UNIVERSITY OF SZEGED

Doctoral School of Biology

PhD Thesis

Expansion of genetic resources of national maize breeding materials with innovative methods.

Rádi Feríz

Supervisors:

Prof. Dr. Dudits Dénes

Dr. Ayaydin Ferhan

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Summary and aims

The importance of corn in the world economy is growing year by year. The growing population of the Earth requires more and more food produced on a continuously-decreasing agricultural lands. Extensive production is being replaced by intensive crop production that is dependent on highly productive hybrids. In order to maintain a competitive status for maize among other crops, there is a need for adaptive potential to intensive cultivation systems and to extreme environmental conditions generated by climate change. These requirements can be interpreted in terms of genetic background, insuring adaptation to weather and soil conditions. It is important to note that these traits are not generated spontaneously in nature and in agriculture, but their result from long and persistent work by breeders. The present dissertation deals with the further development of Hungarian maize lines using the most modern, precision breeding techniques, developing future-oriented and competitive technologies. One of the cornerstones of plant breeding is the time factor, in the shortest period the best varieties must be put on the market. The competition in this respect is extremely fierce and fast-paced. To shorten the breeding process, we have further developed the dihaploid technology and its sub-processes, providing a number of alternatives to solve the problems that arise. In addition to shortening the time, the goal

is to generate genetic variance for special traits as efficiently as possible, regardless of genotype. These methods can provide a solution to effectively improve seed quality values, multiple herbicide resistance, male sterility, and drought tolerance. Having haploid maize plants, our goal was to develop an *in planta* method for oligonucleotide-directed mutagenesis (ODM) using a recessive, albino marker system. By introducing specific oligonucleotides into cells of shoot meristems of haploid seedlings, we were able to induce a point mutation at a targeted site of the phytoene desaturase gene (PDS) in the genome with strong phenotypic feedback. Our technology development program is currently a pioneer in Hungarian precision maize breeding activities, and long-term objectives include the routine application of these methods developed by the present research at the forefront of national maize breeding. Precision breeding methods can provide a solution for breeding corn cultivars with yield stability, improved seed quality, efficient use of water and nutrition, or high adaptation capability. It is very important to emphasize that the technologies we use and develop are genotype independent and do not require the incorporation of a foreign gene. Eliminating these two factors is a key goal that has so far been the biggest barrier to molecular breeding of corn. The results of the proposed experimental developments may contribute to the market introduction of competitive Hungarian maize hybrids.

Materials and methods

Haploid induction

Our experimental garden used for haploid induction program was established in 2015-2016 in the southern part of the Great Plain, in Kiskunhalas. The late-flowering (85-day vegetative growth phase) K405 maize haploid induction line was used as a paternal component in the crossing program with the late K4390 and K4368 mother plants and the early-flowering K4250 hybrid.

Flow cytometry

Ploidy levels of seedlings preselected by anthocianin marker were determined in nuclei suspension from root apex samples by flow cytometry at 532 nm (BD FACS Calibur) at 30 mW.

DNA isolation and genotyping

Genomic DNA was extracted using the MasterPure® Complete DNA and RNA Purification Kit (Epicenter, USA). Using specific primers the SSM marker UMC1152 was amplified by PCR procedure. The generated fragments were separated by agarose gel electrophoresis and lack of male specific fragment indicated parental genome elimination.

Rediploidization of seedlings

Doubling of the chromosome set was induced by colchicine treatment according to the method of Chase and Nanda (1965) with some modifications. Coleoptiles of germinated haploid

seedlings were cut horizontally for 3 to 5 cm in size and immersed in a solution containing 0.1% colchicine in 0.1% DMSO and 0.1% Tween 20 for six hours at 22 ° C.

Injection of synthetic single-stranded DNA molecules into the meristem region of haploid seedlings

The haploid seeds were germinated in wet and rolled filter paper, aligned vertically into a 3-liter beaker filled with 0.5 liters of tap water. After 6 days, the germinated haploid plants had a 2 cm long coleoptile at 16 h light / 8 h dark at 24 ° C. At this stage, a horizontal incision was made in the shoot, approximately 1 cm above the coleoptile mesocotyl junction, and the meristem region was loosened vertically (on the cut side) with a 27 G needle. Synthetic oligonucleotides (SDO) representing the target sequence of the phytoene desaturase (PDS) gene were dissolved in nuclease-free water at a concentration of 100 pM. 3 µl of oligo solution was injected into the meristem region of each plant.

DNA uptake into embryos of maize grains by temporary priming

For these experiments, diploid mature embryos were used. Maize grains containing mature embryos underwent sterilization as a first step. We linked this process to performing seed priming. During sterilization, 70% alcohol was used for 1 minute, followed by washing with distilled water. The seeds were soaked in 1.5% sodium hypochlorite solution for 30 minutes, followed by washing again. A 0.2% HgCl2 solution was used as the final step and a 4x distilled

water wash was performed. Sterile swollen seeds in front of the embryonic shield were removed from sterilization, followed by 48 hours of desiccation. In the next step, the tissues around embryo were removed exposing dry mature embryo. The seeds were embedded in sterile sand in a Petri-dish with the embryo facing up to bring them into a perfect horizontal position. To turn off the maize phytoene desaturase (PDS) gene, the experiment was performed with six synthetic ZmPDS oligos with different structures. Five of these oligos were dissolved in 2 mM sucrose solution, while in one case these molecules were dissolved in water at a concentration of 50 μM. The oligonucleotide solution was instilled into the embryo surface two times in 50 μl doses two hours apart. After the seeds absorbed the oligo solution, they were pre-germinated using wet filter paper overnight and then the samples were planted. A plant was grown from the treated seeds in greenhouse and after their self-pollination, the phenotypes of the plants of the offspring generation were examined. Using DNA isolated from mutant tissues, the affected region of the PDS gene was amplified for sequencing.

DNA uptake through pollen tube during pollination

Isolated inbred lines in 80-100% pistil flowering were self-pollinated. After 12 hours, the tubes were cut horizontally in two ways. Samples in which the tube was cut back to H1 by 3-4 centimeters from the distal end of the tube, where it was designated H2 by 10-13 centimeters. The tubes were on average 20 cm long. The cut surface was treated with an antisense ZmPDS oligo at a concentration of 50 μM in water

in a volume of 300 µl. Oligonucleotides were protected against degradation with phosphothioate (PTO). After the solution was absorbed, the tubes were dusted again and covered with an insulating bag. The offspring generation was sown again the following year, and after self-pollination, the phenotypes of the third generation were examined.

Design and synthesis of FAM-labeled mutagenic oligonucleotides

The 41-mer single-stranded oligonucleotide called SDO-PDS (5 'to ATT ACT GGA GCT AGC TAG ACA AGA TCT TTT GCG ggc C-3′, lowercase letters refer to phosphorothioates) was designed to generate a STOP codon for maize PDS gene. During synthesis of the oligonucleotide, its sequence was designed to recognize the region as close as possible to the starting codon of the PDS gene and the target mutation to be located in the middle of the oligonucleotide. In addition to the CAG-TAG mutation, the GCA-ACA marker mutation was used with the aim of facilitating the confirmation of successful targeted mutagenesis monitored by next generation sequencing (NGS). To facilitate the synthesis and purification of SDO, the GGGG quartet was replaced with GGGC, which can further serve as a marker mutation. Finally, the specificity of the SDO-PDS sequence was checked in the genomic sequence of maize in the NCBI database.

Microscopy and imaging

Apical meristem regions of six-day-old maize seedlings treated with fluorescent oligonucleotide were examined with a Leica SP5 laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) and SDO uptake was detected. For this, the leaves of the potted plants were fixed directly to the slide using a cover plate. Alternatively, we worked in a Petri dish (Fodor and Ayaydin 2018) by excising the leaf and attaching it to a 24 x 50 mm slide.

PCR amplification and sequencing of the phytoen desaturase (PDS) gene

Genomic DNA from untreated control plants and leaf regions with albino phenotype in SDO-treated maize plants were isolated by CTAB-based extraction method. (Doyle 1990). PCR amplifications were performed to analyze the targeted nucleotide exchange with the following phytoene desaturase gene-specific primers:

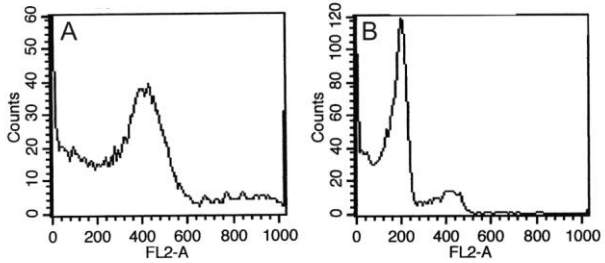
ZmPDS_Forward: 5'CAGTAGTCTGCCTGTACCTATTG-3 'ZmPDS_Reverse: 5'-CGGTGTGTGATCTCC-3'.

Results

Haploid induction and ploidy level determination

In the presented experiments, haploid plants were successfully induced by crossing with the haploid inducer inbred maize line

In our studies, using flow cytometric genome size determination, we determine the induction rate to 10 % what is based on the total number of kernels harvested after crossings (see below). This pre-screening prior to colchicine rediploidization saved time, space, labor, and cost.



Profiles of sorted nuclei with different DNA contents. A: diploid, B:haploid seedlings

Identification of haploid individuals with a microsatellite marker

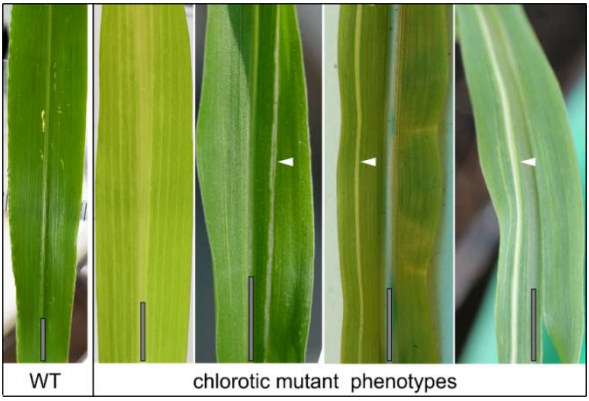
We used the SSR marker for genotyping as an alternative to flow-cytometry in identification of haploid maize plant. Our results show that, depending on the laboratory equipment, both methods can be used for screening.

Production of inbreed lines by doubling haploid genome size

We optimized the process of colchicine treatment of maize seedlings for minimizing mortality and successful restoration of fertility and seed production.

Oligonucleotide-Directed Mutagenesis (ODM) of the phytoene desaturase (PDS) maize gene by treatment of the meristem region in haploid seedlings

Enrichment of FAM-labeled oligonucleotide molecules in the meristem region was successfully detected. As a next step, the oligonucleotide editing the phytoene desaturase gene was introduced into the meristem region by injection, similar to the FAM experiment. Because a recessive mutation marker was used, SDO treatments were performed on haploid seedlings prepared as described previously. As shown in the figure below, chlorotic lesions were observed in the leaves of developing seedlings after SDO treatment. Light green leaves and leaf streaks or albino streaks can be a sign of photosynthetic disturbance.



Untreated and mutant leaves

Directed mutagenesis of phytoene desaturase (PDS) maize gene by treatment of embryos of primed maize grains with oligonucleotide molecules

Based on the U.S. patent (INTRODUCING DNA INTO PLANT CELLS US 8,975,470 B2, Mar. 10, 2015), which demonstrated that foreign DNA can be taken up by primed germinated primed eyes, it was justified to test this method for oligonucleotide uptake as well. As a preliminary experiment for primed seed treatments, similar to meristem injection, we modeled our treatment here using non-specific 5-FAM-labeled oligonucleotides. Preliminary experiments allowed the detection of significant oligonucleotide accumulation in all areas of embryos. On this basis, we carried out editing experiments by incorporation of mutated oligonucleotides into embryogenic tissues. After the treatment, we were able to grow three plants from the transplanted grains, which could be

self-pollinated. Among their off springs, segregation of albino seedlings was recorded in ration as shown by Table below.

Experiment	Treated	Germinated	Pollinated	Planted	Germinated	
					Green	Whiter
P/I	12	10	3	93	86	-
				104	96	19
				105	102	-
Total				302	284	19
Loss		-17%	-70%		-2%	

3. table – Statistical analysis of the P/I experiment

Albino phenotypes in M2 generation

Directed mutagenesis of phytoene desaturase (PDS) maize gene by synthetic oligonucleotide uptake through a pollen tube

Mint Song et al. (2007, Life Science]ournal. 2007;4(1):77-79) and Ajsad et al. (2015, Plant Mol Biol Rep 33:742–747) demonstrate in their review papers that genetic transformation of maize is possible by treatment of pollen tube DNA. This observation supported the treatment of maize pollen tubes with synthetic oligonucleotide molecules. A total of 16 tubes were treated in this experiment, of which 15 could be harvested. 2,946 seeds were sown from the harvested tubes. Generation M2 tube P / VIII / 22 had 145 grains, of which 1 showed an albino phenotype. Since the editing had to take place at an early embryonic stage, we can assume that the edited plant should have shown significant mosaicism. Yet the number of white plants is one, which is 6.2% in terms of the ratio of initial treated tubes to tubes showing albino offspring. Since this one plant is also highly degenerate, we can assume that the editing occurred in a region that affects vitality. Furthermore, it is important to note that in this experiment, the number of seeds hatched after sowing is much lower than in the primed seed- experiment. After sowing, 75% of the seeds germinate. From this phenomenon, we can assume that in the positive successfully edited case, the seeds with already reduced vigor, if still present in the population, belonged to the non-germinated 25%. DNA sequencing is currently underway to verify mutations.

Cut corn cobs pollen tube treatment

Summary and outlook

The primary goal of our experiments carried out at the Biological Research Center of Szeged and the Kiskun Research Center was to create the conditions for the application of precision breeding methods in Hungarian maize breeding. Flow cytometry was used to make haploid induction breeding more reliable. Directed mutagenesis will be a very important tool in future breeding programs. The various methods, of the present research were focused on the development of a method for oligonucleotide-directed mutations. An important reason for this is that the resulting genotypes do not contain a foreign gene and should therefore not be considered GM organisms. At the same time, editing CRISPR-Cas9 is central to our goals, trusting that the EU will allow varieties to be bred.

Publications **MTMT:10073610**

Feríz Rádi, Katalin Török, Marianna Nagymihály, Attila Kereszt, Dénes Dudits (2020) **Improved reliability in production of maize inbred lines by the combination of the R1-navajo marker with flow cytometry or microsatellite genotyping.** Cereal Research Communications DOI 10.1007/s42976-020-00054-9

A. Cseri, P. Borbély, P. Poór, A. Fehér, L. Sass, M. Jancsó, A. Penczi, F. Rádi, Cs. Gyuricza, Tamás Digruber and D. Dudits (2020) **Increased adaptation of an energy willow cultivar to soil salinity by duplication of its genome size.** Biomass and Bioenergy https://doi.org/10.1016/j.biombioe.2020.105655 Reference: JBB_105655

Feríz Rádi, Bettina Nagy, Györgyi Ferenc, Katalin Török, István Nagy, Zoltán Zombori, Dénes Dudits, Ferhan Ayaydin (2020) **Targeted mutagenesis in maize somatic cells by injection of synthetic oligonucleotides into the apical meristem region of seedlings. (Under review)**

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