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# Oligonucleotide-Directed Gene Repair: Promises and Limitations for Plant Gene Modification

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## ABSTRACT

As a new technology for specific mutation induction, oligonucleotide-directed gene repair was investigated for the last decade, in addition to traditional gene transformation and gene targeting based on homologous recombination. This targeted gene repair relies on induction of cellular endogenous DNA repair mechanisms to produce predicted gene alterations, so that the altered gene is expressed under the control of its native promoter. Early successful application of this technology in cultured animal and human cells, and in tobacco and maize cells, promised a new approach for human gene therapy, crop improvement and functional genomics. However, large variations in the repair frequencies and a lack of reproducibility of the early experiments made the technology controversial. Several factors, such as the quality and the delivery of oligonucleotides into cells, were identified as influencing the frequency of repair, while the main obstacle of the technology is a lack of detailed knowledge of gene repair mechanisms at the molecular level. Much research has recently focused on the mechanisms, such as identifying specific DNA repair pathways, and assessing the influence of the cell cycle in the regulation of the repair process. Before the mechanisms are demonstrated and a high repair frequency can be achieved consistently, oligonucleotide-directed gene repair is unlikely to be a favored method for crop improvement and functional genomics in plants. Emerging technologies such as zinc-finger nuclease assisted gene targeting and TILLING may be more efficient methods for crop improvement and functional genomics.

**Keywords:** chimeraplasts, crop improvement, DNA repair, functional genomics, gene targeting, single-stranded oligonucleotides
**Abbreviations: ALS**, acetolactate synthase; **AHAS**, acetohydroxy acid synthase; **BAR/PAT**, phosphinothricin-N-acetyltransferase; **DSB**, double strand break; **GFP/eGFP**, green fluorescent protein/enhanced green fluorescent protein; **ESTs**, expressed sequence tags; **ORF**, open reading frame; **RDO**, RNA/DNA oligonucleotide; **SDO**, single-stranded DNA oligonucleotide; **TILLING**, targeting induced local lesions in genomes

## CONTENTS



## INTRODUCTION

The completion of genome sequencing of *Arabidopsis* and rice, and the availability of large amounts of sequence information from other plant species, including ESTs, allows plant biologists to explore gene functions and to apply the information in crop improvement. However, the rate at which gene functions are discovered is far slower than the pace at which gene sequences are determined. The greatest challenge for plant biologists is a lack of efficient and robust technologies to generate gene replacements, targeted gene knockouts, or site-directed mutagenesis in higher plants. Gene replacement by homologous recombination is routine in yeast, and feasible in mouse embryonic stem cells, but far less efficient in other mammalian cells and in flowering plants (Puchta 2002). Gene transformation by random insertion of foreign genes in plant genomes via nonhomologous end joining events has been successful since the first demonstration over 20 years ago. It has become a major tool for functional genomics as well as crop improvement. However, transgenic plant research has problems, such as epigenetic gene silencing and transgene instability due to the nature of random insertion. Moreover, transgenic crops have limitations concerning commercialization and consumer acceptance, especially in some regions.

Oligonucleotide-directed gene repair was developed for site-specific gene alteration with the ultimate goal to cure human genetic disorders by correcting the causative mutation. Specific chimeric RNA/DNA oligonucleotides (chimeraplasts, RDOs) or chemically modified single-stranded DNA oligonucleotides (SDOs) are designed to pair with the homologous sequence within the genome to introduce single base changes in the specific target gene. The advantage of this approach is that expression of the "corrected" gene is regulated in the same way as a normal gene. Early success of such gene correction using chimeraplasts in cultured mammalian cells (Cole-Strauss *et al*. 1996; Yoon *et al*. 1996) suggested a significant role for this technique in gene

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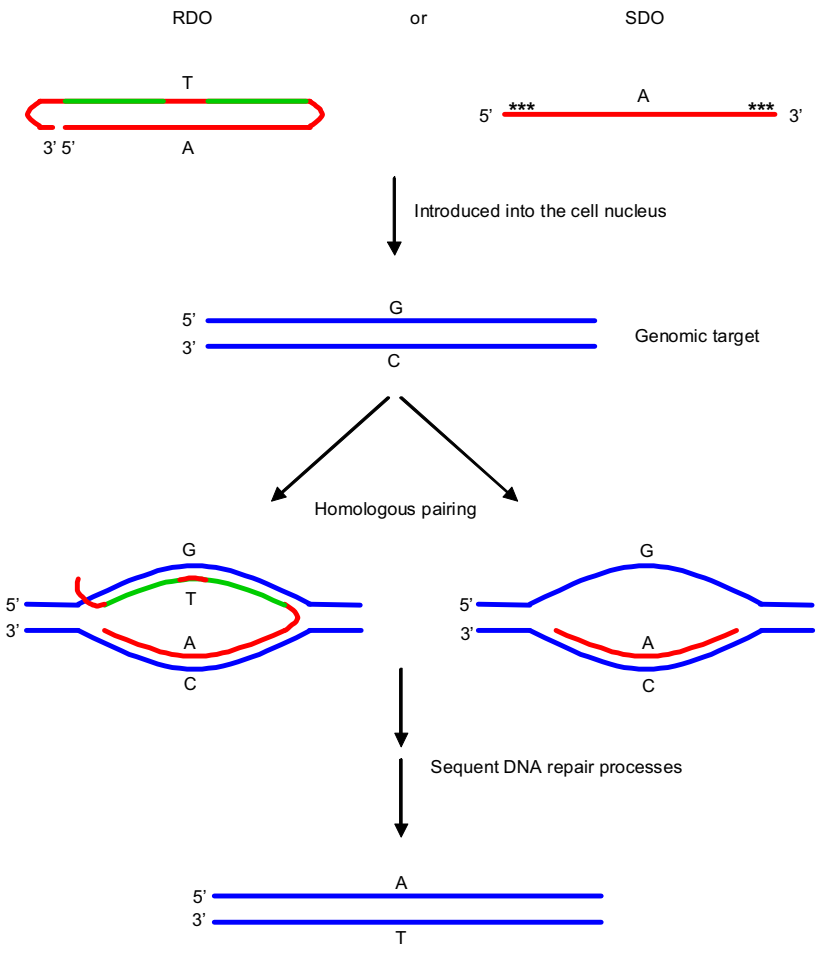


**Fig. 1 Illustration of site-directed gene repair of base pair G/C to A/T at a locus by targeting oligonucleotides.** RDO is a chimeric RNA (green)/ DNA (red) oligonucleotide. SDO is a single-stranded DNA oligonucleotide with chemical modification at the ends (labeled with ***), which can be phosphorothioate linkages, locked nucleic acid residues or other modifications.

therapy, functional genomics and crop improvement. However, large variations and discrepancies of gene repair frequencies were subsequently found. This raised skepticism regarding the reproducibility, consistency and robustness of the method. This minireview focuses on the success and problems of the use of RDOs and SDOs in plants, but does not consider other targeted mutation technologies, such as homologous recombination.

## OLIGONUCLEOTIDE-DIRECTED GENE REPAIR

Gene targeting, or gene replacement via homologous recombination, was the initial approach used to repair defective chromosomal alleles or to produce loss-of-function alleles. Unfortunately, extremely low rates (~$10^{-5}$) of homologous recombination and high levels of non-homologous random insertion of the introduced DNA were found in both mammalian cells and higher plants (Vasquez *et al*. 2001; Puchta 2002). The mechanisms and factors involved in homologous recombination were studied intensely. This provided new knowledge of DNA repair. The development of chimeric RNA/DNA oligonucleotides, chimeraplasts, for targeted gene repair was based on early studies indicating that RNA strands increased the efficiency and stability of homologous pairing (Kotani and Kmiec 1994). The prototypic chimeraplast is a single-stranded 68 nucleotide oligo that forms a double-hairpin conformation by self complementary base pairing. The homologous segment between the chimeraplast and its genomic target is 25 bases in length, with one strand being DNA and the other a combination of ten 2'-O-methyl RNA residues flanking five bases of DNA in the central region, where the DNA double strand region has one base pair mismatch with the DNA sequence of the target gene (**Fig. 1**). This synthetic oligonucleotide, in a target cell, will direct a gene repair event by a two-step reaction; homologous pairing between the oligonucleotide and the target sequence, followed by repair of the mismatch by the endogenous DNA repair machinery. Thus a predetermined base pair change, including a single-nucleotide deletion, insertion or substitution, can be achieved without changing any other sequence. The feasibility of gene repair by chimeraplasts was first demonstrated in Kmiec's laboratory in 1996 by correction of a point mutation in an episomal alkaline phosphatase gene in Chinese hamster ovary cells (Yoon *et al*. 1996). Gene correction restored enzymatic activity, enabling visualization of cells that gained the alkaline phosphatase phenotype by histochemical staining. Following this, a chromosomal gene, a mutant β-globin which causes sickle cell anemia, was corrected by a chimeraplast at a high frequency in cultured human cells (Cole-Strauss *et al*. 1996). Due to a lack of reproducibility found in further similar studies, Gamper *et al*. (2000) reassessed the activity of the chimeraplast and found that the DNA strand of the RDO was responsible for the gene repair activity. The RDO was therefore simplified to a single-stranded DNA oligonucleotide of 25-90 nucleotides with chemical modifications at each end to prevent nuclease degradation (**Fig. 1**). This type of oligonucleotide-directed gene repair had already been demonstrated in yeast in the late 1980s (Moerschell *et al*. 1988).

## SUCCESS STORIES

Following the first demonstration of successful gene repair by chimeric RNA/DNA oligonucleotides in cultured mammalian and human cells with a gene repair frequency up to 50% (Yoon *et al*. 1996; Cole-Strauss *et al*. 1996), several laboratories reported gene alteration in a range of different systems, including *in vivo* animal models, cultured cells, episomal gene repair and *in vitro* assays using cell-free extracts (**Fig. 2**). In 1998, chimeraplasty was first reported in a whole animal model by intravenous injection of RDOs into rats to introduce a point mutation in the rat factor IX gene, which led to reduced factor IX gene activity in liver cells of the injected rats (Kren *et al*. 1998). Similar RDO-directed gene repair was also demonstrated in a variety of


**Fig. 2 Flow-chart of experimental systems for oligonucleotide-directed gene repair.**

tissues, including skin and skeletal muscle. Genetic changes induced by RDOs were shown to be genetically stable for several cell generations, as shown by restoration of pigmentation due to the targeted gene repair of a mutant tyrosinase gene in the skin of albino mice (Alexeev and Yoon 1998; Alexeev *et al*. 2000). Functional rescue of a genetic disease was reported by restoration of the expression of dystrophin in mouse (Rando *et al*. 2000) and in dog (Bartlett *et al*. 2000). Modified single-stranded oligonucleotides were also successfully used in various systems. For example, Igoucheva *et al*. (2001) demonstrated the correction of a mutant inactive β-galactosidase gene by SDOs in mammalian cells. The frequency of correction was 0.05% when using nuclear extracts *in vitro*, 1% for episomal, or 0.1% for the chromosomal transgene of the same mutant β-galactosidase. The simultaneous correction of two endogenous genes in albino mouse melanocytes was achieved using SDOs; the repair frequencies of the tyrosinase gene and c-kit gene ranged from $2x10^{-4}$ to $1x10^{-3}$ (Alexeev *et al*. 2002; for comprehensive reviews, see Liu *et al*. 2003; Igoucheva *et al*. 2004; Parekh-Olmedo *et al*. 2005; de Semir and Aran 2006).

The first successful applications of chimeric oligonucleotide-directed mutagenesis in plants was achieved in tobacco (Beetham *et al*. 1999) and maize (Zhu *et al*. 1999). In both cases, the gene coding for the first enzyme in the biosynthetic pathway of branched amino acids was chosen as the target, which in tobacco is referred to as acetolactate synthase (ALS) and in maize as acetohydroxy acid synthase (AHAS). Mutations of particular amino acids in this protein result in plants resistant to imidazoline and sulfonylurea class herbicides, a selectable phenotype. RDOs designed to produce a predicted amino acid change in the ALS/AHAS enzyme were introduced into tobacco or maize suspension cells by particle bombardment. Resistant callus clones were obtained on selection media containing imazethapyr or chlorsulfurone. The efficiency of recovery of herbicide-resistant tobacco calli was 10 to 20 times above background, and about $10^{-4}$ per cell after receiving oligonucleotides in the case of maize. In both studies, a transgenic, but nonfunctional, GFP gene was also targeted for conversion to functional GFP with separate RDOs. Engineered nontranslatable forms of GFP genes, either by deletion of a single base pair in the ORF (Beetham *et al*. 1999) or introduction of a stop codon at the start codon (Zhu *et al*. 1999), were first genetically transformed into tobacco or maize. Specific RDOs designed to correct those mutations in the GFP genes were then bombarded into transformed tobacco cells or maize cells. Green fluorescent cells were subsequently found among the targeted cells (**Fig. 2**). In the maize study, resistant and fluorescent calli were regenerated into fertile plants, and the progeny of these plants showed Mendelian segregation of herbicide resistance and green fluorescence (Zhu *et al*. 1999, 2000). These were the first plants engineered through oligonucleotide-directed gene repair. Following this success, herbicide resistant tobacco plants were also regenerated through oligonucleotide-directed gene repair using electroporation of tobacco

protoplasts and particle bombardment of cell colonies (Kochevenko and Willmitzer 2003). The frequency of gene repair in this report was ~$10^{-6}$, 10 to 20 times the untreated control, and similar to the report by Beetham *et al*. (1999). However, the DNA sequences from the herbicide resistant tobacco calli or plants (Beetham *et al*. 1999; Kochevenko and Willmitzer 2003) showed there were nonspecific conversions in the targeted base and also changes immediately 5′ to that base. For example, targeting of the tobacco ALS gene at Proline 196 (CCA) resulted in the intended change to CAA (Glutamine) as well as other changes in the same codon, to CTA (Leucine), ACA (Threonine) and TCA (Serine) (Kochevenko and Willmitzer 2003). In the report by Beetham *et al*. (1999), all analyzed cases showed a change to ACA (Threonine) at the Proline 196 (CCA) rather than the projected change to CAA (Glutamine). Analysis of sequences of maize herbicide resistant calli showed 11 of 16 RDO-derived events had the intended change from AGT to AAT at position 621 (Serine to Asparagine), and only 2 of 12 events had the intended change at position 165 (Proline CCG to Alanine GCG) (Zhu *et al*. 1999). This lack of precision was not observed in animal systems, and indicates the possible existence of different DNA repair mechanisms in animals and plants. To help elucidate the factors involved in the conversion mechanism and as a system to study plant DNA repair mechanisms, cell-free assays utilizing protein extracts from either plastids or whole cells were established (Rice *et al*. 2000; Kmiec *et al*. 2001). Nonspecific base changes were also observed during the *in vitro* assay using tobacco cell-free extracts, but not in maize or banana cell-free extracts (Rice *et al*. 2000). Whether this "ectopic" conversion is limited to certain plant species is unknown. Chimeric RNA/DNA oligonucleotides and modified single-stranded DNA oligonuceotides can both convert or insert a targeted base within a plasmid DNA substrate *in vitro* (Gamper *et al*. 2000; Kmiec *et al*. 2001). In rice, Okuzaki and Toriyama (2004) reported RDO induced gene alteration in the rice ALS gene resulting in herbicide resistant plantlets. Recently, *in vivo* conversion of a plasmid nonfunctional GFP gene into a functional GFP in wheat scutellum tissue was reported (Dong *et al*. 2006a). **Table 1** summarizes reports of oligonucleotide-directed gene repair in plants.

## THE PROBLEMS

An enormous interest in the technology for gene therapy and gene targeting was generated due to the early reports of high gene repair frequencies using chimeric RNA/DNA oligonucleotides. However the scientific and biotechnological excitement soon turned to disappointment due to the inconsistencies of results and failures to reproduce the pioneer findings. Several groups reported persistent failures in oligonucleotide-directed gene repair (Strauss 1998; van der Steege *et al*. 2001; Graham *et al*. 2001; Albuquerque-Silva *et al*. 2001; de Semir *et al*. 2003; Manzano *et al*. 2003). There were also many other failed, but not reported, attempts to use this gene repair methodology, as stated by Taubes (2002). Alternative explanations of the initial reports of high frequency gene repair were proposed by Thomas and Capecchi (1997) and Stasiak *et al*. (1997), mainly suggesting possible artifacts in the assessment of gene correction leading to erroneous high values.

In plants, Ruiter *et al*. (2003) reported that the spontaneous mutation frequency in plant cells obscures the effect of chimeraplasty. They targeted an endogenous ALS gene and two transgenes, the bar gene and a fusion between egfp and bar in tobacco and oilseed rape, using chimeric RNA/DNA oligonucleotides. Similar numbers of cells were treated with and without chimeraplasts, and the treatment with chimeraplasts did not lead to increased numbers of herbicide resistant calli or green fluorescent calli. Indeed, a range of sequence changes other than the intended base change was found in the targeted region in both the targeted and control calli, at a similar frequency of ~$10^{-7}$. In rice, Okuzaki and Toriyama (2005) also reported spontaneous mutations in oligonucleotide-directed gene targeting experiments. In our laboratory, proof-of-principle of oligonucleotide-directed gene repair in wheat was achieved by targeting a plasmid nonfunctional GFP gene in wheat scutellum cells (Dong *et al*. 2006a). However, large variations of targeting frequencies were observed among different experiments. When a chromosomal gene, AHAS, was targeted for a predetermined amino acid change conferring resistance to imidazoline and sulfonylurea class herbicides, no herbicide resistant plant was recovered from 12,000 targeted scutella. This was equivalent to ~1.2 x $10^7$ cells receiving targeting oligonucleotides based on the number of cells receiving GFP constructs after bombardment of a scutellum (Dong *et al*. 2006b). It is likely that other laboratories also tried this technology, with failures not published.

Large variations in repair frequencies suggest that oligonucleotide-directed gene repair is a very complicated procedure. Many factors may influence the effectiveness of this type of gene repair. One important factor is that the targeting molecules must reach the nucleus of the target cells. DNA delivery does affect the frequency of targeted gene repair as demonstrated in a number of reports (Liu H *et al*. 2002; Thorpe *et al*. 2002). The competency of the cells to catalyze gene repair is another important factor that contributes to the variable levels of repair. Different cell types have distinct repair competencies (Santana *et al*. 1998; Cole-Strauss *et al*. 1999; Igoucheva *et al*. 1999). Even in the same laboratory using the same batch of oligonucleotide and cell line, Yoon and colleagues observed that in a system for correcting a point mutation in mouse tyrosinase gene, repair frequencies ranged from 0.01% to 15% among at least 30 independent experiments (Alexeev and Yoon 1998; Igoucheva *et al*. 2004). This indeed reflects the complexity of oligonucleotide-directed gene repair. The eluci-

**Table 1** Application of oligonucleotide-directed gene repair in plants.

| Target gene* | Oligo type | Species | System | Targeting method | Frequency | Reference |
|---|---|---|---|---|---|---|
| ALS, GFP | RDO | Tobacco | Suspension cells | *in vivo* chromosomal | Relative to control | Beetham *et al*. 1999 |
| AHAS, PAT/GFP | RDO | Maize | Suspension cells, Immature embryo | *in vivo* chromosomal | 0.01-0.1% | Zhu *et al*. 1999, 2000 |
| Kan, Tet | RDO | Maize, banana, tobacco | Cell-free extracts | *in vitro* | Relative to control | Rice *et al*. 2000 |
| Kan | RDO/SDO | Canola, banana | Cell-free extracts | *in vitro* | Relative to control | Gamper *et al*. 2000 |
| Kan, Tet | RDO/SDO | Spinach | Choloroplast extracts | *in vitro* | Relative to control | Kmiec *et al*. 2001 |
| ALS | RDO | Tobacco | Suspension cells, Protoplasts | *in vivo* chromosomal | ~$10^{-6}$ | Kochevenko and Willmitzer 2003 |
| ALS, EGFP/BAR | RDO | Tobacco, canola | Suspension cells | *in vivo* chromosomal | 0 | Ruiter *et al*. 2003 |
| AHAS | RDO | Rice | Embryonic callus | *in vivo* chromosomal | ~0.01% | Okuzaki and Toriyama 2004 |
| GFP | RDO/SDO | Wheat | Immature embryo | *in vivo* plasmid gene | 0.06-0.62% | Dong *et al*. 2006a |

*Target gene: ALS: acetolactate synthase; GFP: green flurorescent protein; AHAS: acetohydroxy acid synthase; PAT/GFP: phosphinothricin-N-acetyltransferase/green flurorescent protein fusion; Kan: Kanamycin$^R$; Tet: Tetracycline$^R$; EGFP/BAR: enhanced green flurorescent protein/phosphinothricin-N-acetyltransferase fusion

dation of the still unknown mechanism(s) of oligonucleotide-directed gene repair of chromosomal sequences will be important for improvement of the technology.

Targeting of plant genome sequences has differences from mammalian gene targeting or gene therapy. Firstly, delivery of DNA into the nuclei of plant cells is more difficult than animal cells due to the rigid plant cell wall. Using plant protoplasts instead of plant cells may therefore be ideal for DNA delivery. However, Kochevenko and Willmitzer (2003) treated tobacco protoplasts with targeting oligonucleotides and found no increase in repair frequency compared to treatment of intact cells. Secondly, plant gene targeting for functional genomics or breeding requires regeneration of whole plants from targeted somatic cells; whereas in human gene therapy, somatic tissue is the main target. Therefore the regeneration frequency also must be taken into account in plant gene repair experiments. From the published results, the overall gene repair frequency in plants is low ($10^{-4}$ to $10^{-6}$), and the precision of targeted changes is also lower than that in animals. With these frequencies, targeting a gene without a selectable phenotype is impossible. The frequency of gene repair is as low as that of gene targeting via homologous recombination. Both approaches need time-consuming selection procedures and tissue culture. Oligonucleotide-directed gene repair of plant genomes is therefore not advanced over other technologies in regard to frequency, accuracy and procedure.

## MECHANISTIC STUDIES

The molecular mechanism of oligonucleotide-directed gene repair is proposed to be homologous pairing between the corrective molecules and target chromosomal locus as the initial stage, with the subsequent conversion process performed by DNA repair enzymes. The first step is probably rate limiting for the repair frequency (Drury and Kmiec 2003). Genetic studies in yeast revealed that overexpression of RAD51 and RAD54 increased chromosomal gene repair frequency, while RAD52 had some suppression effect (Liu *et al*. 2002a). Yeast RAD51, RAD52 and RAD54 have roles in DNA repair and recombination. Recent studies indicated that RAD54 and RAD51 act coordinately in chromatin remodeling, but not on naked DNA to enhance DNA pairing (Alexiadis and Kadonaga 2002). Other studies also support RAD51 as an important protein facilitating oligonucleotide-directed gene repair (Igoucheva *et al*. 2002; Thorpe *et al*. 2002; Liu *et al*. 2004). Another protein, p53, was found to suppress gene repair. Protein p53 responds to many stresses including DNA damage, and then in turn, it induces growth arrest. Yoon and colleagues found that gene repair activity was elevated when using a p53 defective mouse embryonic cell-free extract in an *in vitro* assay (Igoucheva *et al*. 1999). However, the use of an anti-p53 antibody did not increase gene repair frequency. It was suggested that mismatch repair proteins, such as MSH2 and MSH6, would have greater access to the mismatches in the absence of the p53 protein. The molecular mechanism of the second step of gene correction is still poorly understood. Some results suggest the involvement of the mismatch repair system (MMR) which includes MSH2, MSH3, MSH6, MLH1, PMS2 and others. For example, the addition of an anti-MSH2 antibody in a cell-free extract reduced gene repair activity (Cole-Strauss *et al*. 1999). However, the MSH2 protein inhibited SDO-directed gene repair in mouse embryonic stem cells (Dekker *et al*. 2003, 2006). Other DNA repair pathways such as transcription-coupled repair and nucleotide excision repair (NER) were suggested by Igoucheva *et al*. (2004), and a "replication mode" was suggested by Parekh-Olmedo *et al*. (2005).

The state of transcription and the DNA replication activity of the target gene may be important parameters influencing gene repair frequency. RDOs and SDOs directed to the non-transcribed target strand had more efficient gene repair than those targeting the transcribed strand (Liu *et al*. 2001, 2002b; Brachman and Kmiec 2003; Igoucheva *et al*. 2003). However, this non-transcribed strand conversion bias is not consistent (Bennett and Schaack 2003; Brachman and Kmiec 2003). Brachman and Kmiec (2004) found that DNA replication could increase the level of untranscribed strand preference only if that strand also served as the lagging strand in DNA synthesis. Thus, DNA replication plays an important role in targeted gene repair.

If DNA replication influences the overall process of gene repair, targeting a cell in the S phase could increase the frequency of gene repair. This was found in several studies (Majumdar *et al*. 2003; Brachman and Kmiec 2005; Hu *et al*. 2005). Recently, Olsen *et al*. (2005) showed that the cell cycle influenced the rates of targeted sequence correction *in vivo*, with a peak in the early S phase. Importantly, they also found that the majority of corrected cells were arrested in the G2/M phase and underwent programmed cell death, whereas only 1-2% of the corrected cells formed viable colonies. Ferrara and Kmiec (2006) recently found that oligonucleotides trigger DNA-damage signaling leading to replication stalling, DNA repair and cell cycle arrest. Igoucheva *et al*. (2006) also found that exogenous DNA induced DNA-damage signaling and DNA repair in a cell-type dependant manner, although some results were different from those of Ferrara and Kmiec (2006). It is not surprising that large amounts of exogenous oligonucleotides entering a cell will induce DNA damage/cellular stress response pathways. These important findings may also explain the low and variable frequencies of gene correction often obtained in different experiments in which corrected cells have to survive to varying degrees prior to phenotypic analysis. Understanding the molecular mechanisms and cellular responses of oligonucleotide-directed gene repair will help future development of this technology.

## PERSPECTIVE

In plants there are not many studies of the mechanism of oligonucleotide-directed gene repair. However, findings from mammalian targeted gene repair will help improve this technology in plants. As the efficiency of oligonucleotide-directed gene repair is currently too low, the technology will not replace other biotechnologies used in functional genomics and crop improvement, such as gene transformation, random insertional mutagenesis (Krysan *et al*. 1999) and RNAi approaches (Waterhouse and Helliwell 2003).

Recently, a very elegant approach towards site-specific modification of genome sequences was reported. Based on the knowledge that homologous recombination is enhanced by induction of a DSB (double strand break) at the genomic target site, a chimeric endonuclease was developed with a specific zinc-finger DNA-binding domain fused with the non-specific cleavage endonuclease domain of the *Fok* I restriction enzyme. The binding motif can be engineered to create zinc fingers with different sequence specificities, to recognize virtually any genomic site. Urnov *et al*. (2005) showed that using two four-finger nucleases (recognizing 12 bp of sequence at flanking sites of the target), a mutation in the human interleukin 2 receptor γ gene (IL2Rγ) was corrected by DSB enhanced homologous recombination (HR). The modification frequency reached 18% of the treated cells without selection, a remarkably high frequency compared to a usual HR rate of 0.001% with no DSBs. Zinc-finger nucleases can also work in plants (Lloyd *et al*. 2005; Wright *et al*. 2005).

Another reverse genetic, nontransgenic method, TILLING (targeting induced local lesions in genomes), is showing great potential for functional genomics and crop improvement. As the method uses non-targeted chemical mutation, TILLING has to combine traditional chemical mutagenesis with advanced high-throughput targeted mutation detection techniques to identify allelic series of point mutations including knock-outs and partial inactivations of the gene of interest (Colbert *et al*. 2001; Slade *et al*. 2004).

In our laboratory, we identified 150 mutants of wheat, including some knock-outs, in 4 targeted genes using TILLING in less than four months (unpublished results). The generation of the TILLING population took about six months; this population can be used for screening any gene of interest. In comparison, with oligonucleotide-directed gene repair, we spent one year targeting the wheat AHAS gene, but found no herbicide resistant derivatives. With further development of efficient and low-cost screening methods, TILLING may become a favored method for plant functional genomics and crop improvement.

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## REFERENCES

**Albuquerque-Silva J, Vassart G, Lavinha J, Abramowicz MJ** (2001) Chimeraplasty validation. *Nature Biotechnology* **19**, 1011-1011

**Alexeev V, Igoucheva O, Domashenko A, Cotsarelis G, Yoon K** (2000) Localized *in vivo* genotypic and phenotypic correction of the albino mutation in skin by RNA-DNA oligonucleotide. *Nature Biotechnology* **18**, 43-47

**Alexeev V, Igoucheva O, Yoon K** (2002) Simultaneous targeted alteration of the tyrosinase and c-kit genes by single-stranded oligonucleotides. *Gene Therapy* **9**, 1667-1675

**Alexeev V, Yoon K** (1998) Stable and inheritable changes in genotype and phenotype of albino melanocytes induced by an RNA-DNA oligonucleotide. *Nature Biotechnology* **16**, 1343-1346

**Alexiadis V, Kadonaga JT** (2002) Strand pairing by Rad54 and Rad51 is enhanced by chromatin. *Genes and Development* **16**, 2767-2771

**Bartlett RJ, Stockinger S, Denis MM, Bartlett WT, Inverardi L, Le TT, Man NT, Morris GE, Bogan DJ, Metcalf-Bogan J, Kornegay JN** (2000) *In vivo* targeted repair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide. *Nature Biotechnology* **18**, 615-622

**Beetham PR, Kipp PB, Sawycky XL, Arntzen CJ, May GD** (1999) A tool for functional plant genomics: Chimeric RNA/DNA oligonucleotides cause *in vivo* gene-specific mutations. *Proceedings of the National Academy of Sciences USA* **96**, 8774-8778

**Bennett M, Schaack J** (2003) Development of a dual-luciferase fusion gene as a sensitive marker for site-directed DNA repair strategies. *Journal of Gene Medicine* **5**, 723-732

**Brachman EE, Kmiec EB** (2003) Targeted nucleotide repair of *cyc1* mutations in *Saccharomyces cerevisiae* directed by modified single-stranded DNA oligonucleotides. *Genetics* **163**, 527-538

**Brachman EE, Kmiec EB** (2004) DNA replication and transcription direct a DNA strand bias in the process of targeted gene repair in mammalian cells. *Journal of Cell Science* **117**, 3867-3874

**Brachman EE, Kmiec EB** (2005) Gene repair in mammalian cells is stimulated by the elongation of S phase and transient stalling of replication forks. *DNA Repair* **4**, 445-457

**Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S** (2001) High-throughput screening for induced point mutations. *Plant Physiology* **126**, 480-484

**Cole-Strauss A, Gamper H, Holloman WK, Munoz M, Cheng N, Kmiec EB** (1999) Targeted gene repair directed by the chimeric RNA/DNA oligonucleotide in a mammalian cell-free extract. *Nucleic Acids Research* **27**, 1323-1330

**Cole-Strauss A, Yoon K, Xiang YF, Byrne BC, Rice MC, Gryn J, Holloman WK, Kmiec EB** (1996) Correction of the mutation responsible for sickle cell anemia by an RNA-DNA oligonucleotide. *Science* **273**, 1386-1389

**de Semir D, Aran JM** (2006) Targeted gene repair: The ups and downs of a promising gene therapy approach. *Current Gene Therapy* **6**, 481-504

**de Semir D, Nadal M, Gonzalez JR, Larriba S, Avinyo A, Nunes V, Casals T, Estivill X, Aran JM** (2003) Suitability of oligonucleotide-mediated cystic fibrosis gene repair in airway epithelial cells. *Journal of Gene Medicine* **5**, 625-639

**Dekker M, Brouwers C, Aarts M, van der Torre J, de Vries S, de Vrugt HV, te Riele H** (2006) Effective oligonucleotide-mediated gene disruption in ES cells lacking the mismatch repair protein MSH3. *Gene Therapy* **13**, 686-694

**Dekker M, Brouwers C, te Riele H** (2003) Targeted gene modification in mismatch-repair-deficient embryonic stem cells by single-stranded DNA oligonucleotides. *Nucleic Acids Research* **31**, E27-E27

**Dong CM, Beetham P, Vincent K, Sharp P** (2006a) Oligonucleotide-directed gene repair in wheat using a transient plasmid gene repair assay system. *Plant Cell Reports* **25**, 457-465

**Dong CM, Beetham P, Vincent K, Sharp P** (2006b) Oligonucleotide-directed gene repair in wheat using a transient plasmid gene repair assay system. In: *8th International Congress of Plant Molecular Biology*, Adelaide, Australia, August 20-25, 2006, Book of Abstracts, p 36

**Drury MD, Kmiec EB** (2003) DNA pairing is an important step in the process of targeted nucleotide exchange. *Nucleic Acids Research* **31**, 899-910

**Ferrara L, Kmiec EB** (2006) Targeted gene repair activates Chk1 and Chk2 and stalls replication in corrected cells. *DNA Repair* **5**, 422-431

**Gamper HB, Parekh H, Rice MC, Bruner M, Youkey H, Kmiec EB** (2000) The DNA strand of chimeric RNA/DNA oligonucleotides can direct gene repair/conversion activity in mammalian and plant cell-free extracts. *Nucleic Acids Research* **28**, 4332-4339

**Graham IR, Manzano A, Tagalakis AD, Mohri Z, Sperber G, Hill V, Beattie S, Schepelmann S, Dickson G, Owen JS** (2001) Gene repair validation. *Nature Biotechnology* **19**, 507-508

**Hu YL, Parekh-Olmedo H, Drury M, Skogen M, Kmiec EB** (2005) Reaction parameters of targeted gene repair in mammalian cells. *Molecular Biotechnology* **29**, 197-210

**Igoucheva O, Alexeev V, Pryce M, Yoon K** (2003) Transcription affects formation and processing of intermediates in oligonucleotide-mediated gene alteration. *Nucleic Acids Research* **31**, 2659-2670

**Igoucheva O, Alexeev V, Yoon K** (2001) Targeted gene correction by small single-stranded oligonucleotides in mammalian cells. *Gene Therapy* **8**, 391-399

**Igoucheva O, Alexeev V, Yoon K** (2002) Nuclear extracts promote gene correction and strand pairing of oligonucleotides to the homologous plasmid. *Antisense and Nucleic Acid Drug Development* **12**, 235-246

**Igoucheva O, Alexeev V, Yoon K** (2004) Oligonucleotide-directed mutagenesis and targeted gene correction: A mechanistic point of view. *Current Molecular Medicine* **4**, 445-463

**Igoucheva O, Alexeev V, Yoon K** (2006) Differential cellular responses to exogenous DNA in mammalian cells and its effect on oligonucleotide-directed gene modification. *Gene Therapy* **13**, 266-275

**Igoucheva O, Peritz AE, Levy D, Yoon K** (1999) A sequence-specific gene correction by an RNA-DNA oligonucleotide in mammalian cells characterized by transfection and nuclear extract using a lacZ shuttle system. *Gene Therapy* **6**, 1960-1971

**Kmiec EB, Johnson C, May GD** (2001) Chloroplast lysates support directed mutagenesis via modified DNA and chimeric RNA/DNA oligonucleotides. *Plant Journal* **27**, 267-274

**Kochevenko A, Willmitzer L** (2003) Chimeric RNA/DNA oligonucleotide-based site-specific modification of the tobacco acetolactate synthase gene. *Plant Physiology* **132**, 174-184

**Kotani H, Kmiec EB** (1994) A role for RNA synthesis in homologous pairing events. *Molecular and Cellular Biology* **14**, 6097-6106

**Kren BT, Bandyopadhyay P, Steer CJ** (1998) *In vivo* site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. *Nature Medicine* **4**, 285-290

**Krysan PJ, Young JC, Sussman MR** (1999) T-DNA as an insertional mutagen in Arabidopsis. *The Plant Cell* **11**, 2283-2290

**Liu H, Agarwal S, Kmiec E, Davis BR** (2002) Targeted β-globin gene conversion in human hematopoietic CD34(+) and Lin(-)CD38(-) cells. *Gene Therapy* **9**, 118-126

**Liu L, Cheng SQ, van Brabant AJ, Kmiec EB** (2002a) Rad51p and Rad54p, but not Rad52p, elevate gene repair in *Saccharomyces cerevisiae* directed by modified single-stranded oligonucleotide vectors. *Nucleic Acids Research* **30**, 2742-2750

**Liu L, Maguire KK, Kmiec EB** (2004) Genetic re-engineering of *Saccharomyces cerevisiae* RAD51 leads to a significant increase in the frequency of gene repair *in vivo*. *Nucleic Acids Research* **32**, 2093-2101

**Liu L, Parekh-Olmedo H, Kmiec EB** (2003) The development and regulation of gene repair. *Nature Reviews Genetics* **4**, 679-689

**Liu L, Rice MC, Drury M, Cheng SQ, Gamper H, Kmiec EB** (2002b) Strand bias in targeted gene repair is influenced by transcriptional activity. *Molecular and Cellular Biology* **22**, 3852-3863

**Liu L, Rice MC, Kmiec EB** (2001) *In vivo* gene repair of point and frameshift mutations directed by chimeric RNA/DNA oligonucleotides and modified single-stranded oligonucleotides. *Nucleic Acids Research* **29**, 4238-4250

**Lloyd A, Plaisier CL, Carroll D, Drews GN** (2005) Targeted mutagenesis using zinc-finger nucleases in Arabidopsis. *Proceedings of the National Academy of Sciences USA* **102**, 2232-2237

**Majumdar A, Puri N, Cuenoud B, Natt F, Martin P, Khorlin A, Dyatkina N, George AJ, Miller PS, Seidman MM** (2003) Cell cycle modulation of gene targeting by a triple helix-forming oligonucleotide. *The Journal of Biological Chemistry* **278**, 11072-11077

**Manzano A, Mohri Z, Sperber G, Ogris M, Graham I, Dickson G, Owen JS** (2003) Failure to generate atheroprotective apolipoprotein AI phenotypes using synthetic RNA/DNA oligonucleotides (chimeraplasts). *Journal of Gene Medicine* **5**, 795-802

**Moerschell RP, Tsunasawa S, Sherman F** (1988) Transformation of yeast with synthetic oligonucleotides. *Proceedings of the National Academy of Sciences USA* **85**, 524-528

**Okuzaki A, Toriyama K** (2004) Chimeric RNA/DNA oligonucleotide-directed gene targeting in rice. *Plant Cell Reports* **22**, 509-512

**Okuzaki A, Toriyama K** (2005) Producing rice plants with a site-specific base change in the acetolactate synthase gene by chimeraplast-directed gene targeting. In: Toriyama K, Heong KL, Hardy B (Eds) *Rice is Life: Scientific Perspectives for the 21st Century*, http://www.irri.org/publications/wrrc/

wrrcPDF/session3-07.pdf

**Olsen PA, Randol M, Krauss S** (2005) Implications of cell cycle progression on functional sequence correction by short single-stranded DNA oligonucleotides. *Gene Therapy* **12**, 546-551

**Parekh-Olmedo H, Ferrara L, Brachman E, Kmiec EB** (2005) Gene therapy progress and prospects: targeted gene repair. *Gene Therapy* **12**, 639-646

**Puchta H** (2002) Gene replacement by homologous recombination in plants. *Plant Molecular Biology* **48**, 173-182

**Rando TA, Disatnik MH, Zhou LZH** (2000) Rescue of dystrophin expression in mdx mouse muscle by RNA/DNA oligonucleotides. *Proceedings of the National Academy of Sciences USA* **97**, 5363-5368

**Rice MC, May GD, Kipp PB, Parekh H, Kmiec EB** (2000) Genetic repair of mutations in plant cell-free extracts directed by specific chimeric oligonucleotides. *Plant Physiology* **123**, 427-437

**Ruiter R, Brande IVB, Stals E, Delaure S, Cornelissen M, D'Halluin K** (2003) Spontaneous mutation frequency in plants obscures the effect of chimeraplasty. *Plant Molecular Biology* **53**, 675-689

**Santana E, Peritz AE, Iyer S, Uitto J, Yoon K** (1998) Different frequency of gene targeting events by the RNA-DNA oligonucleotide among epithelial cells. *Journal of Investigative Dermatology* **111**, 1172-1177

**Slade AJ, Fuerstenberg SI, Loeffler D, Steine MN, Facciotti D** (2005) A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotechnology* **23**, 75-81

**Stasiak A, West SC, Egelman EH** (1997) Sickle cell anemia research and a recombinant DNA technique. *Science* **277**, 460-462

**Strauss M** (1998) The site-specific correction of genetic defects. *Nature Medicine* **4**, 274-275

**Taubes G** (2002) Gene therapy: The strange case of chimeraplasty. *Science* **298**, 2116-2120

**Thomas KR, Capecchi MR** (1997) Recombinant DNA technique and sickle cell anemia research. *Science* **275**, 1404-1405

**Thorpe P, Stevenson BJ, Porteous DJ** (2002) Optimising gene repair strategies in cell culture. *Gene Therapy* **9**, 700-702

**Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC** (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**, 646-651

**van der Steege G, Schuilenga-Hut PHL, Buys C, Scheffer H, Pas HH, Jonkman MF** (2001) Persistent failures in gene repair. *Nature Biotechnology* **19**, 305-306

**Vasquez KM, Marburger K, Intody Z, Wilson JH** (2001) Manipulating the mammalian genome by homologous recombination. *Proceedings of the National Academy of Sciences USA* **98**, 8403-8410

**Waterhouse PM, Helliwell CA** (2003) Exploring plant genomes by RNA-induced gene silencing. *Nature Reviews Genetics* **4**, 29-38

**Wright DA, Townsend JA, Winfrey RJ, Irwin PA, Rajagopal J, Lonosky PM, Hall BD, Jondle MD, Voytas DF** (2005) High-frequency homologous recombination in plants mediated by zinc-finger nucleases. *Plant Journal* **44**, 693-705

**Yoon K, Cole-Strauss A, Kmiec EB** (1996) Targeted gene correction of episomal DNA in mammalian cell mediated by a chimeric RNA/DNA oligonucleotide. *Proceedings of the National Academy of Sciences USA* **93**, 2071-2076

**Zhu T, Mettenburg K, Peterson DJ, Tagliani L, Baszczynski CL** (2000) Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. *Nature Biotechnology* **18**, 555-558

**Zhu T, Peterson DJ, Tagliani L, St. Clair G, Baszczynski CL, Bowen B** (1999) Targeted manipulation of maize genes *in vivo* using chimeric RNA/DNA oligonucleotides. *Proceedings of the National Academy of Sciences USA* **96**, 8768-8773