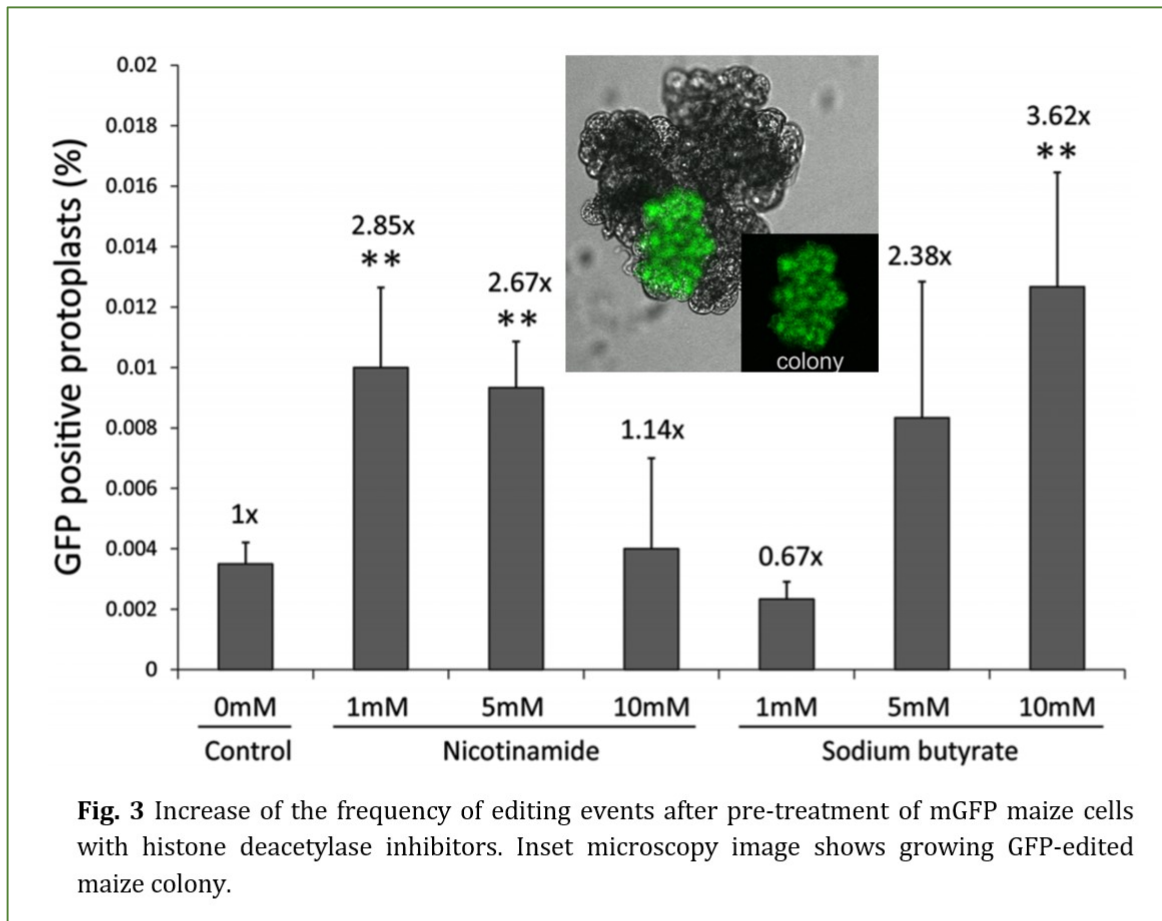
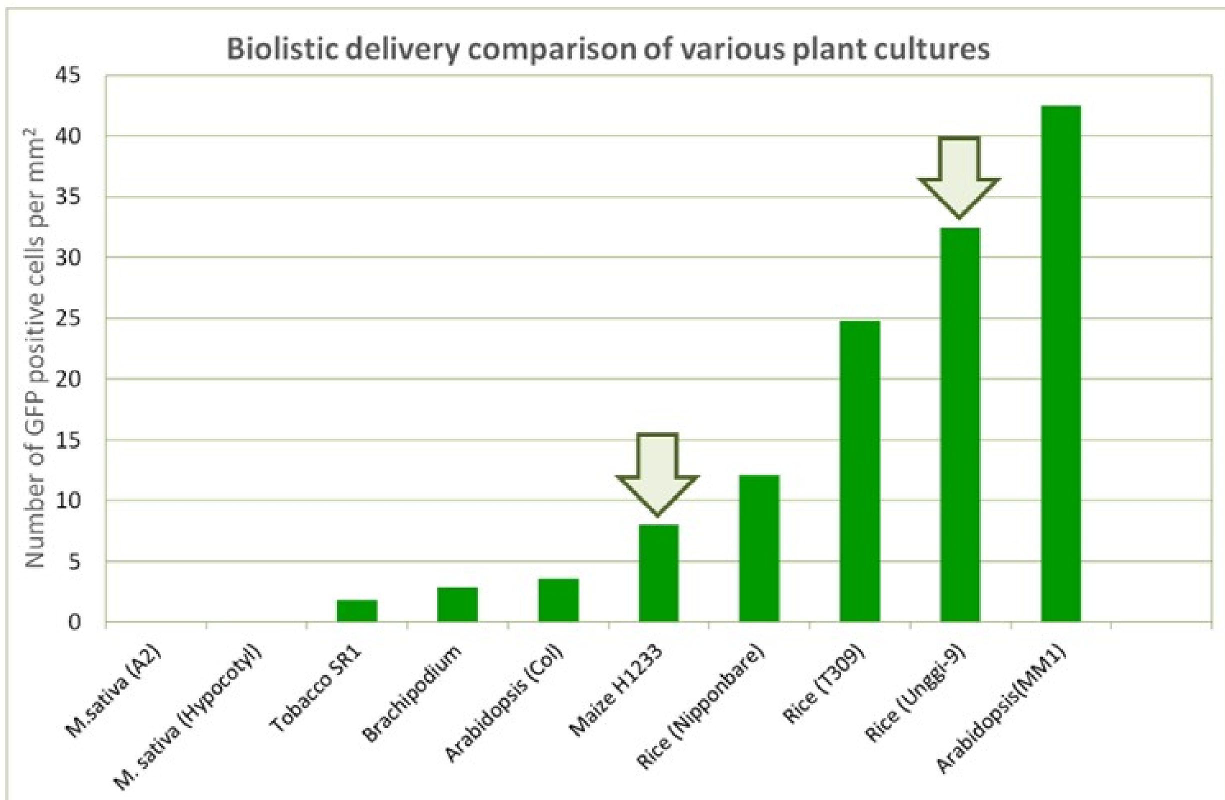
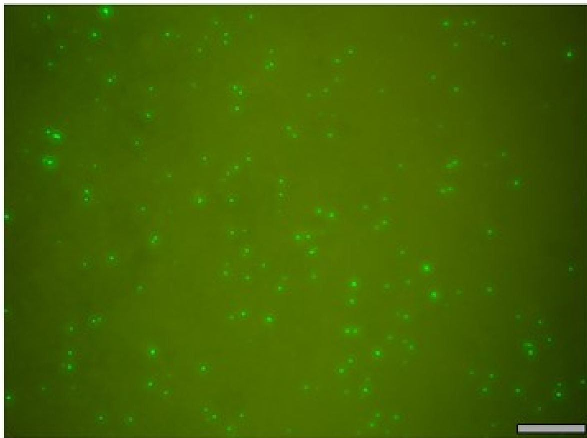
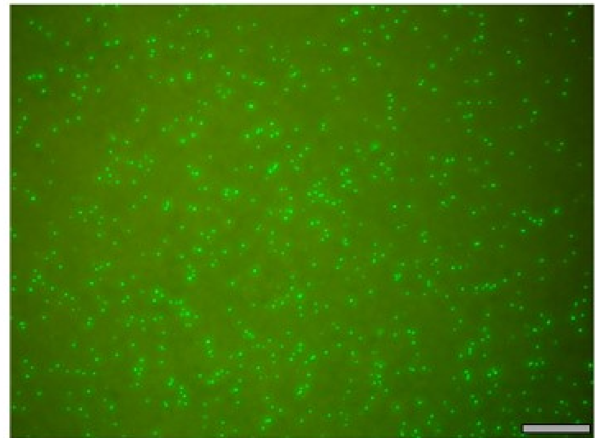


Fig. 3 Increase of the frequency of editing events after pre-treatment of mGFP maize cells with histone deacetylase inhibitors. Inset microscopy image shows growing GFP-edited maize colony.







Zea mays (H1233)

8 GFP^+ cell/mm^2

Oryza sativa (Unggi-9)

32.4 GFP^+ cell/mm^2

Fig. 4 Comparison of various monocot and dicot plant cultures for biolistic delivery efficiency using GFP plasmid bombardment. Fluorescence microscopy images of GFP plasmid-bombarded plates are shown for maize (cv. H1233) and rice (cv. Unggi-9). Scale bars 500 μm (unpublished results).

2.3. Co-editing of acetolactate synthase (ALS) and mGFP genes

In editing genes of agronomic traits, we can face with the problem of how to identify and select mutant cells in *in vitro* cultures. This limitation urges experiments to optimize a protocol that is based on the use of co-editing technology. As an OTNE-based approach, we bombarded the mGFP-containing maize suspension cultures with a mixture of SDOs designed to edit both mGFP gene and ALS gene that can provide chlorsulfuron resistance as a selectable marker. To have a comparative efficiency data, we have also included CRISPR/Cas9-based editing approach to co-edit the same pair of genes (ALS and mGFP) on transgenic maize cultures using Cas9 and purpose-built gRNA plasmids. Since we have discovered that our rice cultivar Unggi-9 is exceptionally efficient in biolistic-based delivery experiments (4x more efficient than maize, **Fig. 4**), we have included this cereal cultivar in our experiments as well. Mutant GFP plasmid was delivered transiently in co-editing experiments to maize and rice. Short and long SDOs with or without PTO modification were used in these co-editing experiments to assess the contribution of oligonucleotide chemistry. Based on the number of GFP-positive cells, we have found that CRISPR/Cas9 system prefers longer SDOs while the OTNE method is more efficient with shorter SDOs. For both editing approaches, PTO-modified SDOs scored significantly better as compared to native SDOs. We have presented these results in poster format nationally and internationally (Posters # 7, 10).

To test the ALS editing events, 1-week-old bombarded cells were transferred to selective media containing 10 μg/L chlorsulfuron (1 μg/L was the threshold for control cultures). We have identified several bright-green fluorescent and/or chlorsulfuron-resistant colonies (**Fig. 5A**). All resistant colonies were picked up from bombarded plates and transferred to new plates while keeping chlorsulfuron selection pressure (**Fig. 5B**). Thirteen independent resistant colonies were sampled and sent for capillary sequencing to assess the point mutations that occurred on ALS gene.

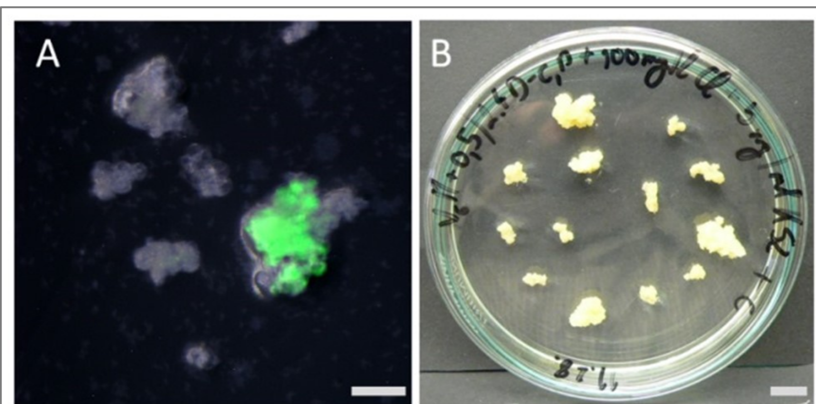


Fig. 5 CRISPR/Cas9 and OTNE co-editing experiments to target genomically integrated mGFP and maize ALS genes. **(A)** mGFP-edited green fluorescent colony growing on chlorsulfuron medium. **(B)** Selected resistant colonies growing on 10 μg/L chlorsulfuron and 100 mg/L antibacterial claforan. Scale bars 100 μm (panel A) and 1 cm (panel B).

Even though the selected colonies were growing on selection media, none of the samples indicated the expected point mutation. To be able to screen a much larger number of resistant colonies with low cost, we have used an alternative approach as well. Designed

ALS mutation imparts a unique NruI restriction site within the target region. Hence, we used the restriction digestion approach for the PCR fragments amplified from the target region. A total of 98 independent resistant colonies were screened this way and none were positive for the expected point mutation. Chlorsulfuron resistance is known to occur due to several other mutation points in ALS gene. Such alternative mutations induced by *in vitro* culturing might be the culprit of this unexpected result. At the 3rd year of the grant period, our original plan of "co-editing ALS and a fluorescent protein gene" has become less "original" due to a report of CRISPR/Cas9-based ALS gene editing in maize with co-delivery of dsRED gene as a marker [Chilcoat *et al*. 2017 Prog. Mol. Biol. Transl. Sci. 149, 27–46]. Therefore, at the last year of the project, we shifted our attention to use maize PDS gene and meristem injection of SDOs as a novel, transgene-free editing approach for maize (see Section 2.5 below).

2.4. Cationic polymer delivery as a novel nucleic acid uptake/gene-editing protocol for protoplasts

Starting with various liposome compositions, we have tried several alternative approaches to increase the delivery efficiency of nucleic acids and editing reagents into maize protoplasts. Various tested liposome formulations and alternative methods such as PEG, PEI, chitosan and poly-L-lysine resulted in relatively high toxicity and low level of delivery efficiency for our maize protoplasts. During a literature search, we have encountered poly (2-hydroxy propylene imine) (pHP) approach which has been reported as an efficient, hydrophilic, low toxicity cationic DNA delivery method for mammalian cells (Zaliauskiene *et al*., 2010; Bioconjug. Chem., 21, 1602–1611). Since this polymer was not commercially available for purchase, our chemist team member Dr. Ferenc has synthesized this polymer which is then dialyzed, lyophilized, solubilized and filter-sterilized. Using GFP plasmid, fluorescent SDOs and advanced confocal microscopes available at our disposal, we have successfully optimized plant-specific buffer components and delivery conditions for this lesser-known nucleic acid delivery reagent with potential applicability in OTNE and CRISPR/Cas9-based gene-editing in plant protoplast-based methodologies (**Fig. 6, 7, 8**, Posters #1, 9). Currently, a manuscript is in preparation regarding this work (Ms. in preparation #2).

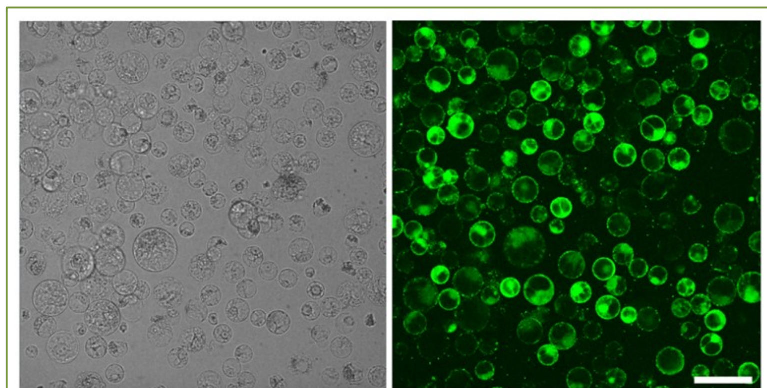


Fig. 6 Highly efficient fluorescent oligonucleotide delivery to maize protoplasts using in-house synthesized enhanced-hydrophilic derivative of cationic polyethylene imine polymer. Scale bar 60 µm.

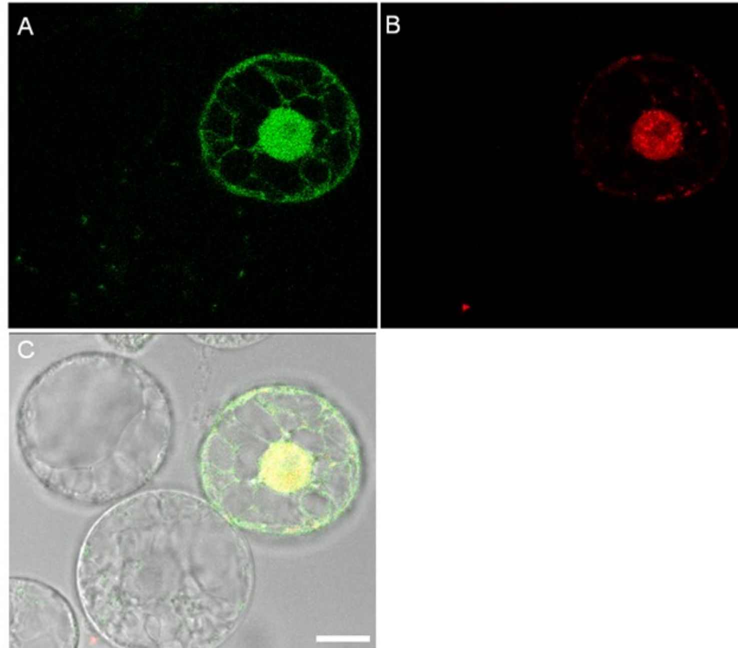


Fig. 7 GFP plasmid and oligonucleotide co-delivery using cationic polymer method on maize protoplasts. Panel **(A)** shows a GFP protein accumulation in cytoplasm and nucleus of a successfully targeted cell. **(B)** Red fluorescent-conjugated (Cy3-labeled) oligonucleotide accumulation in nucleus. Panel **(C)** shows merged fluorescent and bright field images. Scale bar 10 µm.

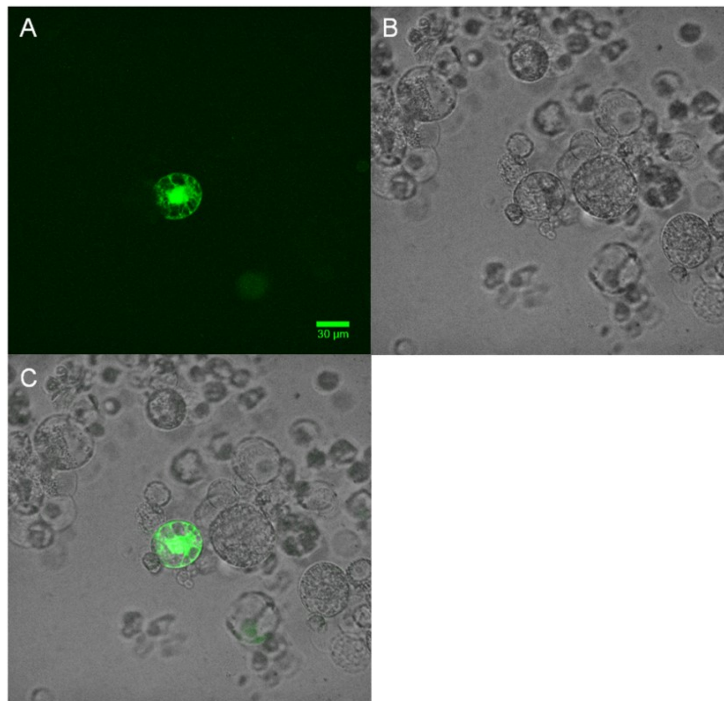


Fig. 8 OTNE-based GFP editing of mGFP transgenic maize protoplasts using the cationic polymer delivery method. **(A)** GFP expressing cell. **(B)** Bright field imaging. **(C)** Merged images. Scale bar 30 µm.

2.5. Direct injection of SDOs into maize meristems as a novel *in planta* gene-editing protocol

Plant regeneration in tissue cultures is an essential step in maize genome editing protocols, currently widely used for both research and breeding. This process can be responsible for the high frequency of off-target mutations; therefore, we have tested an alternative approach, an *in planta* oligonucleotide treatment by injection of the DNA solution into apical meristematic region of haploid maize seedlings. 5′-fluorescein-labeled oligonucleotides were found to be accumulated in cells of the shoot apical meristem and of the vascular bundles of leaf primordia (**Fig. 9**). Introduction of a specific mutation, leading to a STOP codon in the phytoene desaturase (PDS) gene of chlorophyll biogenesis served as a phenotypic marker after the application of SDOs. We have obtained several maize plants, leaves of which were showing distinct white stripes suggesting impaired synthesis of chlorophyll (**Fig. 10**). The white stripes formed in the plants have encompassed the full length of the maize leaves, indicating that a clonal and stable origin of editing event occurred early in the development during meristem injections with mutagenic SDOs. Apart from the white stripes, in one of the injected plants, we have also observed a complete fading of green leaf color with a distinct pale appearance as compared to untreated wild-type leaves (**Fig. 10**). Using a nondestructive maize leaf imaging method developed in the course of this project (Fodor and Ayaydin, 2018), we have imaged the chlorophyll fluorescence of these mutants in high resolution using oil immersion objective and fluorescence confocal laser scanning microscopy. In accordance with the lack of green color, these mutant leaves displayed a significant reduction of chlorophyll fluorescence at regions of white stripes or, in case of pale mutant, at the level of a whole leaf (**Fig. 11**). To confirm the successful editing of PDS gene at the molecular level, we have taken samples from chlorotic regions of the leaves, amplified the related gene sequence using polymerase chain reaction (PCR) and performed next-generation sequencing (NGS) analysis of the target PDS gene. NGS results were in complete accordance with imaging data (**Table1**) and conclusively indicated that the PDS gene editing occurred at the designed target site (Rádi *et al.* 2018, submitted manuscript)

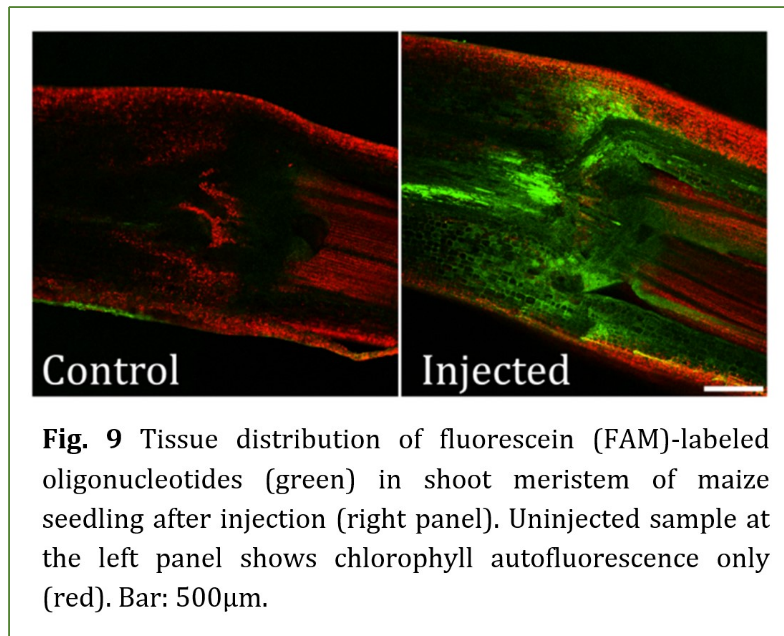


Fig. 9 Tissue distribution of fluorescein (FAM)-labeled oligonucleotides (green) in shoot meristem of maize seedling after injection (right panel). Uninjected sample at the left panel shows chlorophyll autofluorescence only (red). Bar: 500µm.

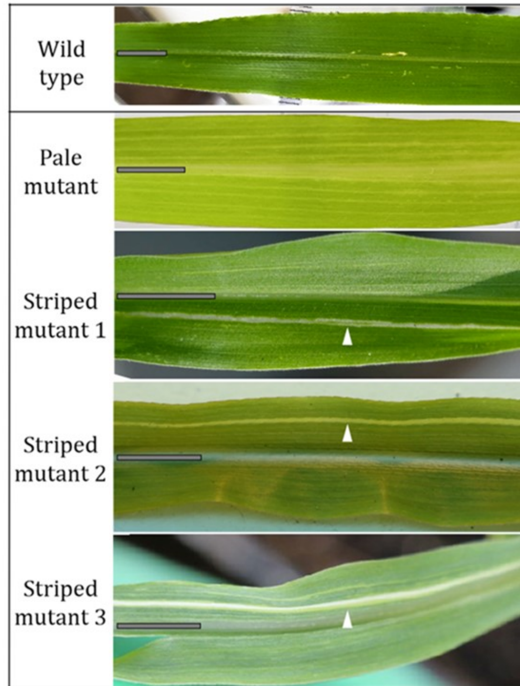


Fig. 10 Chlorophyll deficiency phenotypes in gene-edited maize plants after treatment of shoot apical meristems with mutagenic oligonucleotides specific for the phytoene desaturase (PDS) gene. Pale mutant (2nd from top) displays a complete reduction of chlorophyll synthesis (see also Fig. 7). Arrowheads indicate white stripes formed in edited plants. Gray rectangular scale bars (1 cm) are positioned to mark the position of the midveins of the leaves.

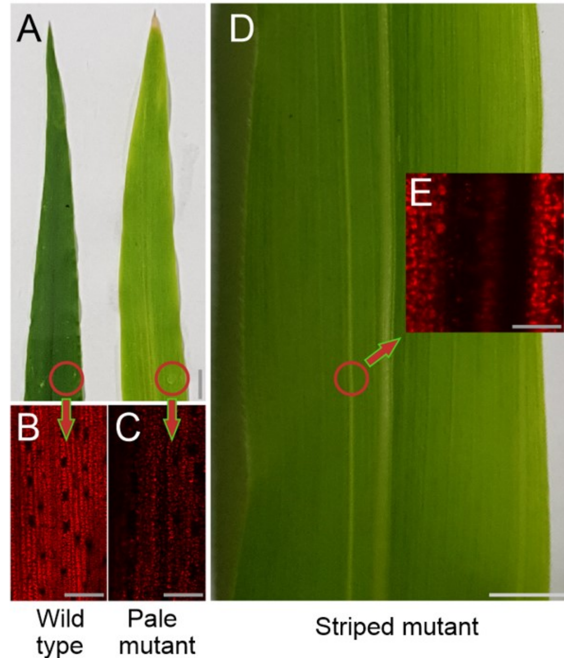


Fig. 11 Reduced chlorophyll fluorescence in maize leaves after oligonucleotide-directed mutagenesis of the phytoene desaturase (PDS) gene. **(A)** A comparative image of wild-type leaf (dark green, left) and oligo-nucleotide-treated, pale green (right) leaf. **(B, C)** Confocal microscopy imaging of parenchyma chlorophyll fluorescence of wild type and pale-green leaf samples shown in panel A. **(D)** Formation of white stripe in a leaf of oligonucleotide-treated sample. **(E)** High-resolution confocal microscopy close-up image of chlorophyll autofluorescence of the white stripe shown in panel D. Note the lack of chlorophyll fluorescence at the mid-region corresponding to the white stripe. Scale bars are 250 mm (A, D), 150 µm (B, C) and 50 µm (E).

A

Mutagenic oligonucleotide	5´- G AAT ATT ACT GGA GCT AGC **TAG** ACA AGA TCT TTT GCG GGC C - 3´
ZmPDS wild type:	5´- ...CT ATG AAT ATT ACT GGA GCT AGC **CAG** **G**CA AGA TCT TTT GCG GGG CAA CTT...- 3´
Mutant sequence variants:	
Target gene read with TAG	5´- ...CT ATG AAT ATT ACT GGA GCT AGC **TAG** **(G/A)**CA AGA TCT TTT GCG GGG CAA CTT...- 3´
Target gene read with ACA	5´- ...CT ATG AAT ATT ACT GGA GCT AGC **(T/C)AG** **ACA** AGA TCT TTT GCG GGG CAA CTT...- 3´
Double mutation reads	5´- ...CT ATG AAT ATT ACT GGA GCT AGC **TAG** **ACA** AGA TCT TTT GCG GGG CAA CTT...- 3´

B

Genotype	Target gene reads with CAG	Target gene reads with TAG	Target gene reads with ACA	Double mutation reads
ZmPDS wild type	14884 (99.966%)	5 (0.033%)	21 (0.141%)	1 (0.007%)
Zmpds mutant N° 1	29488 (99.763%)	66 (0.223%)	115 (0.389%)	52 (0.176%)
Zmpds mutant N° 2	15925 (99.868%)	21 (0.132%)	97 (0.608%)	17 (0.107%)
Zmpds mutant N° 3	13297 (98.459%)	203 (1.503%)	210 (1.554%)	161 (1.192%)
Zmpds mutant N° 4	30629 (97.953%)	627 (2.005%)	640 (2.047%)	491 (1.570%)

Table 1. Ion Torrent sequencing shows targeted nucleotide conversions by injection of synthetic oligonucleotide molecules into the meristematic region of maize seedlings. **(A)** Nucleotide sequence of mutagenic oligonucleotide and wild-type and mutant variant reads of target ZmPDS gene. **(B)** The total number and frequency of reads with characteristic triplets.

Expected results vs. achieved results of TASK 2

- We have optimized conditions and published our results regarding the use of HDAC inhibitors to significantly improve oligonucleotide-directed gene editing in maize.
- Our grant proposal idea of "cationic liposome delivery" approach has successfully evolved into "cationic polymer-based delivery" method for plant protoplasts. We have shown not only efficient plasmid delivery but also SDO-based gene editing while using this new approach.
- We have achieved chlorsulfuron-resistant maize colonies, but mutations did not occur at the expected site. In the case of maize PDS gene editing, however, we have successfully confirmed desired sequence changes by NGS analysis.
- As a novel, "transgene-free genome editing approach for maize", we used for the first time a direct meristem injection technique for maize seedlings. Due to effective oligonucleotide chemistry optimizations of TASK 1 and intensive trial work, we have successfully optimized and applied this unique editing approach for plants, and it has a major benefit of bypassing the somaclonal variation-prone tissue culturing step. Recently, we have submitted our work for publication, and currently, experiments are in progress to use PDS/meristem editing approach for "co-editing" of agronomically important genes.

Dissemination of results related to TASK 2: Tiricz et al. (2017), Fodor and Ayaydin (2018), Rádi et al. (2018, submitted), Posters #3, 7, 9, 10, 11, 12, Oral presentations #2, 4

Workplan for TASK 3

***1. Year**: Based on information from Task 1 and Task 2, various SDO^{AS} molecules will be introduced into maize cells and the frequency of 5-MT-resistant colonies will serve as a parameter in optimizing various protocols. Establishment of cell lines from 5-MT-resistant micro-colonies, DNA extraction for PCR amplification of AS gene and sequencing AS genes. For in vivo expression of functional units of the CRISPR/Cas9 system transient vector molecules will be constructed by integrating elements from the pTaU6-sgRNA (designed for targeting the mGFP or the AS sequences) and pJIT163-2NLSCas9 plasmids. **2. Year:** Transient expression of Cas9 nuclease and sgRNAs from the constructed CRISPR/Cas9 plasmids: the first one is to target "T" to be changed by "G" in mGFP gene and the second one is to cause a "TA" to "GC" nucleotide change for the induction of Y365A mutation in AS maize gene. For TNE by homology-directed repair (HDR), these plasmids encoding the Cas9 nuclease and sgRNAs will be co-bombarded with SDO^{GFP} or SDO^{AS} into the transgenic (mGFP) maize cells. As reference the SDO molecules will be bombarded without plasmids. The efficiency of TNE will be monitored by quantification of GFP-positive cells and of the number of the 5-MT-resistant colonies. Cell lines will be established from the resistant colonies and used for molecular characterization by sequencing of PCR products from the AS genes. **3.Year:** In vitro synthesis of Cas9 protein and sgRNA-targeted for mGFP gene. Production of recombinant Cas9 protein and synthesis of specific sgRNA to construct ribonucleoprotein complex (RNP) from these components. Establish the parameters for proteolistics delivery of this RNP complex with SDO^{GFP} into maize cells. Preparation of cationic liposomes packaged with the RNP complex and with SDO^{GFP} and optimization of the parameters for their efficient delivery into protoplasts. Alternatively, the delivery of the ribonucleoprotein and SDO^{GFP} will be based on mesoporous silica nanoparticles as described by Martin-Ortigosa et al. (2014). Functionality will be tested by monitoring the number of GFP-positive cells for each delivery method.*

TASK 3: Combinatorial use of SDOs and CRISPR/Cas9 system for increasing the efficiency of TNE in mutant GFP gene and the production of feedback insensitive AS (anthranilate synthase) mutants in cultured transgenic maize cells

Researchers : Dénes Dudits, Bettina Nagy Hilda Tiricz, Györgyi Ferenc, Ferhan Ayaydin, Zoltán Zombori, Elfrieda Fodor; Technician: Katalin Török

In the present stage of development/improvement of genome editing tools in plants, the majority of research efforts are being devoted to the CRISPR/Cas9 methodology. The oligonucleotide-directed mutagenesis (ODM) can gain increasing significance if novel protocols can be established, especially for the generation of new agronomic traits. It is a well-established fact that the synthesis of feedback-insensitive anthranilate synthase (AS) in crop plants can result in elevated tryptophan levels. Different amino acid changes in the α-subunit of anthranilate synthase (AS) enzyme can be responsible for insensitivity.
As outlined in the project plan of the grant proposal, 5–MT selection marker, in combination with the correction of mutant GFP gene, can serve as a test system for co-editing selectable and non-selectable traits on cultured plant cells.

3. 1. Targeting anthranilate synthase gene for 5-MT resistance in maize and rice cultures

As shown by the results of TASK2 section, when compared to maize H1233 cultivar, the rice cultivar Unggi-9 displayed 4x higher bombardment efficiency in “biolistic delivery comparison” experiments. Therefore, we included this rice variety in our TASK 3 experiments as well. We have tested and optimized inhibitory concentrations of the 5-MT, both for maize and rice cultures and found that

200 μM 5-MT is the inhibitory threshold concentration for the recipient cells. Since 5-MT is a "false feedback inhibitor" of tryptophan synthesis, in all related experiments, we omitted using media components that may provide external tryptophan for the tested cultures.
We determined the exact DNA sequence of the α-subunit of the anthranilate synthase gene in our maize and rice cultivars by using PCR amplification and Sanger sequencing. In both maize and rice, potential mutation sites that would confer putative tryptophan (Trp) insensitivity could be identified (Maize: S124F, Y365A, Q528D; Rice: S126F, Y367A, L530D). Afterwards, the corresponding mutagenic SDOs of various lengths and modifications were synthesized accordingly. Several factors were taken into consideration during SDO designs, such as the absence of strong secondary structures (e.g., hairpins), favorable GC content and minimized polynucleotide repeats (e.g., GGGG).
Using Benchling CRISPR design software, we have designed appropriate gRNA sequences with minimal off-target hits and built these sequences into maize U3 promoter-bearing pZmU3-gRNA plasmid construct (#53061 from Addgene). We confirmed successful cloning of all inserts by sequencing.

3.2 Transient expression and immunodetection of SpCas9 in maize

To be used in CRISPR/Cas9 experiments, we have ordered two different plasmid constructs harboring the *Streptococcus pyogenes* Cas9 (SpCas9) gene from the Addgene nonprofit plasmid repository. Plasmid #66187 (pYLCRISPR/Cas9Pubi-H) is for the expression of SpCas9 in plants and have maize ubiquitin promoter (Pubi) as well as hygromycin selection. The plasmid #52256 (pFGC-pcoCas9), on the other hand, is a binary plasmid (suitable for both biolistic and Agrobacterium-based delivery) containing 35SPPDK:pcoCas9:NOS cassette for plant expression of SpCas9 and multiple cloning sites for insertion of guide RNA.
Before beginning the delivery experiments, we wanted to be certain that these Cas9 plasmid constructs express the Cas9 protein in our plant cultures when using our biolistic delivery conditions. A free-of-charge Cas-9 antibody sample has been requested from plant antibody specialist Agrisera company in exchange for providing immunolocalization images for the product leaflet of this new product. Each of the Cas9 plasmid constructs together with a GFP plasmid (as a marker) were co-bombarded into maize cells. Following aldehyde-based fixation and a plant cell immunolocalization protocol that has been optimized in our laboratory (Ayaydin *et al*., 2000, Plant Journal, 23:85-96), we successfully detected the expressed SpCas9 protein for both Cas9 constructs in bombarded maize cells using anti-Cas9 antibody. Our representative images are currently on display at Agrisera website (see below) and are being used in the company's Cas9 product leaflet (**Fig. 12**):

https://www.agrisera.com/en/artiklar/cas9-csn1-crispr-associated-endonuclease.html

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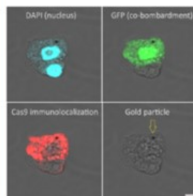
product AS16 3690

Cas9 | CRISPR-associated endonuclease 9 (polyclonal)

Application example



Immunolocalization of Cas9 on maize (*Zea mays*, cv. H1233) plant suspension cultures. Rabbit anti-Cas9 antibody (Agrisera AS16 3690), diluted 1:100 and DyLight 550 anti-rabbit secondary antibody (Agrisera AS11 1782) diluted 1:300, (red) are used for immunodetection following formaldehyde fixation, cell wall digestion and detergent permeabilization of cells. DAPI is used as nuclear marker (blue). Gold particle used for microprojectile bombardment of plasmids (GFP and Cas9) is indicated with an arrow at the last column. Scalebar is 5µm.

Courtesy of Dr. Ferhan Ayaydin, Hungarian Academy of Science, Hungary

Fig. 12 Agrisera product leaflet showing our immunolocalization data regarding Cas9 protein detection after the biolistic delivery of Addgene (#52256) Cas9 plasmid into maize (cv. H1233). Red fluorescent secondary antibody shows Cas9 protein. GFP plasmid (pEGAD) is used as a marker to locate co-bombarded cells.

3. 3. Generation of 5-MT resistant cells using both OTNE and CRISPR/Cas9 approach

During OTNE experiments (where SDOs are used for editing), both maize and rice cultures were bombarded with SDOs of different lengths (38-mer, 41-mer and 81-mer) and chemical modifications (native or PTO) to assess co-editing events. For AS editing, we employed both single-point as well as double-point editing using SDOs that were specifically tailored to the sequenced H1233 maize and Unggi-9 rice AS genes (See section 3.1). Based on the number of GFP positive cells, we have found that, as in the case of ALS co-editing experiments, correction of mutant GFP gene is significantly more efficient in OTNE with shorter, 38-mer PTO-modified SDOs than with longer PTO-modified or native SDOs.

We have successfully edited transiently-delivered mutant GFP plasmids in rice cultures using both OTNE and CRISPR/Cas9 techniques (**Fig. 13**). We have found a significantly higher number of mGFP-edited green fluorescent cells in rice cell cultures than in maize

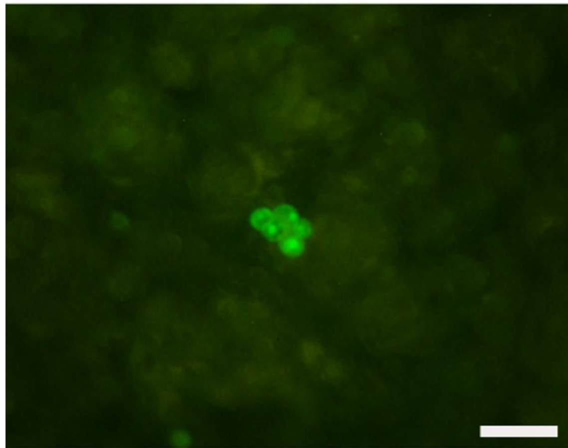


Fig. 13 A dividing GFP-edited rice (Unggi-9) cluster growing on 5-MT plate after the bombardment of cells with SDO^{GFP} (38-mer) and SDO^{AS} (S126F, L530D). Scale bar 100 μm.

cultures. Similar "rice-aptness" were experienced in comparison experiments where the non-transgenic mother maize cell line (H1233) was bombarded in the presence of mGFP plasmid with a variety of different mGFP-editing SDOs with or without CRISPR/Cas9 components. These results revealed that in our experimental conditions, H1233 maize cells, with which we have established mGFP stable cell lines earlier, are much less amenable to biolistic-based gene editing as compared to Unggi-9 cultivar of rice cells regardless of genomic- or transient-editing of mGFP gene. **This piece of data firmly verifies that our grant proposal approach of implementing fluorescent proteins and fluorescence microscopy in plant gene editing studies can be of immense value**. Without the inclusion of mGFP as a visual fluorescent marker of gene editing efficiency or without the results of GFP plasmid bombardments on different plant species and cultivars (see Fig. 4), we would not have been able to get an immediate quantitative data about the inferiority or superiority of certain cell lines with respect to the efficiency of nucleic acid delivery or gene editing. Fluorescence techniques were also highly instrumental in judging the potency and delivery efficiency of specific SDOs, RNAs and Cas9 plasmid variants.

In co-delivery experiments, although a significant fraction of mGFP-edited fluorescent cells was lost during the late phases of 5-MT selection process, the use of mGFP editing in AS experiments proved to be very beneficial, too, as it helped us to quickly pinpoint successfully-bombarded Petri dishes containing "gene editing-receptive" cells (e.g.: unstressed cells with properly functioning cell division cycle in a medium free of competing and inhibitory microorganisms).

Owing to a considerable number of bombardment and optimization experiments and application of a stringent 5-MT selection regime, at the end of the third year of the project, we successfully obtained rice colonies growing on tryptophan-starved, 5-MT containing plates using both OTNE and CRISPR/Cas9 methods (**Fig. 14**). Recently, we have also taken samples from these colonies to be sent for NGS sequencing (in progress).

Despite the obvious inferiority of H1233 maize cell line in our biolistic delivery experiments, we did not cease editing experiments with maize cells and, in agreement with the original project plan, we performed co-delivery experiments on maize using protoplast-based methods. Using the cationic polymer method, we were very successful in delivering plasmids and SDOs to maize protoplasts (section 2.4). In addition to adapting this method

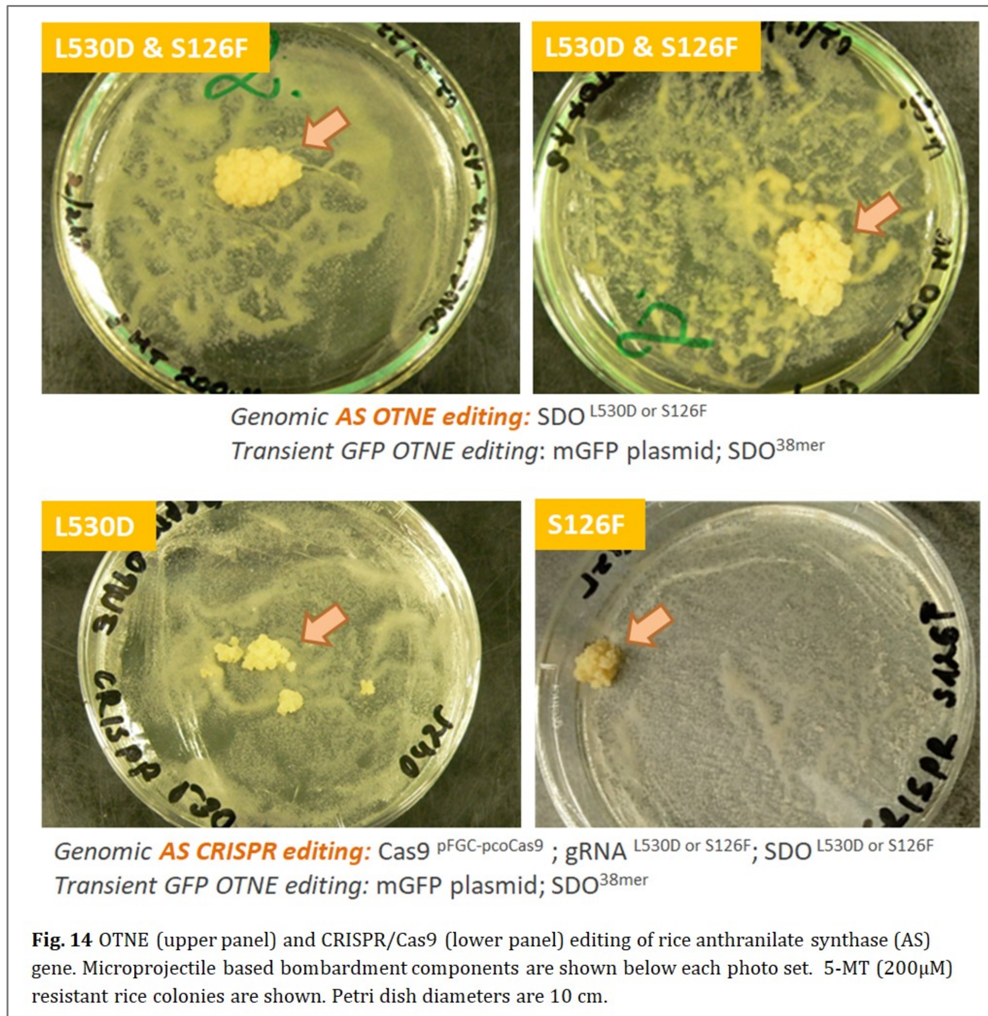


Fig. 14 OTNE (upper panel) and CRISPR/Cas9 (lower panel) editing of rice anthranilate synthase (AS) gene. Microprojectile based bombardment components are shown below each photo set. 5-MT (200μM) resistant rice colonies are shown. Petri dish diameters are 10 cm.

for co-editing of AS and ALS genes, a novel silica-based approach is also currently in progress in our laboratory (see section 3.4 below).

3.4. *In vitro* synthesized CRISPR components for ribonucleoprotein (RNP) complex delivery

The CRISPR/Cas9 system offers two options for using guide RNAs. The single guide RNA (sgRNA) option combines the crRNA and tracrRNA segments into one long RNA molecule, hence reducing the number of components and simplifying the CRISPR editing protocol. On the other hand, *in vitro* syntheses of these long molecules are costly, especially if several versions are needed for protocol optimizations. The two-part system, which we used in our experiments, pairs an optimized, shortened universal tracrRNA oligonucleotide with a shortened target-specific crRNA oligonucleotide for improved targeting of Cas9 to dsDNA targets. Due to its universal sequence, we purchased tracrRNA component from IDT (Integrated DNA technologies) and synthesized various crRNA versions by ourselves. The two RNAs were then annealed at 1:1 molar ratio to generate active guide RNAs which were then mixed with SpCas9 protein to obtain ribonucleoprotein (RNP) complexes.

We have used several sources of Cas9 nuclease. We purchased commercial nuclease (from IDT) as well as mRNA of SpCas9 (Trilink). Additionally, we have also bacterially expressed and purified SpCas9 protein in our laboratory as a third alternative source of Cas9 (Based on Liu *et al*., 2015, Mbio, 6, e01714-15). The classical ethanol precipitation-based nucleic acid delivery method did not result in editing events by bombardment of SDOs, Cas9 mRNA and gRNA. As an alternative approach, we have applied a cationic lipid-based biolistic delivery (TransIT-2020, Mirus), using either our purified SpCas9 or the commercially purchased SpCas9 nuclease. This alternative approach has not resulted in GFP positive or 5-MT resistant colonies, either. We suspect that this result may be partly due to suboptimal suitability of H1233 maize cultivar for biolistic-based delivery methodologies (see **Fig. 4**). Due to its better suitability, experiments are currently in progress to test RNP delivery-based editing in Unggi-9 cultivar of rice.
Since biolistic delivery to our maize cultivar was less efficient, we also tested protoplast-based approaches. mCherry RNA (from Trilink) was used as a marker in initial protoplast delivery experiments with liposomes, which caused reduced protoplast viability. Currently, we are testing in-house synthesized pHP cationic polymer approach (See 2.4) to deliver RNP components to mGFP-transgenic maize protoplasts. Development of a novel delivery method incorporating mesostructured, hexagonal silica particles (kindly provided by Prof. H.A. Öktem, METU University, Turkey) doped with in-house synthesized pHP cationic polymers is also underway.

Expected results vs. achieved results of TASK 3

- Our research plan had been built around the extensive use of fluorescence techniques for maize gene editing. We successfully disseminated several data (publications, poster, talks, even product leaflets) that proved the validity of this strategy.
- While the RNP approach or AS-editing of maize has not yet met our expectations, we could achieve 5MT-resistance with rice using both OTNE and CRISPR techniques. NGS analyses of these clones will conclusively determine whether desired mutations occurred or observed resistance is due to somaclonal variation and random mutations which happen to occur during *in vitro* culturing (e.g., see section 2.3, ALS editing).
- Given the drawbacks involved with *in vitro* culturing and in the light of "the title and main aim" of our grant proposal, we can conclude that direct meristem injection of SDOs to seedlings (Section 2.5) may be a much more viable option for maize (and possibly for other cereals) to obtain "**Transgene-free gene-specific editing with synthetic oligonucleotides"**

Dissemination of results related to TASK 3: Ferenc and Dudits (2017), Fodor and Ayaydin (2018), Posters #4 6, 7, 10 Oral presentations #2, 4.

DISSEMINATION OF RESULTS

Grant-supported publications (grant participants underlined)

1. Tiricz H, Nagy B, Ferenc G, Török K, Nagy I, Dudits D, Ayaydin F (2017) Relaxed chromatin induced by histone deacetylase inhibitors improves the oligonucleotide-directed gene editing in plant cells *Journal of Plant Research* 131: 179.
2. Ferenc G and Dudits D (2017): Génspecifikus mutagenezis rövid szintetikus DNS-molekulákkal, *Precíziós nemesítés* – Kulcs az agrárinnovációhoz. Eds: Ervin Balázs and Dénes Dudits. Publisher: Agroinform, 95-104. (English translation of book chapter title: Gene-specific mutagenesis with short synthetic oligonucleotides)
3. Ayaydin F, Kotogány E, Ábrahám E, Horváth GV (2017) Detection of Changes in the *Medicago sativa* Retinoblastoma-Related Protein (MsRBR1) Phosphorylation During Cell Cycle Progression in Synchronized Cell Suspension Culture, "Methods Molecular Biology" Cell Cycle Synchronization, Ed: Gáspár Bánfalvi. Publisher: Springer, Vol. 1524, 267-285. (Related to grant due to "Flow cytometry analysis of nuclei")
4. Fodor E and Ayaydin F (2018) Fluorescent Probes and Live Imaging of Plant Cells. In: Sánchez-Moreiras A., Reigosa M. (eds) *Advances in Plant Ecophysiology Techniques*. Springer, Cham. 241-251 (Related to grant due to "Noninvasive, high resolution live imaging of potted maize plants and PPT-resistant transgenic maize cultures")
5. Rádi F, Nagy B, Ferenc G, Török K, Nagy I, Zombori Z, Dudits D, Ayaydin F (2018, submitted) Targeted mutagenesis in maize somatic cells by injection of synthetic oligonucleotides into the apical meristem region of seedlings. (See abstract of submitted manuscript at the end of document)

Grant-supported poster presentations (Presenter in bold, grant participants underlined)

1. Rakk D, Bodnár B, Kincses F, **Ferenc G**, Nyerges Á , Mészáros M, Veszelka Sz, Deli M, Dudits D, Kovács L, Kupihár Z (2015) Lipid derivatives of oligonucleotides-Preliminary Results. *EuroTIDES conference, Berlin, Germany*
2. **Ferenc G**, Váradi Z, Bokros A, Kupihár Z, Fodor E, Dudits D and Ayaydin F (2017) Studies on Uptake of Oligonucleotide-Lipid Conjugates. *TIDES Oligonucleotide & Peptide Therapeutics conference*, San Diego, *CA, USA*
3. Öktem A, Ferenc G, Nagy B, Kalac A, Fodor E, Dudits D, **Ayaydin F** (2018) Delivery of nucleic acids and gene editing components by using a modified cationic polymer. *6th Plant Genomics and Gene Editing Congress: Europe, Rotterdam, Netherlands*
4. **Ferenc G**, Kupihár Z, Fodor E, Kukli T, Szamosvölgyi Á, Nagy B, Zombori Z, Öktem A, Nagy I, Dudits D and Ayaydin F (2018). Chemically modified template ssDNAs and gRNAs for increasing the efficiency of plant gene editing. *6th Plant Genomics and Gene Editing Congress: Europe, Rotterdam, Netherlands*
5. **Ferenc G**, Kerner K, Váradi Z, Bokros A, Kupihár Z, Fodor E, Dudits D and Ayaydin F (2017) Studies on Uptake of Oligonucleotide-Lipid Conjugates, *Straub Days conference, Szeged, Hungary*
6. **Nagy B**, Ferenc G, Török K, Nagy I, Dudits D and Ayaydin F (2018) GFP-based transient gene editing marker system to support selection of agronomic traits: A case study of targeting elevated

tryptophan content in maize. *6th Plant Genomics and Gene Editing Congress: Europe, Rotterdam, Netherlands*

7. **Zombori Z,** Török SP, Nagy B, Ferenc G, Török K, Dudits D and Ayaydin F (2018) Comparison of two different genome-editing technologies (OTNE and CRISPR/Cas9) in maize (*Zea mays* L.) and rice (*Oryza sativa* L.) cells. *6th Plant Genomics and Gene Editing Congress: Europe, Rotterdam, Netherlands*
8. **Ferenc G**, Kupihár Z, Kukli T, Szamosvölgyi A, Kóczán L, Nagy B, Öktem A, Dudits D and Ayaydin F (2018) The effect of chemical modifications of oligonucleotides on gene editing efficiency in plant cells. *Straub days conference, Szeged, Hungary*
9. **Kelemen-Válkony I**, Ayaydin F (2018) Efficient long term cryopreservation of plant cultures. *Straub days conference, Szeged, Hungary* (Related to grant due to "Successful long-term freezing and viable thawing of GFP transgenic maize cultures")
10. Török SP, Török K, Dudits D, Ayaydin F, **Zombori Z** (2018) Comparison of different genome-editing technologies (CRISPR/Cas9 and OTNE) in maize (*Zea mays* L.) and rice (*Oryza sativa* L.) *Straub days conference, Szeged, Hungary*
11. **Nagy B**, Tiricz H, Ferenc G, Török K, Nagy I, Dudits D, Ayaydin F (2018) Relaxed chromatin induced by histone deacetylase inhibitors improves oligonucleotide-directed gene editing in plant cells. *Straub days conference, Szeged, Hungary*
12. **Öktem A**, Ferenc G, Kalac A, Fodor E, Dudits D, Ayaydin F (2018) Novel polymer-based delivery of gene editing components in plants. *Straub days conference, Szeged, Hungary*

Grant-supported oral presentations (Presenter in bold, grant participants underlined)

1. **Ferenc G**, Váradi Z, Bokros A, Kupihár Z, Fodor E, Dudits D, Ayaydin F (2017) Studies on uptake of oligonucleotide-lipid conjugates. *Annual meeting of the Working Committee for Carbohydrates, Nucleic Acids and Antibiotics of the Hungarian Academy of Sciences, Mátraháza, Hungary.*
2. **Dudits D** (2017) Genome editing in precision breeding for health benefits. *21. Word Congress of Clinical Nutrition. Budapest*.
3. **Ferenc G**, Kupihár Z, Nagy B, Zombori Z, Öktem A, Fodor E, Nagy I, Dudits D, Ayaydin F (2018) Chemically modified template ssDNAs and gRNAs for increasing the efficiency of plant gene editing. *Annual meeting of the Working Committee for Carbohydrates, Nucleic Acids and Antibiotics of the Hungarian Academy of Sciences, Mátrafüred, Hungary.*
4. **Ayaydin F**, Nagy B, Ferenc G, Öktem A, Zombori Z, Fodor E, Dudits D (2018) Use of fluorescence microscopy and mutant green fluorescent protein in plant gene editing. *Annual Meeting of Hungarian Society for Microscopy, Siófok, Hungary.*

Grant-supported manuscripts in preparation (grant participants underlined)

1. Ferenc G, Kupihár Z, Nagy B, Zombori Z, Öktem A, Fodor E, Nagy I, Dudits D, Ayaydin F
Chemically modified template ssDNAs and gRNAs for increasing the efficiency of plant gene editing
2. Öktem A, Ferenc G, Nagy B, Kalac A, Fodor E, Dudits D, Ayaydin F
Delivery of nucleic acids and gene editing components by using a modified cationic polymer

Grant-results inspired oral presentations in Hungarian language for the general public as well as for the experts in the field

1. **Dudits D**: (13 Dec 2016) Precíziós nemesítés az élhetőbb világért. *Mindenki Akadémiája előadás, Budapest* [Translation: Precision breeding for a livable world, Presentation at *Everybody's Academy, Budapest*]
2. **Dudits D** (18 Oct 2017). Precíziós nemesítés, mint biogazdálkodási innováció. *SZIE kertészeti Kar előadás, Budapest.* [Translation: Precision breeding as an innovation of organic farming. Presentation at *SZIU Faculty of Horticultural Science, Budapest*]
3. **Dudits D** (6 Nov 2017). Precíziós nemesítés, mint biogazdálkodási innováció. *Magyar Tudomány Ünnepe MTA székház előadás, Budapest* [Translation: Precision breeding as an innovation of organic farming. Presentation at *Celebration of Hungarian Science, Hall of Hungarian Academy of Sciences, Budapest*]
4. **Dudits D** (12 Jan 2018) Új utakon a rezisztencia génekre alapozott növényvédelem. *KITE Zrt. Továbbképzés Hévíz előadás* [Translation: New ways of pest control based on resistance genes. Presentation at *KITE Zrt. Training, Hévíz*]
5. **Dudits D** (7 Feb 2018) Precíziós nemesítés: a géntechnológia és az agrár-innováció kapcsolata. *Agrárkonferencia Visegrád, előadás* [Translation: Precision breeding: the relationship between genetic engineering and agroinnovation. Presentation at *Agrarian Conference, Visegrád*]
6. **Dudits D** (28 Feb 2018)) Precíziós nemesítés az élhetőbb világért. *Miskolci Akadémiai Bizottság előadás* [Translation: Precision breeding for a livable world. Presentation at *Academy Committee, Miskolc*]
7. **Dudits D** (7 May 2018) A genetikai kód nagy pontosságú átprogramozása. *MTA Közgyülési előadás, Budapest* [Translation: Highly accurate reprogramming of the genetic code. Presentation at *General Meeting at Hungarian Academy of Sciences, Budapest*]
8. **Dudits D** (11 Sep 2018). Egészséges élelmiszer precíziós nemesítéssel. *Jövő Nemzedék Bizottság előadás, Budapest* [Translation: Healthy food with precision breeding. Presentation at *Future Generations Commissioner, Budapest*]

Abstract of the manuscript submitted to the Plant Journal

Targeted mutagenesis in maize somatic cells by injection of synthetic oligonucleotides into the apical meristem region of seedlings

Feríz Rádi, Bettina Nagy, Györgyi Ferenc, Katalin Török, István Nagy, Zoltán Zombori, Dénes Dudits, Ferhan Ayaydin

Summary

Genome editing tools include the use of synthetic oligonucleotides for targeted exchange of nucleotides in plants, too. A lack of plant regeneration capability in in vitro tissue cultures can limit the development of maize genome editing protocols for research and breeding. We tested an alternative, in planta oligonucleotide treatment by injection of DNA solution into apical meristematic region of haploid maize seedlings. Using 5′-fluorescein-labeled oligonucleotides, we detected accumulation of synthetic DNA molecules in cells of the shoot apical meristem and of the vascular bundles of leaf primordia. Introduction of a specific mutation in the phytoene desaturase gene of chlorophyll biogenesis served as a phenotypic marker after the application of single-stranded oligonucleotides. The 41 nucleotide long molecule was synthesized with TAG stop codon and a marker ACA triplet. Confocal microscopy of leaves from M1 maize plants showed a lack of chlorophyll fluorescence in white leaf stripes, or reduced fluorescence signal in pale-green leaves on the outgrowing plantlet. Sequence confirmation of gene editing by Ion Torrent sequencing indicated the presence of mutant and wild type cell populations indicating chimeric tissue formation in meristematic region. Double mutations with the TAG stop codon and the ACA marker triplet were detected in the phytoene desaturase gene from white or pale-green sectors from fully developed leaves. The experiments presented show the mutagenic nature of oligonucleotide molecules after application into the shoot meristematic region of maize seedling. This genome editing methodology can overcome limitations of tissue culture systems and prevent the generation of somaclonal variations.

Significance statement

The described protocol for the oligonucleotide directed mutagenesis is based on *in vivo* treatment of shoot apical meristem, which eliminates the use of tissue culture system and induction of somaclonal variations.