***In Vivo* Precision Genetic Change of Soybean $\Delta 9$ -Stearoyl (18:0)-ACP Desaturase Gene for Increase Palmitoyl (16:0)-ACP Specificity and along with Change of Endogenous ALS Genes as Selectable Markers and Yeast Strain “EY 957” as a Model**

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DOI: <http://doi.org/10.38177/ajast.2021.5321>

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Article Received: 28 June 2021

Article Accepted: 29 August 2021

Article Published: 30 September 2021

ABSTRACT

Altering genes in their native environment is a powerful tool for biologists and breeders to study gene function and to genetically modify or redesign plant metabolism toward production of specific higher –value products. Even though gene targeting has been widely applied in organisms such as yeast and mammals, its efficiency in plants still is not high enough for routine application. The strategy used in this work consists of using ssDNA oligonucleotide-directed gene targeting to generate a site-specific base conversion or amino acid conversion in the soybean $\Delta 9$ -stearoyl (18:0)-ACP desaturase and ALS (acetolactate synthase) genes to make the former specific to (16:0)-ACP (in order to produce 16:1) and the latter to make it resistant to a sulfonurea herbicide (for selection). In the same manner, yeast *Saccharomyces cerevisiae* was used as a model to test the approach since advantages of using such a model were well recognized. Though there were reports of success and reproducibility of such an approach in certain agronomical crops where most targeted genes for repair were transient plasmid genes or episomal genes (Gamper, 2000), this was the first time such a strategy was applied to soybean. The approach was not a success with the soybean; however, positive results were recorded with the yeast model.

Keywords: Soybean, $\Delta 9$ -stearoyl (18:0)-ACP desaturase, ALS (acetolactate synthase), Sulfonurea herbicide.

Introduction

Providing the world's people with sufficient food and fiber while minimizing the environmental footprint of agriculture is one of the greatest challenges of our time. Genetic improvement of crop plants is an important component of enhancing the sustainability of global agricultural systems and also a way of providing the world with enough food while minimizing the environmental tread (Stainwand and Ronald, 2010). During the past 30 years, various methods for targeted DNA insertion in plants have been established. Many of these methods have been improved for higher efficiencies and a broader range of genomic sites that can be targeted.

One strategy of introducing agronomically or industrially important traits into plants is through genetic engineering which directly manipulates the genetic makeup of the plants using molecular genetic tools which still is of the greatest challenges of our time (Blumwald, 2010).

Oligonucleotide-directed gene targeting is a potential technique for agricultural trait modification in economically important crops (Dong, 2006). The successful use of small synthetic oligonucleotides such as chimeric RNA/DNA and single stranded DNA to make targeted, specific, and permanent changes to homologous regions within genomes for understanding gene function in yeast and mammalian cells have been reported during the 1990's (Cole-Strauss, 1996; Lui, 2002; Bonawitz et al. 2019). Through this approach, the corrected gene can be maintained, expressed, and regulated as a normal endogenous gene. In addition, this approach presents the possibility to correct dominant or gain-of-function mutations that are not amenable to gene replacement strategy and also could be used to make site-directed mutations in the genome, thus leading to the potential to be an important tool in functional analysis of proteins. Even though the potential of oligonucleotide-directed gene

modification is promising, it is currently limited by low frequency and large variability. However, early reports on chimeric RNA/DNA oligonucleotides (Cole-Strauss, 1999; Cole-Strauss, 1996; Kren, 1998) created high expectations due to the approach's high frequencies of gene modification, arguing it could be used as a quick and easy alternative method in making targeted gene modification without selection procedures. Poor reproducibility, difficulties in systematic frequencies, or explanations of large variations among experiments triggered controversy and skepticism among different research groups, as researchers used different species, cell types, genes, methods of delivery, and assays (Albuquerque-Sila, 2001; Graham, 2001; Igoucheva, 2004; Liu, 2001; Stasiak, 1997; Taubes, 2002; Thomas, 1997; Van der Steege, 2001).

New approaches have been systematically taken to define the mechanism by which targeted genes are repaired (Brachman, 2003; Gamper, 2000; Liu, 2001; Lui, 2002). Gamper et al., (Gamper, 2000) demonstrated that the overall length of the chimeric oligo (CO), particularly in the region of complementarities or homologies, was an important factor in successful repair. Lui et al., (Liu, 2001) reported that when a certain number of phosphodiester bonds connecting the terminal residues were modified in single-stranded oligonucleotides, the all-DNA section was involved in directing gene repair as an isolated single molecule. Furthermore, Gamper et al., (Gamper, 2000), using mammalian and plant cell-free extracts, demonstrated that single-stranded oligo-nucleotides containing three phosphorothioate bonds at the 3' and 5' termini were at least three times more effective than the chimeric oligonucleotides (CO) in gene repair assays.

Transformation of the yeast, *Saccharomyces cerevisiae*, directly with synthetic oligonucleotides has been proven to be a useful technique for making site-specific mutations especially of the CYC1 gene encoding iso-1-cytochrome c (Brachman, 2003; Moerschell, 1991; Moerschell, 1988; Moerschell, 1990; Yamamoto, 1992).

A number of procedures to increase the frequency of transformation with synthetic oligonucleotides using yeast- *S. cerevisiae* as a model has been investigated by (Yamamoto, 1992). Moerschell et al., (Moerschell, 1988) demonstrated transformation of LiAc-treated cells was found to be more efficient than transformation of spheroplasts. *In vivo* site-directed mutagenesis by transformation of yeast using single-stranded oligonucleotides was investigated by these authors with the cyc1-31 allele that has a TC → A change with a mutation that reverts spontaneously at a low frequency of $\approx 10^{-9}$ and a cyc1-812 which has T deletion. Using various concentrations of oligonucleotides, they showed that transformation frequencies increase with higher amounts of oligonucleotides (with oligonucleotides of 40 to 50 nucleotides highly effective and an optimum of 60 nucleotides yielding 1580 transformants / $5-7 \times 10^7$ cells or a frequency of $\sim 2.6 \times 10^{-5}$) and decreases with increasing numbers of mismatches. Electroporation was further tried for improving the transformation frequencies and found to be lower than the LiAc procedure, albeit effective.

Liu et al., (Liu, 2001) successfully corrected an episome containing a mutated fusion gene encoding hygromycin resistance by using eGFP expression as the target for repair in *Saccharomyces cerevisiae* LSY678 mata. Using both RNA/DNA chimeric and single-stranded DNA molecules of different lengths (25, 40, 60, 74 or 100 bases) and doses (1, 2.5, 5, 7.5 and 10 $\mu\text{g}/\text{ml}$) designed to convert TAG → TAC that contain three phosphorothioate linkages (single-stranded DNA) at each end, these authors succeeded in restoring function (resistance to hygromycin and

expression of eGFP). The yeast strain containing the mutated plasmid was treated with various targeting vectors by electroporation. After electroporating 10^5 cells, their results showed the number of colonies that arose with the DNA vector of 74 nt was > 90-fold higher than the chimeric oligonucleotide, with a targeting efficiency of approximately 0.016%. They also indicated the efficiency of correction was dose-dependent, with an optimum at 5 $\mu\text{g/ml}$ and decreasing at the highest level of 10 $\mu\text{g/ml}$.

The primary aim of this study is to introduce a synthetic fragment of DNA molecule (e.g., a single-stranded DNA oligo) into soybean cells to replace or change the corresponding chromosomal segment of the endogenous soybean $\Delta 9$ stearoyl (18:0)-ACP desaturase gene by homologous recombination or pairing. This approach presents a precise way of manipulating the genome and does not have to include a complete protein coding sequence or separate signals to ensure its expression. As such, we attempted to change the endogenous soybean $\Delta 9$ stearoyl (18:0)-ACP desaturase gene by making it specific to 16:0-ACP using single-stranded DNA (ssDNA) oligos by converting G188/L and T117/R because such a mutation in the castor 18:0-ACP desaturase has resulted in such a change in substrate specificity.

However, this approach requires selection strategies that would allow identifying the recombinants or mutant embryos. The selection strategy chosen here is to target the key enzyme in the branched chain amino acids metabolic pathway. Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathway to the branched chain amino acids (BCAAs) valine, leucine and isoleucine. It has been reported the metabolic control of BCAA biosynthesis is controlled by the end products through allosteric regulation of ALS, and its inhibition (ALS) causes growth inhibition in higher plants (Hervieu, 1999). ALS is also inhibited by sulfonylurea herbicides, resulting in complete inhibition of plant growth.

Thus, mutation of proline196 to alanine and serine 656 to asparagine of the tobacco and maize aceto-lactate synthase (ALS) gene was found to result in conferring resistance of this gene to a sulfonylurea herbicide (Beetham, 1999; Zhu, 1999). In the same fashion, we created a mutation in the corresponding amino acid proline 183 to alanine and serine 639 to asparagine of the soybean ALS proteins. The mutated ALS oligo were used along with the soybean $\Delta 9$ ssDNA oligos to transform soybean somatic embryos.

In the same line or in addition to the soybean transformation, yeast *S. cerevisiae* strain “EY 957” as a model was tested for a better appreciation, degree of feasibility, frequency, and efficiency of the approach.

Materials and Methods

Soybeans

Soybeans [*Glycine max* (L.) Merrill cv. Jack] were grown in the greenhouse at University of Kentucky, Lexington, under a 16 hr photoperiod at $26 \pm 2^\circ \text{C}$. Pods with immature seeds were surface sterilized by immersing for 30 sec in 70% 2-iso-propyl alcohol followed by a 10 min immersion in 25 % commercial bleach (6.00 % hypochlorite) with a few drops of Liquinox (detergent). The pods were then rinsed three times in sterile water for 5 min each time. Immature seeds 3-6 mm in length were removed from the pods. The end containing the embryonic axis was cut off and discarded. The two cotyledons were then pushed out from the seed coat, separated, and placed with abaxial

side (round side) down on MSD40 medium (Hangsik, 2003; Trick, 1997). The media used during the various stages of soybean somatic embryogenesis are listed in (Table 1) (refer Table 1).

Cultures were then incubated at 25° C at a 23 hrs photoperiod (low light intensity, 5-10 mEm⁻²s⁻¹). Globular-staged somatic embryo clusters were harvested from the explant tissues 4 to 6 weeks after induction and then placed on MSD20 solid medium (Trick, 1997) for a period of one month for proliferation. Embryogenic tissues were then transferred from MSD20 to FNL (Finer and Nagasawa “lite”) “liquid medium” (Samoylov, 1998a) for further proliferation. Suspension cultures were agitated at 100 rpm and maintained with a 2 weeks subculture period at 25° C with a 23 hrs. photoperiod.

Selection Agents

Exceed® or 3-[4, 5-bis (difluoromethoxy)-pyrimidin-2-yl]-1-(2-methoxycarbonyl-phenylsulfonyl) urea from Syngenta Crop Protection, Inc. Greensboro, NC USA.

Determination of Exceed Concentrations for Selection

Non-transformed globular stage soybean somatic embryos were plated on a 1/5 D20 medium (1/5 of normal D20 full concentration minus asparagine) (Finer, 1988) with different concentrations of Exceed. The concentration at which the SS embryos turned brown and ceased any further proliferation was determined, and this concentration was used for selection of soybean somatic embryos transformants.

Design of Oligo Primers

Genbank was searched to find full-length sequence data for the both soybean Δ9 and ALS genes. After blast searching and alignment (using vector NTI) against castor Δ9 desaturase (for soybean Δ9 desaturase) and tobacco and maize ALS gene (for soybean ALS gene), four (4) synthetic DNA oligo primers (74 and 80 base pairs long containing three phosphorothioate bonds* at each terminus) were designed to change the endogenous soybean stearoyl ACP-desaturase and the ALS gene. Δ9 desaturase gene: primer A: 5'-G*A*A*TG-GAT-CCT-CGA-ACC-GAG-AAC-AGC-CCC-TAC-CTT-CTT-TTC-ATT-TAC-ACT-TCA-TTT-CAA-GAG-AGG-GCA-ACC-*T*T*C-3' and primer B: 5'A*T*C*-GAG-GAA-GCC-CTG-CCT-ACT-TAC-CAA-ACA-ATG-TTA-AAT-**AGG**-TTG-GAT-GGA-GTT-CGT-GAT-GAA-ACA-GGT-GCC-AGC-CTT*G*A*C-3'. ALS gene: primer C: 5'-A*T*G*GA-CAG-CGT-CCC-AGT-CGT-CGC-CAT-CAC-CGG-CCA-GGT-**CGC**-CCG-CCG-GAT-GAT-CGG-CAC-CGA-CGC-CTT-CCA-*A*G*A-3' and primer D: 5'-T*G*-C*CC-CAT-CAG-GAG-CAT-GTG-TTG-CCG-ATG-ATT-CCC-**AAT**-AAT-GGA-TCC-TTC-AAG-GAT-GTG-ATA-A CT-GAG-GGT-*G*A*T-3'.

In almost the same fashion, we designed two standard desalted primers (74 and 40 bp) to reverse a mutation and create a deletion in two different genes in *S. cerevisiae*: Trp1 gene (two primers A and B to make it wild type) and Ole1 gene (to create a mutation). For Trp1 A: A*A*A*ATTCAAGTCTT GTAAAAGCATATAAAAA TAGT**TCAG** GC ACTCCGAAATACTTGGTT GGCGT GTTTC G*T* A*A; primer B: 5'AAAGCAT ATAAA AATAGT**TCAGG**CACTCCGA AATACTTGG-3', primer C: 5'-TTGAAT AACT**G**A CACCAACATTTGAA CTGATTGAACATGG-3' for Ole1 gene. All the oligo primers used in this work were synthesized by Integrated DNA Technologies, Inc (IDT). The mutated amino acids are underlined and in bold.

Microprojectile Bombardment

Slightly mashed green embryo clumps were placed in the center of a moist filter paper in sterile petri plates (approximately 1000 to 1500 mg of somatic embryos per plate) and dried in a laminar flow hood for 15 min prior to bombardment. Transformation was carried out via particle bombardment with a gene gun (Dupont PDS1000; Bio-Rad Laboratories, Hercules, and CA) by gold/DNA microprojectile preparations as described by (Trick, 1997). Briefly, for nine shots, 25 µg of DNA was used to coat 7.5 mg of 0.6 µm gold. Cultures were bombarded at 10,686.9 kPa (1,550 psi) helium gas pressure under 91 kPa (27 in) of Hg vacuum, at a shooting distance of 11 cm from rupture disk to target tissue. A combination primer A&C, A&D, B&C and B& D were used to transform the soybean somatic embryos. Immediately after bombardment, embryogenic cultures were placed on D20 proliferation media without any selective agent for seven days.

Selection and Regeneration of Transformants

After transformation of soybean somatic embryos, selection was accomplished with selection medium containing “Exceed” an ALS inhibitor herbicide. A total of more than 32 sessions of gene gun shootings (more than 320 plates) were made using different amount of oligos (400-1800 µg). Bombarded globular soybean somatic embryos (SSE) that were cultured on D20 were transferred (approximately 100 clumps of 0.3–0.4 cm diameter per plate) onto selection medium containing the selection agent “Exceed” at different concentration levels (1.5 – 8 mg). Every 2 weeks, visibly growing clumps were moved to fresh selection medium.

Yeast Auxotroph

Genotype of strain used: Yeast *S. cerevisiae* strain EY 957 from Dr. D. Micheal Mendenhall (University of Kentucky, Department of Biochemistry) and NSF award EPS-0132295. The strain has a single-site mutation in the *Trp1* gene that reverts at a low frequency.

Selection Media

Standard YPD and synthetic media were used for growing and testing the yeast strain YE957. W⁽⁻⁾ medium is a standard synthetic medium lacking tryptophan but containing all the other amino acids and 2% agarose. W⁽⁻⁾/oleic acid is a W⁽⁻⁾ medium supplemented with oleic acid and Igepal CA-630 (Sigma) at final concentrations of 250 µg/ml and 0.2% (v/v), respectively.

Yeast Transformation

(A) Lithium Acetate Method

The following procedure is the method of (Elble, 1992). A glob of cells from a plate was scraped with the broad end of a toothpick and grown overnight. 0.5 mL of the culture was spun in a microfuge for 10 seconds and the supernatant was decanted. 10 µl (100µg) of single-stranded carrier DNA plus (x) µg of transforming DNA were added and vortexed. Then 500 µl of PLATE (40% PEG 4000, 0.1 M Li-acetate, 0.1 M Tris-HCL pH 7.5-, and 10-mM Na-EDTA) was added to the culture, vortex and incubated overnight on the benchtop. 50 µl (from the bottom) of the mixture were spread onto selective plates.

(B) Electroporation of Saccharomyces cerevisiae

Electroporation of yeast cells was performed in a Biorad Gene Pulser as described by (Meilhoc, 1990; Yamamoto, 1992) with a small modification. Cells were grown at 30°C to a density of $\sim 1.5 \times 10^7$ cells mL⁻¹ in YPD medium, washed with 25 mL cold dH₂O twice and 1 mL of 1M sorbitol once. The harvest cells were incubated at 30°C for 10 minutes in YPD containing 20 mM-HEPES (pH 8.0) and 25 mM-dithiothreitol and then resuspended in electroporation buffer (EB).

Aliquots (200 µL) were electroporated with different concentrations of oligos in a 0.2 cm electrode gap cuvette, with the Gene Pulser. Following electroporation, 1 mL of pre-warmed (30°C) YPD medium was added immediately to the well. The cell suspension was then incubated for three hours at (30°C). Cells were harvested by centrifugation for 30 seconds in a microfuge and resuspended.

Approximately 200 µL of this suspension were plated on selective medium. Two controls were assays in which the DNA was omitted in both and one electroporated and the other not. The yeast experiment was repeated three times in the same conditions.

Results

In this work, it was examined whether the single-strand DNA oligonucleotides approach for gene targeting could be applied to soybean somatic embryos. Similar approaches have been reported with certain agronomically important crops such as tobacco, maize, and rice, even though different vectors were used. I therefore endeavored the *in vivo* targeting of two different genes using different combinations (Table 1).

A total of more than 300 plates were transformed and none of the transformants stood the selection. In this study it was hard to determine whether the approach was a success in the case of the endogenous $\Delta 9$ gene since no material survived selection for allowing SNP (single nucleotide polymorphism) analysis.

However, the second targeted gene apparently did not mutate because if it had, then it should have survived the selection.

When looking at the data gathered through literature review on this approach and its frequency of transformation (between 10^{-4} and 10^{-6}) among eukaryotes tested and the size of soybean genome (1-1.15 Gb), it was not surprising that a single event of homology or any recombination did not register since we targeted genomic sequences.

Results of the yeast model, however, differed from the soybean study and confirmed in sort what was previously reported when yeast was used as a model organism for analyzing oligonucleotide-directed base changes in plasmids. In these early pioneer studies, (Moerschell, 1988); and (Yamamoto, 1992) utilized a mutation in the *cyc1* gene as a target for repair directed by transfected (unmodified) DNA oligonucleotides.

In this study I used a strain of yeast “Yeast 975”, a yeast auxotroph for the amino acid tryptophan, as a model using different levels of ssDNA concentrations. I targeted for repair the *trp1* gene that has a point mutation in the *trp1* gene using single-stranded DNA oligonucleotides.

Table 1. Summary of nucleotides or amino acid conversion for the soybean somatic embryos (SSE) experiment

Soybean Targeted Genes	Predicted Change		# of plates bombarded	SSE that survived selection	Positives for target genes	Total # of plates bombarded
	Nucleotides	A. A.				
$\Delta 9$ -ACP-DES ^A	GGT→CTT	G → L				
$\Delta 9$ -ACP-DES ^B	ACT→ AGG	T → R				
ALSI	CCC → CGC*	P → A				
ALSII	AGT → AAT	S → N				
$\Delta 9$ -ACP-DES ^A & ALSI				0	0	80
$\Delta 9$ -ACP-DES ^A & ALSII				0	0	80
$\Delta 9$ -ACP-DES ^B & ALSI				0	0	80
$\Delta 9$ -ACP-DES ^B & ALSII				0	0	80
Total				0	0	320

* Proline converted to alanine for selection (Zhu, 1999).

Both procedures (LiAc and electroporation) were tested in Dr. Mendenhall's lab using different concentrations of single-stranded oligonucleotides (from 75 to 300 µg). Several attempts were made to restore the yeast-Trp-1 heterotrophy using the Li-acetate transformation method. However, the frequency of transformation was too low each time; results were around three colonies with the control plate and around 5 to 6 colonies with the transformed plate (Fig.1).

Due to these low results, we resolved to use the electroporation method (refer to the materials and methods section) of testing for the remainder of the study. The attempt was successful in reverting the mutated strain to the wild type form, and results showed the efficiency of transformation was ssDNA concentration-dependent (Table 2). For the same amount of oligonucleotide used, the number of transformants obtained with electroporation was higher than the LiAc procedure (refer Fig.1C).

The transformation efficiency represented as the number of transformants obtained per microgram of oligonucleotide was reported in (Fig.2). The average number of transformants obtained in our experiment was very low in comparison to that obtained in different laboratories. However, our results were in con-cordance with the

frequency of transformation trends as reported by Yamamoto's group (Yamamoto, 1992). Since one of our objectives was to reverse the auxotrophy (to make it wild type) using the single-stranded DNA oligo to determine whether the system would work, different amounts of oligo (in an incremental order) were assayed, and the experiment proved to be a success even though the level of transformants obtained were much lower than reported in the literature (123-210 transformants or a frequency of $6 \times 10^{-6} - 1 \times 10^{-5}$) (Yamamoto, 1992).

Fig.1. (A) Control plate: yeast cells plated on minimal media without oligonucleotide. (B) LiAc procedure: yeast cells treated or transformed with Trp (tryptophan) oligonucleotide plated on minimal media. (C) Electroporation procedure: yeast cells electroporated with Trp oligonucleotide only plated on minimal media

Table 2. Frequencies of transformant of yeast strain EY957 with electroporation procedure

Reaction Mixture (μ l)	Oligonucleotides (μ g) ^a		Transformants or Colonies grown on minimal media	
	Trp	Ole	Trp	Ole
255	50	200	30	-
249.5	75	150	42	-
244	100	100	49	-
249.5	150	75	65	-
255	200	50	112	-

*The reaction mixture contained 200 μ l of resuspended yeast cells (~) plus combination with the corresponding volume of oligonucleotides (Trp and Ole) in microliters. Amount of oligonucleotide (μ g) used in each reaction mixture is given as such.

**The selection is done first on plate YPD plate containing 18:1 minus the amino acid tryptophan and then printed on plate YPD plate without both tryptophan and 18:1.

^aResults from treatment with 300 μ l (and above) oligonucleotides were discarded due high toxicity.

Trp: tryptophan single stranded oligonucleotide.

Ole: yeast $\Delta 9$ -CoA-desaturase single stranded oligonucleotide.

Data were average of three experiments.

Fig.2. Frequency of transformation: number of colonies on minimal media per mg of oligonucleotide

However, based on these results, a co-mutagenesis experiment using two different single-stranded DNA oligos targeting two different genes-the tryptophan gene to reverse the auxotrophy (to use it as a positive selection system) and the OLE1 gene (that codes for yeast $\Delta 9$ desaturase) was done by creating a point mutation that results in a premature stop codon in the middle of the gene. The co-mutagenesis experiment has been repeated more than three times with different levels of oligos and has not yielded any positive result.

However, the number of transformants or colonies (a frequency of 2×10^{-6} - 7×10^{-6}) for the tryptophan gene happened to increase with the amount of oligos. Literature reported efficiency of modified single-stranded DNA oligonucleotides-directed gene repair varies with DNA and cell concentration and also differs considerably with per example repair of an insertion being 5-fold better than a deletion, and can be schemed as follow: replacement > insertion > deletion (Liu, 2001; Meilhoc, 1990). In most reviewed literature, researchers targeted plasmid or episomal genes, and in very few cases genomic sequences, and here we targeted a genomic DNA to test the approach. To better appreciate whether the mutation in the Ole1 gene was created or not, we proceeded to the

printing of each transformed plate, and furthermore all transformants from each level had been streaked on a plate that contained oleic acid and without tryptophan and on a plate without oleic acid or tryptophan. After 48 hours incubation at 30° C, we observed that there is growth in both plates suggesting that one of the transformations did not work. Normally if the OLE1 mutation has occurred, there will not be any growth in the plate without oleic acid or tryptophan. It was unfortunate to report my different attempts in creating that point mutation were unsuccessful.

Discussion

To facilitate gene function studies as well as gene engineering, it is important to have technologies to induce specific changes in the genome available (Ruiter, 2003). In this regard, oligonucleotide-directed gene repair can be considered a precision genetic or reverse genetic for more rapid trait development than traditional gene transformation or gene targeting via homologous recombination (Britt, 2003; Puchta, 2002). When oligonucleotide-directed DNA conversion of point, insertion, or deletion mutations on the targeted chromosomal position occurred, the problems associated with transgene instability and variable expression levels due to the random nature of transgene integration could be overcome. Several studies (Hanin, 2003; Puchta, 2002) reported success in homologous recombination, especially in higher plants, is limited and characterized by low frequency. Although homologous recombination as a tool of directed sequence modification in plant as well as in mammalian cells has been hampered by a low frequency in most cell types, it will hopefully become a routine method and a powerful tool for precisely modifying genomic sequences into designed or engineered sequences of individual genes or multigene families.

Site-specific alteration of DNA is not only useful for studying function of proteins *in vivo*, but it is also useful for creating plants with desired phenotypes, including, for example, environmental stress tolerance, improved nutritional value, herbicide resistance, disease resistance, modified oil production, etc. The early success of oligonucleotide-directed gene conversion was reported in tobacco (Beetham, 1999) and maize (Zhu, 1999; Zhu, 2000). Even though the frequency of the targeted gene repair was low (approximately 1 in 10⁴ in maize (Zhu, 1999) and 1 in 10⁶ (Kochevenko, 2003) in tobacco), it was demonstrated that altered genes can be stably maintained through mitosis and transmitted through meiosis in a Mendelian fashion to subsequent generations (Dong, 2006).

While most researchers used chimeric RNA/DNA oligo, triplex-forming oligonucleotides (TPOs) or single-stranded DNA oligonucleotides to target episomal or plasmid genes in the host transformants, we, in this study, have taken a similar approach to mutation correction using ssDNA to targeted genomic genes. Despite a limited success, our data suggests a point mutation in genomic sequence is corrected by ssDNA having a length between 40 and 74 nt at least in the yeast model. Worth mentioning, however, is the existence of several factors that might affect successful gene targeting.

The quality and quantity of oligonucleotides delivered to cells are among important factors cited in achieving a successful gene repair (Moerschell, 1988; Yamamoto, 1992). Furthermore, the large variation in reported repair frequency (in mammalian cells) within the same lab and between labs (Alexeev, 2002; Van der Steege, 2001) suggests that many other factors may be involved since different cell types and different physiological stages of cells may have different repair activity. It is also reported that the biological activities of both DNA pairing and

repair in a target cell and the status of DNA replication and transcription of the targeted gene are known to influence the gene repair process (Brachman, 2004; Drury, 2003; Igoucheva, 2002; Igoucheva, 2003). Oligonucleotide-directed gene repair is a complex biological process which is not easily controllable but also needs for the oligo to reach the targeted DNA in a cell which has DNA pairing and DNA repair activity. Thus the biological activity of the targeted cells is important for successful targeting since oligonucleotide-directed gene targeting involves several pathways that include homologous pairing, mismatch recognition and DNA repair (Dong, 2006; Igoucheva, 2004).

Independent of the differences in data, the frequency and efficiency of transformation with regard to the amount and length of vector used as well as the transfection procedure used (LiAc or electroporation) among the different groups, yeast can provide at some point an important system in which one can examine oligonucleotide-directed gene repair. With regard to our experiment, even though one could see it as a pure speculation, the difference in results (although not very important) in within the procedures used (LiAc and electroporation) could be attributed to the difference in the strain used in addition to the factors mentioned above. However, the problem with the cotransformation with the *Ole 1* gene and the lack of positive results are more complex for interpretation than the reversion of the *Trp1* gene alone since we didn't have a positive control for *Ole1* gene to better assess the efficiency of the cotransformation. Due to lack of availability of yeast mutant or auxotroph with one point mutation for *Ole 1* gene, we, therefore, used the only yeast auxotroph strain available at the time and try to introduce a mutation in the target gene through the oligonucleotides based mechanism and then use tryptophan as a positive selection system for the cotransformation experimentation.

The results proved the cotransformation unsuccessful since the transformed cells grew in the minimal medium. As for the soybean experiments, this is the first attempt. Taken into account the difficulty involved in the plant homology recombination (Hanin, 2003; Reiss, 2003; Wright, 2005)), and also considering the difficulty in soybean transformation, the results were not that surprising. There is an increasing need to engineer complex genetic traits into plants. With over three decades of advancements in plant genome engineering, targeted mutation at a single nucleotide at defined genomic sites is still technically challenging, despite the remarkable progress in targeted insertion in plants.

It is also important to test the repair ability of the target cells before extensive gene-targeting experiments are undertaken. For example, one can introduce a mutated nonfunctional plasmid GFP into cultured tobacco line (as model plant) or soybean suspension line and then check whether its functionality can be restored using single-stranded DNA oligonucleotide. This kind of assay system can be useful in testing the feasibility of oligonucleotide-directed gene alteration. Such a positive selection system was used by Beetham's group (Beetham, 1999) to restore GFP function.

Furthermore, while the single-stranded DNA oligos happened to not working for us because of the very low frequency in higher plants as reported in the literature (Britt, 2003; Puchta, 2002), there is progress in new tools and approaches in this area of gene targeting such as targeted mutagenesis using zinc-finger nucleases (ZFNs) (Bibikova, 2003; Porteus, 2005; Segal, 2003, Bonawitz et al. 2019). ZFNs are artificial fusion proteins that link a

zinc finger DNA-binding domain to a nonspecific nuclease domain. Evidence with *Arabidopsis thaliana* experiments using ZFNs to create targeted mutations indicated that the strategy is efficient enough that there is no need for positive-and negative-selection schemes for individual mutant identification (Lloyd, 2005).

ZFNs are highly programmable: The amino acids of the zinc finger domain can be adjusted for a variety of genomic targets. Therefore, the application of ZFNs does not rely on the creation of recipient plant lines carrying preintroduced target sites. Specific zinc finger repeats have been developed for most nucleotide triplets, but the modular combination of these repeats for an effective, sequence-specific ZFN requires laborious screening and optimization (Urnov et al., 2010; Donga et al., 2021).

Thus, technological advances such as ZFN-based targeted-mutagenesis strategy among others will contribute to improved efficiency of targeted DNA mutation in plants.

Acknowledgements

This work was supported by the United Soybean Board, the Kentucky Soybean Promotion Board and the Kentucky Agricultural Experiment Station. Erin Yost provided very helpful technical support.

Declarations

Competing Interests Statement

The authors declare no competing financial, professional and personal interests.

Consent for publication

Authors declare that they consented for the publication of this research work.

Data Availability

Authors are willing to share the data and materials based on relevant needs.

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