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SHORT COMMUNICATION

In planta test system for targeted cellular mutagenesis by injection of oligonucleotides to apical meristem of maize seedlings

Feríz Rádi[1,2,3] · Bettina Nagy[1,7] · Györgyi Ferenc[1] · Katalin Török[1] · István Nagy[4,5] · Zoltán Zombori[1] · Dénes Dudits[1,2] · Ferhan Ayaydin[6,7]

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Abstract

Genome-editing tools from Oligonucleotide-Directed Mutagenesis (ODM) to CRISPR system use synthetic oligonucleotides for targeted exchange of nucleotides. Presently, majority of genome-editing protocols are dependent on the in vitro cell or tissue culture systems with somaclonal variation, and limitations in plant regeneration. Therefore, here, we report an alternative *in planta* cellular test system for optimization of the ODM, based on the injection of oligonucleotide solution into the 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. For silencing or knocking down of the phytoene desaturase gene in somatic cells, 41-mer long single-stranded oligonucleotides with TAG stop codon were injected into maize seedlings. We detected out-growing M1 plantlets that developed leaves with white stripes or pale-green color. Confocal microscopy of white stripes showed that in addition to the chlorophyll fluorescence-deficient tissue region, chlorophyll containing cells are present in white stripes. The Ion Torrent sequencing of DNA samples from the white stripes indicated 0.13–1.50% read frequency for the TAG stop codon in the phytoene desaturase gene. Appearance of chlorotic abnormalities supports the mutagenic nature of oligonucleotide molecules after injection into the shoot apical meristem region of maize seedling. The described protocol provides basis for early seedling stage characterization of functionality of a mutagenic oligonucleotide with different chemistry and testing efficiency of various treatment combinations at plant level.

Keywords Chlorotic mutant · Maize · Oligonucleotide-directed mutagenesis (ODM) · Phytoene desaturase gene · Shoot meristem · Test system · *In planta*

Introduction

Current genome-editing technologies including the most preferred CRISPR/Cas9 system introduce double-stranded (ds) DNA breaks by nuclease enzymes at a target locus in plant genomes (Jaganathan et al. 2018). Alternatively, editing of plant genes via generation of single base mutation was successfully achieved by introducing single-stranded DNA oligonucleotides (SDOs) into plant cells or protoplasts and regeneration of mutant plants (Rivera-Torres and Kmiec 2016; Sauer et al. 2016). The oligonucleotide-directed mutagenesis (ODM) and the use of programmable nucleases represent alternative as well as complementary approaches with different molecular mechanisms (Gocal 2015). The template-directed repair of double-stranded breaks in the presence of exogenously supplied SDOs was also tested in plant systems (Svitashev et al. 2015; Wang et al. 2015; Sauer et al. 2016).

In practice, for ODM, the chemically synthesized oligonucleotide molecules are introduced into plant protoplasts, cells, embryo-derived callus tissues cultured in vitro. Most frequently, SDO molecules are precipitated onto gold microprojectiles and bombarded into cultured cells. As pioneering work, Beetham et al. (1999) bombarded cells from tobacco suspension culture with chimeric oligonucleotides composed of DNA and modified RNA residues and the mutated acetolactate synthase gene imparted chlorsulfuron resistance.

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Feríz Rádi and Bettina Nagy contributed equally to this work.

✉ Györgyi Ferenc
ferenc.gyorgyi@brc.hu

Extended author information available on the last page of the article

In maize, both immature embryo-derived callus tissues and cultured cells were bombarded with synthetic SDOs. Plant protoplasts as membrane surrounded cells can serve as recipient for SDO molecules. Either PEG-mediated uptake or electroporation was successfully used for oligonucleotide delivery into tobacco, oilseed rape, and Arabidopsis protoplasts (Kochevenko and Willmitzer 2003; Ruiter et al. 2003; Kim and Kim 2014; Gocal 2015).

Thus far, the reported methodologies for ODM are based on different tissue culture techniques that ensure fertile plant regeneration from the mutated cells cultured in vitro. Since the morphogenic or embryogenic potentials of dedifferentiated callus tissues are highly dependent on the genotype also in maize (Armstrong et al. 1991), this component in editing protocols can limit wider use of ODM as a new breeding technique. Therefore, in attempts to extend the application of this technology, various *in planta*-editing protocols may gain preference.

Uptake of SDO molecules into cells of plant organs and their functionality were primarily studied using antisense oligonucleotides. There are several reports about the uptake of these molecules into intact plant tissues such as leaves (Sun et al. 2005; Dinc et al. 2011), fast-growing pollen tubes (Liao et al. 2013) or whole plants (Xie et al. 2014). One candidate for ODM treatment can be the shoot apical meristem (SAM) that was extensively used explant for transformation protocols (Sticklen and Oraby 2005; Baskaran and Dasgupta 2012; Baskaran et al. 2016).

As an initial step, here, we report a test system for ODM in somatic haploid cells of maize seedlings. Albino marker mutations were induced in shoot apical meristem region of the seedlings by injection of premature STOP codon-bearing synthetic oligonucleotides that are homologous to the phytoene desaturase gene. Development of leaves with white stripes or reduced fluorescence signal in pale-green leaves served as phenotypic indication for the functionality of mutagenic SDOs. Statistical analysis of NGS datasets confirmed specific mutation events. The experimental approach described here opens a way for functional characterization of synthetic oligonucleotides with chemical modifications or optimizing cellular stage by modification of chromatin structure (Tiricz et al. 2018).

Materials and methods

Growth and treatment conditions of maize haploid seedlings

For the present test system's development, we used haploid maize genotype produced by crossings between the maize haploid inducer line and the female parent. The marker system using the R1-nj allele of the r1 gene locus allowed visual separation of haploids and diploids by seed phenotyping of anthocyanin coloration. The maternal haploid progenies showed purple pigmentation in the endosperm tissue on the crown of the kernel and colorless embryos. At the whole plant level, the haploid genome size was confirmed by the flow cytometry of nuclei isolated from leaves as described before (Tiricz et al. 2018).

Haploid kernels were germinated in wet and rolled filter paper aligned vertically in 3 L beaker filled with 0.5 L tap water. The germinated haploid seedlings reached about 2 cm long coleoptile size after 6 days in 16 h light/8 h dark at 24 °C room temperature. At this stage, they were cut horizontally approximately 1 cm above the coleoptile-mesocotyl junction, and the meristem region was pierced vertically (through the cut side) with a 27G needle. The synthetic oligonucleotides representing the target sequence of *PDS* gene were dissolved in nuclease-free water at 100 µM concentration, selected in several test experiments. Oligo solution (3 µL) was injected into the meristem region of each plant. These treated seedlings were planted in styrofoam trays containing peat moss and grown in a glasshouse with 16 h light/8 h dark at 28 °C up to maturity.

Design and synthesis of FAM-labeled or mutagenic oligonucleotides

The 41-mer single-stranded DNA oligonucleotide named SDOPDS (5′-g aaT ATT ACT GGA GCT AGC **TAG** ACA AGA TCT TTT GC*G ggc* C-3′, lowercase letters stand for phosphorothioates) was designed for creating a STOP codon into maize *PDS* gene. The oligonucleotide was placed as close to the start codon as possible with the targeted mutation in the middle of the oligonucleotide. To help the synthesis and purification of the SDO, the *GGGG* quartet was changed to *GGGC*. Finally, the specificity of the SDO^{PDS} sequence was checked in maize genome using NCBI blast.

The chemical synthesis of the SDO^{PDS} molecule was performed using a DNA/RNA/LNA H-16 synthesizer (K&A Laborgeraete) by standard β-cyanoethyl phosphoramidite chemistry at a nominal scale of 0.2 µmol. For oligonucleotide uptake studies, a 40-mer SDOs representing random nonsense sequences were labeled at the 5′-end by fluorescein (6-FAM). SDO^{PDS} was purified by reverse phase HPLC followed by cation exchange using Dowex 50 resin.

The reagents for automated SDO synthesis and fluorescent labeling were from Link Technologies, Chemgenes, Sigma Aldrich, and Molar Chemicals Kft.

PCR amplification and sequencing of the phytoene desaturase gene

Genomic DNA from the untreated control and the SDO-treated maize plant's leaves with phenotypic symptoms were

isolated with a CTAB-based extraction method according to Doyle (1990).

For analysis of the oligonucleotide-targeted nucleotide exchange, PCR amplifications were performed with the following phytoene desaturase gene-specific primers: *ZmPDS*_Forward: 5′-CAGTAGTCTGCCTGTACCTATTG-3′, *ZmPDS*_Reverse: 5′-CGGTGTGATCTCCTTCCTTTAT-3′) with Ion P1 and different barcode adaptors. Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Scientific) was used for the PCR reactions with the following cycle conditions: 1. Initial denaturation: 98 °C for 3 min. 2. Denaturation: 98 °C for 30 s. 3. Annealing: 63 °C for 45 s. 4. Extension: 72 °C for 30 s. (2–4 steps: 30 cycles) 5. Final extension: 72 °C for 10 min. Amplified fragments were purified by GeneJet Gel Extraction kit (Thermo Scientific) according to the manufacturer's recommendation. Fragments of 312 nucleotides were used for Ion Torrent sequencing, which were performed as described earlier in Tiricz et al. (2018).

Microscopy and imaging

Fluorescent oligonucleotide-injected apical meristem regions of 6-day-old hand sectioned maize seedlings were imaged using Leica SP5 laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) either by directly layering intact potted plants' leaf over the immersion objective using a coverslip-bottom Petri dish (Fodor and Ayaydin 2018) or by excision of the leaf and mounting on 24 × 50 mm coverslip. For chlorophyll imaging, 543-nm laser and a detection range of 650–750 nm were used. For cell wall autofluorescence, 405-nm laser excitation and 415–530-nm emission range were used. Macroscopic leaf images were captured using Samsung Galaxy Note 8 mobile phone camera (Samsung Electronics, Seoul, South Korea). Plot profile measurement of selected rectangular areas was performed using Fiji software (Schindelin et al. 2012). Exported intensity values of plot profiles were used to plot the charts in Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Composite images were prepared using CorelDraw Graphics Suite X7 (Corel Corporation, Ottawa, Canada).

Results and discussion

Detection of FAM-labeled oligonucleotides in maize meristematic tissues after injection into the basal region of seedling

To monitor the uptake and tissue distribution of SDO molecules injected into the basal section of seedlings, 40-mer SDOs nonsense sequences were synthesized with covalently attached 5′-fluorescein (6-FAM representing random) dye. The localization of these molecules was analyzed by confocal laser scanning microscopy. In the longitudinal sections of seedlings after 24 h of injection, the green fluorescence intensity was high in shoot apical meristem and vascular bundles of leaf primordia (Fig. 1a, c). Close-up imaging of the meristem region showed fluorescent material to be accumulated inside and around the cells (Fig. 1d, e with arrowheads). Lack of green fluorescence in water-injected control samples indicated that the observed green fluorescence of injected samples was not due to artifactual wounding-induced autofluorescence (Fig. 1b).

Experimental evidence for introducing synthetic oligonucleotide molecules into the tissues and cells of different plant organs are only known from studies with antisense oligonucleotides (asSDOs). These molecules could be infiltrated in intact tobacco leaves with the help of a syringe or by vacuum treatment (Dinc et al. 2011; Wojtasik et al. 2014). The wheat leaves were cut, and the lower 1- to 2 cm part was submerged in asSDO water solution. For the present ODM treatment, we target maize shoot apical meristem at an early developmental stage of seedlings. Cereal shoot apical meristems were widely used for different genetic transformation protocols including Agrobacterium infection or microinjection of the shoot apical meristem explants (reviewed in (Sticklen and Oraby 2005). Cells of the L2 layers of isolated shoot meristems from maize were microinjected with vector constructs for anthocyanin production (Lusardi et al. 1994). In the present study, the high-resolution confocal laser scanning microscopy showed that the SDO molecules could be accumulated in meristematic cells (Fig. 1).

Chlorotic phenotypes of leaves from the outgrowing M1 haploid maize plants

The primary goal of the present study was to establish a gene-specific mutagenesis test system by injection of synthetic plus-strand SDO molecule solution into the meristematic region of young maize seedlings. For phenotypic monitoring of mutagenic events in haploid somatic cells, we selected the phytoene desaturase (*ZmPDS*) gene, encoding a key enzyme in carotenoid biosynthesis, and that was widely used in genome-editing experiments with CRISPR/Cas9 system (Jia and Wang 2014; Wang et al. 2015; Nishitani et al. 2016; Odipio et al. 2017). T-DNA insertion mutant of Arabidopsis *PDS3* gene showed dwarfism and albino phenotypes due to impairment in chlorophyll, carotenoid, and gibberellin biosynthesis (Qin et al. 2007). For functionality test of SDO molecules, we used haploid maize seedlings to detect mutation events by appearance of chlorotic phenotype in the SDO-treated M1 generation plants. After crossing with the maize haploid inducer line, haploid kernels were selected on the basis of the anthocyanin pigmentation encoded by the

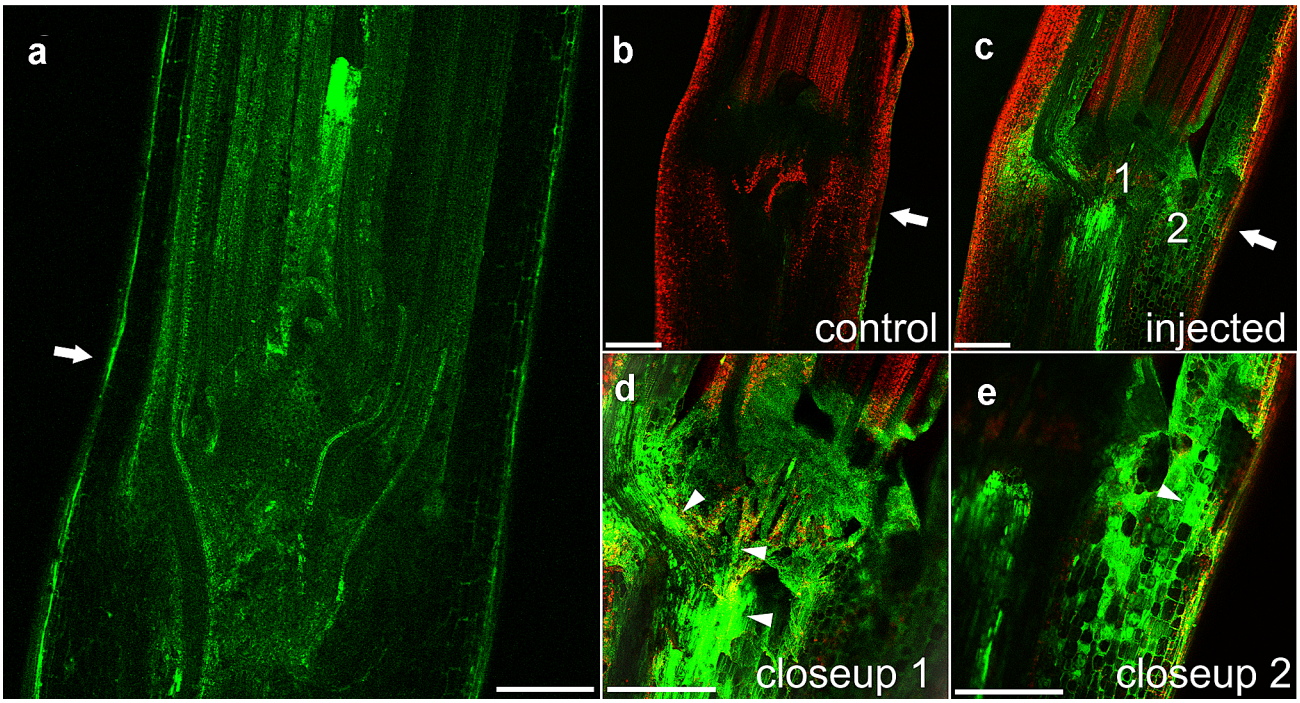

Fig. 1 Tissue distribution of fluorescein-labeled oligonucleotides in shoot meristem of maize seedling after the injection. **a** and **c** Low magnification imaging of cross-sections showing higher green fluorescence intensity in the oligonucleotide-injected region and in shoot apical meristem. Arrows point to the approximate position of shoot apical meristem. **b** Water-injected control sample. **d** and **e** Higher magnification confocal microscopy images captured from the sample shown in (**c**). The numbers 1 and 2 are placed to indicate the regions from which the close-up images were prepared. Arrowheads highlight some of the oligonucleotide-accumulated regions. Chlorophyll autofluorescence of samples are displayed in red for panels (**b–e**). Scale bars: 500 µm

R1-nj gene. The haploid nature of treated plants was confirmed at a later developmental stage by isolation of nuclei from leaves, and identification of genome size with flow cytometry (Rádi et al. 2020).

For designing and synthesis of mutagenic SDO molecules, *ZmPDS* gene was sequenced from the recipient maize genotype. According to the BLAST search in Phytozome database, this gene is located on chromosome 1 (LOC542329). In addition, the truncated 15-cis phytoene desaturase gene (LOC103633372) is located on chromosome 7. Therefore, targeted mutagenesis of *ZmPDS* gene in haploid maize plants can serve as a test system with phenotypic marker. After identification of the target region, the 41 nucleotide- long molecule was synthesized with TAG stop codon (Table 1a). Out of 90 treated plants, 4 of them developed leaves with different patterns of white or pale-green stripe (Fig. 2c,d) and 1 plant developed pale-green leaves (Fig. 2b). M1 plant with white stripes located on different leaves could also been identified (Fig. 2f). One of these leaves showed two mutant cell lineages represented by the parallel white stripes (Fig. 2g). Quantification of fluorescence signal intensities in a rectangular selection area revealed average pixel intensity values of 111.8 and 52.4 for wild-type and pale-green mutant leaf, respectively (Fig. 3a,c). Moreover, we could recognize cells with high signal intensity in mutant leaf, as well. This pattern suggests a chimeric nature of pale-green leaf with mixture of wild-type and mutant cells. Confocal microscopy of white leaf stripe revealed both chlorophyll fluorescence minus and plus cells with dominant presence of albino cells (Fig. 3d,f). The appearance of white stripes in green leaf tissues can be an indication for the targeted mutagenic function of SDO molecules, resulting in a limited number of mutation events in dividing cells of leaf primordia. Dry maize seeds can contain four to five embryonic leaves that are activated during germination (Liua and Chang 2013). Uptake of mutagenic SDO molecules into cells of early leaf primordia (Fig. 1e) can be a starting point for initiation of albino stripe. Mutant phenotypes detected here show a close similarity with maize plants edited in phytoene synthase gene by CRISPR/Cas9 system (Zhu et al. 2016). An early study by Lusardi et al. (1994) can help to interpret the observed pattern of mutant tissue regions. These authors microinjected five individual cells of the L2 layer in isolated maize shoot apical meristems with transformation vectors expressing the Lc gene of anthocyanin biosynthesis. The cells showing anthocyanin accumulation were organized in sectors consisting of a stripe located along the vein, with a length ranging between 0.5 and 1.5 cm. In some cases, more than one stripe was visible on the same leaf running parallel along different veins. The complete

Table 1 Ion torrent sequencing shows targeted nucleotide conversions by injection of synthetic oligonucleotide molecules into the meristematic region of maize seedlings

(a)

Mutagenic oligonucleotide	5′-G AAT ATT ACT GGA GCT AGC **TAG** ACA AGA TCT TTT GCG GGC C -3′
ZmPDS wild type	5′-… ATT ACT GGA GCT AGC **CAG** ACA AGA TCT TTT GCG …- 3′
Target gene read with TAG	5′-… ATT ACT GGA GCT AGC **TAG** ACA AGA TCT TTT GCG …- 3′

(b)

Genotype	Target gene reads with CAG	Target gene reads with TAG
Wild type	14,904	5 (0.0335%)
Mutant No. 1	29,551	66 (0.223%)
Mutant No. 2	16,172	21 (0.130%)
Mutant No. 3	13,346	203 (1.50%)
Mutant No. 4	30,778	627 (1.20%)

(a) Nucleotide sequence of mutagenic oligonucleotide, wild-type and mutant variant reads of target *ZmPDS* gene fragment. Critical residues are highlighted (bold and underlined). (b) The total number and frequency of reads with characteristic triplets

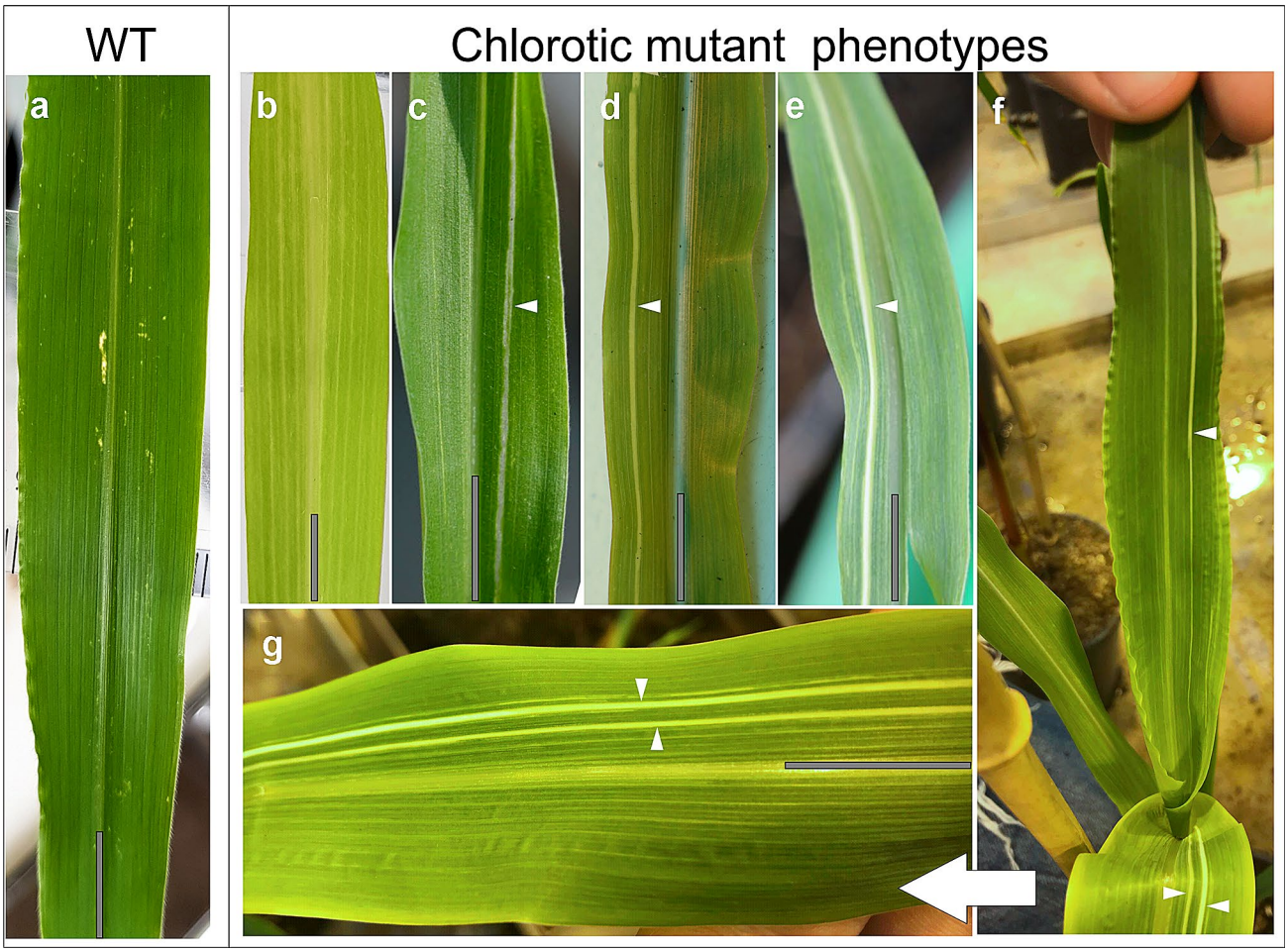

Fig. 2 Variation in the chlorotic phenotype in M1 mutant haploid maize plants after treatment of shoot apical meristem region of seedlings with mutagenic oligonucleotides specific for the phytoene desaturase (*PDS*) gene. **a** Wild-type leaf, **b** pale-green leaf, **c** leaf with pale stripe, **d**,**e** leaves with white stripe, **f**, **g** maize plant with single or doubled white stripes on two different leaves. Arrowheads indicate white stripes. Grey rectangular scale bars (1 cm) are positioned vertically to mark the position of the mid-veins of the leaves

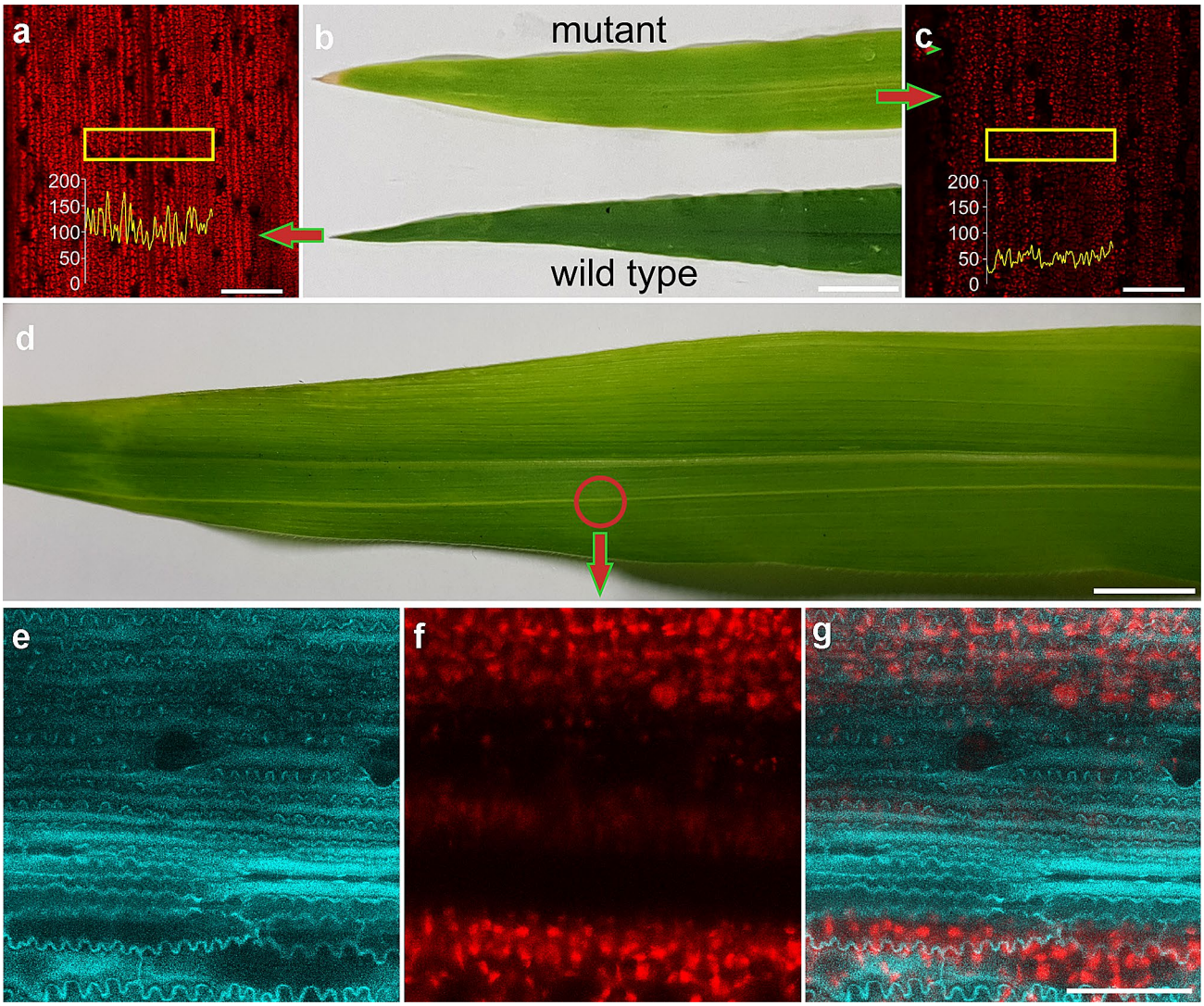

Fig. 3 Reduced chlorophyll fluorescence in leaves after oligonucleotide-directed mutagenesis of the phytoene desaturase (*PDS*) gene in haploid maize. **a**, **c** Confocal microscopy imaging of parenchyma chlorophyll fluorescence of mutant and wild-type leaf samples shown in panel (**b**). Intensity profile of a rectangular selection area is shown as image-embedded chart. *Y*-axis represents intensity values (arbitrary units), *x*-axis corresponds to the width of the selected rectangle. **b** Macroscopic image of oligonucleotide-treated (pale green) and wild-type leaf. **d** Formation of white stripe in a leaf of oligonucleotide-treated sample. **e**–**g** Close-up confocal microscope imaging of cell wall autofluorescence (blue) and chlorophyll autofluorescence (red) of the white stripe shown in panel **d**. Scale bars: 150 µm (**a**, **c**), 1 cm (**b**, **d**), 50 µm (**e**–**g**)

pale-green phenotype of several fully developed leaves from one of *Zmpds* mutants can indicate a different mutation mechanism. Pale-green leaf mutant of Cavendish dessert banana was generated by the CRISPR/Cas9 gene editing of the PDS gene (Naim et al. 2018).

Deep sequencing of PCR amplicons representing the target region of the ZmPDS gene from the chlorotic leaf sectors

Selective amplification and sequencing of wild-type and mutagenized *ZmPDS* genes were used to verify targeted genome engineering in maize leaf cells by injection of mutagenic oligonucleotide molecules into the meristematic region of seedlings (Table 1). Genomic DNA samples were isolated from the green leaf of an untreated plant and the pale-green leaf of the *Zmpds* mutant plant, or from the white stripe sectors of mutant leaves. As shown in Table 1a, the wild-type *ZmPDS* gene in the recipient maize genotype has CAG codon. Here, we used the synthetic oligonucleotides to generate TAG stop codon by C to T conversion at the position 236. The Ion Torrent analysis of the amplified PCR fragment from *Zmpds* mutant tissues revealed 3.94–45.4 times higher number of target reads with TAG than in the

wild-type *ZmPDS* gene. The presented frequency data in Table 1b are different in various leaf samples of treated seedlings. The presented sequence information shows the occurrence of oligo-directed editing events in some cells of the treated leaf primordium tissues. In these samples, the presence of the wild-type target reads with CAG can be interpreted as a sign of chimeric nature of mature leaves with mixture of wild-type and mutant cells. Treatment of a complex organ, the shoot apical meristem, with SDO molecules generated genetically heterogeneous cell population with a variable frequency of wild-type and mutant cells.

Nucleotide sequencing data presented in Table 1b revealed that ODM approach did not induce deletions or insertions in the gene region studied. Preference of specific nucleotide exchange during ODM can be an advantage for this technology in comparison to CRISPR/Cas9 system. As an example, the percentage of deletions ranged from 23.42 to 60.41% in mutation types induced by CRISPR/Cas9 mutagenesis in maize phytoene synthase gene (Zhu et al. 2016). Genetic diversity represented by functional single nucleotide polymorphisms (SNPs) is a very useful source for trait improvement in plant breeding. The directed conversion of a single nucleotide or the repair of a single base by SDO molecules gain increasing significance in genome editing (Rivera-Torres and Kmiec 2016; Sauer et al. 2016).

In summary, both the detection of chlorophyll content alterations and the Ion Torrent sequencing data support the mutagenic nature of synthetic SDO molecules injected into the meristematic region of maize seedlings. As it is expected, exposition of heterogenic somatic cell population to these synthetic molecules resulted in chimeric shoot apical meristems that could be reflected during subsequent organ development. Despite of this complication, this technology can serve as a test system for functional characterization of SDO molecules *in planta*. Use of proper marker such as the albino phenotype and haploid seedling allowed recognition of mutant cells or tissue regions in leaves of the M1 generation plant. This approach is far more efficient and faster than editing cultured cells or tissues. Furthermore, in vitro techniques can induce spontaneous mutations, resulting in somaclonal variations (Evans 1989). Indeed, the recent large-scale whole-genome sequencing of rice plants showed that the tissue culture process creates the most off-target mutations in edited plants (Tang et al. 2018). Further studies are required to test whether the mutated cells of shoot meristems can be involved in sperm cell formation. If this is the case, the induced mutations will be inherited, and the described methodology will have extended applications in plant research.

Author contribution statement Feríz Rádi: conceptualization, methodology, investigation, writing—review and editing. Bettina Nagy: investigation, data curation, writing—review and editing. Györgyi Ferenc: investigation, data curation, supervision, writing—review and editing. Katalin Török: investigation. István Nagy: Data curation, writing—review and editing. Zoltán Zombori: investigation. Dénes Dudits: conceptualization, supervision, writing—original draft. Ferhan Ayaydin: microscopy and image analysis, supervision, writing—review and editing.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors and Affiliations

Feríz Rádi[1,2,3] · Bettina Nagy[1,7] · Györgyi Ferenc[1] · Katalin Török[1] · István Nagy[4,5] · Zoltán Zombori[1] · Dénes Dudits[1,2] · Ferhan Ayaydin[6,7]

Feríz Rádi
rferiz92@gmail.com

Bettina Nagy
nagy.bettina@brc.hu

Katalin Török
torok.karolyne@brc.hu

István Nagy
nagyi@baygen.hu; nagyi@atgandco.hu

Zoltán Zombori
zombori.zoltan@brc.hu

Dénes Dudits
dudits.denes@brc.hu

Ferhan Ayaydin
ayaydin.ferhan@brc.hu; ferhan.ayaydin@hcemm.eu

1 Institute of Plant Biology, Biological Research Centre, Eötvös Loránd Research Network, Szeged, Hungary

2 Kiskun Research Center Ltd, Kiskunhalas, Hungary

3 Doctoral School in Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

4 Institute of Biochemistry, Biological Research Centre, Eötvös Loránd Research Network, Szeged, Hungary

5 ATGandCo Biotechnology Ltd, Mórahalom, Hungary

6 Hungarian Centre of Excellence for Molecular Medicine (HCMM) Nonprofit Ltd, Szeged, Hungary

7 Laboratory of Cellular Imaging, Biological Research Centre, Eötvös Loránd Research Network, Szeged, Hungary