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Oligonucleotide-directed mutagenesis for precision gene editing

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Summary

Differences in gene sequences, many of which are single nucleotide polymorphisms, underlie some of the most important traits in plants. With humanity facing significant challenges to increase global agricultural productivity, there is an urgent need to accelerate the development of these traits in plants. oligonucleotide-directed mutagenesis (ODM), one of the many tools of Cibus' Rapid Trait Development System (***RTDS*** ™) technology, offers a rapid, precise and non-transgenic breeding alternative for trait improvement in agriculture to address this urgent need. This review explores the application of ODM as a precision genome editing technology, with emphasis on using oligonucleotides to make targeted edits in plasmid, episomal and chromosomal DNA of bacterial, fungal, mammalian and plant systems. The process of employing ODM by way of ***RTDS*** technology has been improved in many ways by utilizing a fluorescence conversion system wherein a blue fluorescent protein (BFP) can be changed to a green fluorescent protein (GFP) by editing a single nucleotide of the BFP gene (CAC→TAC; H66 to Y66). For example, dependent on oligonucleotide length, applying oligonucleotide-mediated technology to target the BFP transgene in *Arabidopsis thaliana* protoplasts resulted in up to 0.05% precisely edited GFP loci. Here, the development of traits in commercially relevant plant varieties to improve crop performance by genome editing technologies such as ODM, and by extension ***RTDS***, is reviewed.

Keywords: oligonucleotide-directed mutagenesis, precision gene editing, ***RTDS***™, CRISPR, TALEN

Introduction

A major challenge in biology, particularly plant biology, is gene conversion. Almost a decade ago, this challenge was also known as directed gene modification or gene targeting and more recently has been termed precision gene editing. Irrespective of the name, the intent of gene conversion is to augment the genetic diversity of a specific genotype by precisely altering the sequence of a particular genome target(s) by one or more bases. These alterations may also include precise insertions or deletions in the target sequence. The oligonucleotide-directed mutagenesis (ODM) technique for genome editing has been successfully em in bacterial, yeast, mammalian and plant systems (Aarts *et al*., 2006; Gocal *et al*., 2015; Moerschell *et* 1988; Yoon *et al*., 1996). This review will specifically focus on using chemically synthesized oligonucl as a template for making targeted changes in these four systems.

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The oligonucleotide template

Once oligonucleotide synthesis became commercially available, the automated production of short nucleic acid strands became routine. This advancement allowed not only site-directed mutagenesis to progress beyond just editing plasmids in *Escherichia coli*, but to making precise changes in the nuclear genome of a variety of organisms. In the late 70s, it had been shown using site-directed mutagenesis that one or a few nucleotides could be precisely exchanged within a plasmid template (Hutchison *et al*., 1978). Studies correcting the non-sense/frameshift mutations at various positions along the *cyc1* gene in *Saccharomyces cerevisiae* using targeted oligonucleotides extended this result to the nuclear genome (Moerschell *et al*., 1988; Yamamoto *et al*., 1992).

Less than a decade later, a technique known as chimeraplasty, a gene editing technique that utilized an RNA/DNA chimeric oligonucleotide, was developed to introduce site-specific genomic alterations (Cole-Strauss *et al*., 1996). Chimeraplasty-mediated modification of a target sequence is accomplished using an exogenous polynucleotide, the so-called chimeraplast, which locates its complementary sequence in the genome and harnesses the cell's inherent DNA repair system to direct the change in the gene target. The original chimeraplast ranged between 68 and 88 nucleotides in length and comprised both DNA and 2′-*O*-methyl-modified RNA residues (see Figure 1a). These molecules were designed so that complementary bases fold to form a duplex region (homology region). The information strand (lower strand) of this molecule consists of DNA having a sequence identical to the target region except for the specific base(s) to be changed. This strand directs conversion(s) within its target. Improved conversion frequencies were achieved when modified RNA bases complementary to the target sequence were included on the top strand of the duplex (Metz, R., Frank, B., Walker, K.A., Avissar, P., Sawycky, X.L. and Beetham, P.R. unpublished data). This ‘classic’ chimeraplast design contained 5 nucleotides of DNA on the top strand between the 2′-*O*-methyl RNA bases (Figure 1a). The 2′-*O*-methyl-modified RNA bases facilitated higher binding affinity to the target locus (Kmiec, E.B., Frank, B. and Holloman, B. unpublished data). Hundreds of chimeraplast designs have been evaluated both *in vitro* and *in vivo* for their ability to effect targeted conversions in plasmid, episomal and chromosomal targets of bacterial and eukaryotic systems (Metz, R., Frank, B., DiCola, M., Kurihara, T., Bailey, A., Walker, K.A., Avissar, P., Sawycky, X.L., and Beetham, P.R. unpublished data). Metz *et al*. (2002) was one of the first groups to characterize the chimeraplast and define its optimal properties. When the top strand of the chimeraplast was made completely of 2′-*O*-methyl modified RNA, its binding affinity was maximized and conversion efficiency was increased. Addition of a 5-bp GC-clamp on one side bordering the homology region increased exonuclease resistance and a nick between the 5′ and 3′ ends of the chimeraplast allowed topological unwinding of the molecule. Additionally, when both ends of the duplex were flanked by single-stranded hairpin loops, concatamerization was prevented. These improvements aided in chemical and thermal stability as well as resistance to nucleases.

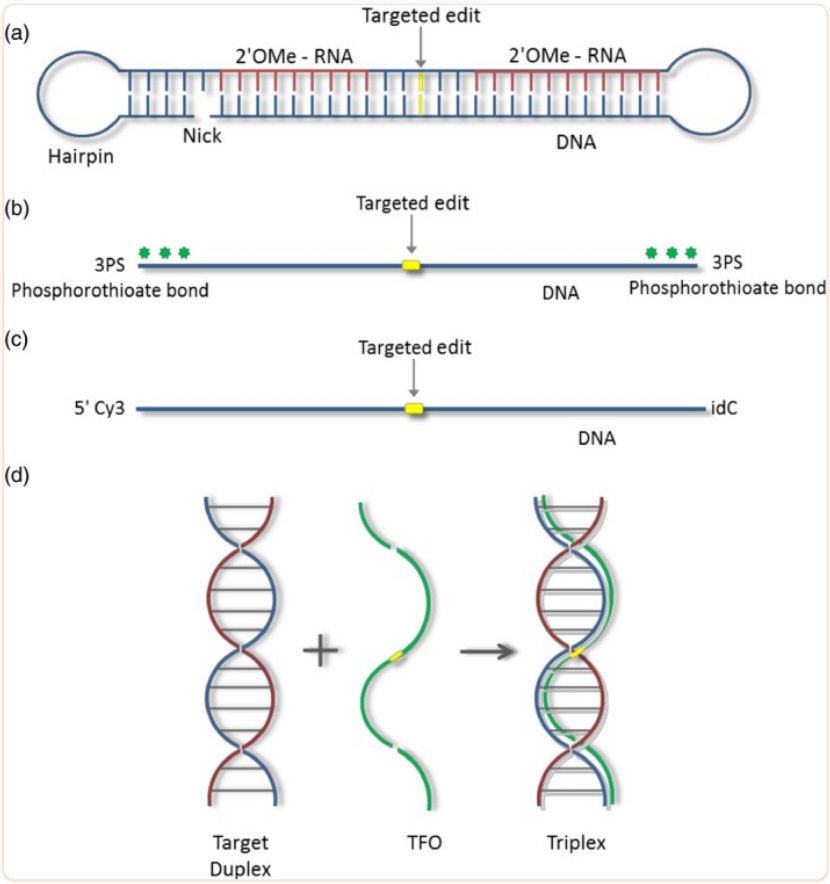

Figure 1

Oligonucleotide designs. (a) chimeraplast schematic showing regions of DNA (blue) and RNA (red; 2′-*O*-methyl modified), a nick and hairpin (total chimeraplast is ~68 nucleobases). (b) A single-stranded oligonucleotide modified with 3PS (3 phosphorothioate bonds) at both the 5′ and 3′ ends (total oligonucleotide length is 41, 101 or 201 nucleobases). (c) A single-stranded oligonucleotide modified with a Cy3 dye at the 5′ end and a reverse base (idC) at the 3′ end (total oligonucleotide is 41 nucleobases). (d) Triplex-forming oligonucleotide (TFO). The target duplex homopurine and homopyrimidine strands are shown in blue and red. The TFO, which binds the homopurine strand, is indicated in green. The location of the targeted nucleotide in all oligonucleotides is shown in yellow.

As the lower strand of the chimeraplast consisted entirely of DNA and because the synthesis of high-quality, long (generally 68–88mer) oligonucleotides at the time was inefficient, shorter single-strand oligonucleotide designs mimicking the information strand were tested. Early efforts focused on interrogating thousands of these single-strand designs in various bacterial, yeast, mammalian and plant systems to identify the design that was most efficacious (Metz, R., DiCola, M., Bailey, A., Metz, R., Kurihara, T., Frank, B. and Walther, D. unpublished data). Oligonucleotide structure and chemistries were designed so that they could be used as DNA templates to promote gene conversion through ODM technology. Oligonucleotides may be short in le contain a centrally located mismatch in one or a few bases to the target sequence. The oligonucleotid chemically synthesized and consisted of both DNA and modified nucleotides or other end-protective chemistries. These modifications prevented the oligonucleotides from undergoing recombination, bu allowed them to act as a mutagen and DNA template. For this reason, ODM is considered a targeted m esis system (Dong *et al.*, 2006; Gocal *et al.*, 2015). Based on peer-reviewed literature, the most frequently used designs were modified with phosphorothioate linkages on the terminal bases (Figure 1b; Andrieu-Soler

et al., 2005; De Piédoue *et al*., 2007; Radecke *et al*., 2006). In mammalian cells, these end linkages can be toxic, with the degree of toxicity being correlated to the number of phosphorothioate linkages as measured as fractional cell survival or γ-H2AX phosphorylation (Olsen *et al*., 2005; Rios *et al*., 2012). As the number of phosphorothioate linkages decreased, the corrected portion of the population increased (Rios *et al*., 2012). An alternative single-strand design that has been used successfully to mediate conversion of an inactive GFP target in wheat and AHAS in canola contains a 5′Cy3 label and a 3′idC reverse base (Figure 1c; Dong *et al*., 2006; Gocal *et al*., 2015). The Cy3 label allows for the visualization of oligonucleotides within cells and in most plant varieties is less toxic than the phosphorothioate end-protective linkages (C. Schöpke unpublished data). Triplex-forming oligonucleotides have also been used for gene correction, but generally they require homopurine or homopyrimidine stretches for triplex formation and thus have been less used (Figure 1d; Havre and Glazer, 1993; Wang *et al*., 1996).

Precision in gene conversion

ODM employing the chimeraplast design was first used to correct the alkaline phosphatase gene on episomal DNA in mammalian CHO cells, yielding a correction frequency of 30% (Yoon *et al*., 1996). Shortly thereafter, this technique was successfully used to correct a mutation in the β-globin gene that is responsible for sickle-cell anemia, while at the same time the highly (90%) homologous locus δ-globin remained unaltered, proving that the base change occurred specifically in the targeted gene (Cole-Strauss *et al*., 1996). Although an overall conversion frequency was not reported by the authors, the correction of the mutated β-globin gene was shown to be dose dependent. Oligonucleotide-directed mutagenesis alterations in the X chromosome gene, hypoxanthine-guanine phosphoribosyl-transferase (HPRT), were measured using phosphorothioate-protected single-strand oligonucleotides targeting single point loss-of-function mutations in the HPRT gene previously generated by ethyl methanesulfonate (EMS) in the V79 male Chinese hamster lung cell line (Kenner *et al*., 2002). Hypoxanthine-guanine phosphoribosyl-transferase encodes an enzyme in the purine salvage pathway, which when mutated becomes resistant to 6-thioguanine. Because there are many possible loss-of-function HPRT alleles that will lead to 6-thioguanine resistance and because it is an X-linked gene, it is possible to measure both conversion and random mutagenesis events of a single allele. The study found no significant difference in sensitivity to 6-thioguanine between the wild-type control cell populations and the converted cell populations treated with the oligonucleotides, suggesting no detectable random mutagenesis occurred at this locus. Additional work targeting the β-globin locus in CD34+-enriched cell population showed a targeting efficiency range from 5% to 13%, while the closely related homologue δ-globin remained unaltered, thereby providing additional evidence for the specificity of targeting (Xiang *et al*., 1997). In summary, the data from Cole-Strauss *et al*. (1996), Kenner *et al*. (2002) and Xiang *et al*. (1997) provide proof that ODM is capable of precisely targeting a single-base pair mutation in genomic DNA at a reasonable frequency and in a highly specific manner.

Conversion is independent of the transcriptional state of the gene

Additional studies using chimeraplasts have been carried out on target genes with different transcriptional states. For example, Kren *et al*. (1997) converted the functional, transcriptionally active alkaline phosphatase gene in the human hepatoma cell line HuH-7 to a mutant form. This was accomplished with a relative fr
quency of approximately 11.9%, which, when corrected for transfection efficiency, approached nearly
In this report, an alternate chimeraplast design where the mismatch is present only in the DNA stran
also examined. This design differed from the original chimeraplast design structure wherein the mis
on both the DNA and the RNA/DNA strands of the chimeraplast. The efficiency of conversion using th
tered chimeraplast was only about 2%, which is substantially less than the original design. While previous
studies reported success in editing transcriptionally active targets, Kren *et al*. (1997) were able to show that a chimeraplast was also capable of altering the genomic sequence in a transcriptionally inactive gene. For

this work, they targeted the β-globin locus, which is silent in HuH-7 cells, and converted it from the wild-type allele to the sickle-cell allele, demonstrating that the activity of the chimeraplast is independent of the transcriptional state of the target gene (Kren *et al*., 1997). Similarly, Bertoni *et al*. (2005) showed successful targeting of the dystrophin gene proliferating myoblasts, where it is transcriptionally silent.

Stability and heritability of the nucleotide change

Alexeev and Yoon (1998) were the first to demonstrate the stability and heritability of a nucleotide change induced by a chimeraplast. They targeted the tyrosinase gene, which encodes a key enzyme for melanin synthesis and pigmentation in melanocytes derived from albino mice. These melanocytes harboured a point mutation in the tyrosinase gene, resulting in an amino acid change that abolished enzymatic activity. Correction of the point mutation restores the enzymatic activity, enabling cell pigmentation by melanin synthesis. Transfection of albino melanocytes with a chimeraplast resulted in black-pigmented cells, which were cloned and continued to exhibit the pigmentation throughout several generations. Analysis of the tyrosinase alleles in the clones demonstrated that at least one locus had been corrected, thereby restoring the full-length enzymatically active protein in these clones. Thus, the phenotypic and genotypic changes effected by chimeraplasts were shown to be permanent and stable. The permanence, stability and heritability of precise changes were later demonstrated, as will be discussed below, in various plant systems for both transgenic marker genes as well as the acetohydroxy acid synthase (AHAS) gene target conferring resistance to various herbicides (Beetham *et al*., 1999; Gocal *et al*., 2015; Kochevenko and Willmitzer, 2003; Okuzaki and Toriyama, 2004; Zhu *et al*., 1999, 2000).

Conversion in bacteria

Single-strand oligonucleotides have also been used to successfully convert genes in bacteria, specifically *E. coli* (*rpsL* and *rpoB* genes, Swingle *et al*., 2010; various targets, Wang *et al*., 2009) and in *Pseudomonas syringae* (*rpsL* gene, Swingle *et al*., 2010). In *E. coli*, both RecA and MutS activities were required for efficient conversion. This was illustrated by the fact that conversion in the recA-WM1100 strain was indistinguishable from background, and conversion activity was fully restored by complementation with a RecA-expressing plasmid (Metz *et al*., 2002). In this same study, similar data was presented for MutS, suggesting a two-step mechanism of pairing followed by repair. An improvement to the conversion efficiency in bacteria was achieved using the beta protein, a recombinase from phage λ, combined with chemically synthesized single-strand oligonucleotides to correct the galK restoring auxotrophy (Ellis *et al*., 2001). The beta protein is encoded by bet, one of the three lambda Red functions. These results were dramatically extended in 2009 by Wang *et al*. using a technique called multiplex automated genome engineering (MAGE) that enabled a handful of mutations to be obtained within a single *E. coli* genome. Building on this multiplex approach, Isaacs *et al*. (2011) showed that using conjugative assembly genome engineering (CAGE) technology, they were able to site specifically replace more than 300 TAG stop codons with synonymous TAA codons to produce a completely recoded *E. coli*.

Plants

oligonucleotide-directed mutagenesis (ODM) is a non-transgenic base pair-specific precision gene ed
platform that has been significantly advanced through ***RTDS*** (Gocal *et al*., 2015) to achieve novel and
mercially valuable traits in agriculturally important crops. The ***RTDS*** technology harnesses the cell's
DNA repair system to edit specific targeted bases within the genome through the use of chemically sy
sized oligonucleotides. These oligonucleotides are used as repair templates to generate mismatches in the DNA at the target site. Through homology-directed pairing between the oligonucleotide and the DNA of the

target region, the cell's repair machinery is directed to those sites to correct the mismatched base(s) guided by the oligonucleotide sequence. Once the correction process is completed, the oligonucleotide is degraded by the cell through natural processes.

An initial foray of this technology in a plant system involved using cell free extracts to correct a plasmid bearing a non-sense mutation in the coding sequence of an *nptII* gene, thereby restoring the active coding sequence and function to confer kanamycin resistance to bacteria into which these corrected plasmids were transformed (Gamper *et al*., 2000). Using extracts from both mammalian and plant cells, this genetic (phenotypic) readout system in bacteria has been heavily used to dissect the structure/activity relationship of chimeraplasts and later single-strand oligonucleotide designs (Metz, R., Frank, B., DiCola, M., Kurihara, T., Bailey, A., Rice, M.C., May, G.D., Kipp, P.B., and Kmiec, E.B. unpublished data). The most effective designs were tested for their ability to correct transiently expressed plasmid targets with *in vivo* readout in various plant cells (Walker, K.A., Avissar, P., Sawycky, X.L., Beetham, P.R. unpublished data).

Several years after the first successful use of ODM in mammalian systems, this gene editing technology was employed in plants. Most applications of ODM in plants to convert endogenous loci have targeted single point mutations in the acetolactate synthase (ALS) gene(s), also known as the acetohydroxy acid synthase (AHAS) gene(s). This enzyme catalyses the first step in the biosynthesis of the essential branched chain amino acids isoleucine, leucine and valine, and mutant enzymes is readily selectable with herbicides that inhibit them; imidazolinones (Imis), sulfonylureas (SUs), chlorsulfuron (CS), pyrimidinylthiobenzoates and bispyribac-sodium (BS) (Tan *et al*., 2005). These are Group 2 herbicides based on the nomenclature from the Canadian herbicide classification system – HRAC Group B and Australian Group B. To achieve resistance to the aforementioned herbicide chemistries, one of three amino acid positions was targeted, specifically P197, W574 and S653, with the numbering based on the sequence of the *Arabidopsis* AHAS protein. The first published study describing the successful application of ODM technology was done in a tobacco cell line known as Nt-1 (Beetham *et al*., 1999; Ruiter *et al*., 2003), followed closely by maize (Zhu *et al*., 1999, 2000), then *Arabidopsis* (Kochevenko and Willmitzer, 2003), rice (Okuzaki and Toriyama, 2004) and oil seed rape (*Brassica napus*) (Gocal *et al*., 2015; Ruiter *et al*., 2003). In tobacco, maize, *Arabidopsis* and rice, chimeraplasts were employed to target the conversions, whereas a 5′Cy3 label and an 3′idC reverse base protected single-strand oligonucleotide design was used to target conversions in oil seed rape (Gocal *et al*., 2015). Furthermore, the method used to deliver the oligonucleotides varied between plants, with PEG-mediated delivery to protoplasts being utilized in *Arabidopsis* (Kochevenko and Willmitzer, 2003), tobacco (Ruiter *et al*., 2003) and oil seed rape (Gocal *et al*., 2015; Ruiter *et al*., 2003), and biolistics being used as the delivery method for tobacco (Beetham *et al*., 1999; Ruiter *et al*., 2003), maize (Zhu *et al*., 1999, 2000), oil seed rape (Ruiter *et al*., 2003) and rice (Okuzaki and Toriyama, 2004). In both maize and rice, based on the number of cells receiving oligonucleotides, the biolistics method resulted in a conversion rate of 1×10^{-4}, similar to reported conversion frequencies for PEG-mediated delivery (Okuzaki and Toriyama, 2004; Zhu *et al*., 1999, 2000). However, it is difficult to compare different oligonucleotide delivery methods because conversion rates varied depending on the crop, the cell biology system, the oligonucleotide type, its concentration, the strand being targeted (coding or non-coding) and the targeted mutation being made.

RTDS technology applied to the conversion of BFP to GFP in *Arabidopsis*

Oligonucleotide-mediated conversions have been improved in many ways by utilizing a fluorescence
sion system wherein a blue fluorescent protein (BFP) can be changed to a green fluorescent protein (
editing a single nucleotide of the BFP gene. For instance, optimization of oligonucleotide length and e
tective chemistries has shown promise in enhancing conversion efficiency. To illustrate the effectiven
oligonucleotide-mediated conversions in *Arabidopsis,* protoplasts derived from a BFP transgenic line were tested for BFP to GFP gene editing. Protoplasts were transfected with either a 41, 101, or 201 nucleobase

(nb) oligonucleotide (BFP/41, BFP/101, BFP/201), each containing the C→T edit required to convert BFP to GFP, and monitored for GFP fluorescence 72 h after oligonucleotide introduction using cytometry. All three oligonucleotide lengths tested resulted in a notably higher percentage of GFP-positive cells when compared to the control treatment (Figure 2; Warburg, Z.J., Miller, R., Mozoruk, J. and Sauer, N.J. unpublished data). Oligonucleotide length had a positive correlation with respect to GFP fluorescing cells, with oligonucleotide BFP/201 resulting in nearly five times more GFP-positive cells than oligonucleotide BFP/41 (Figure 2). This result demonstrates that oligonucleotide-mediated conversions are an effective method to make precise changes in *Arabidopsis*, and further that oligonucleotide optimization can play an important role with respect to the frequency of targeted edits.

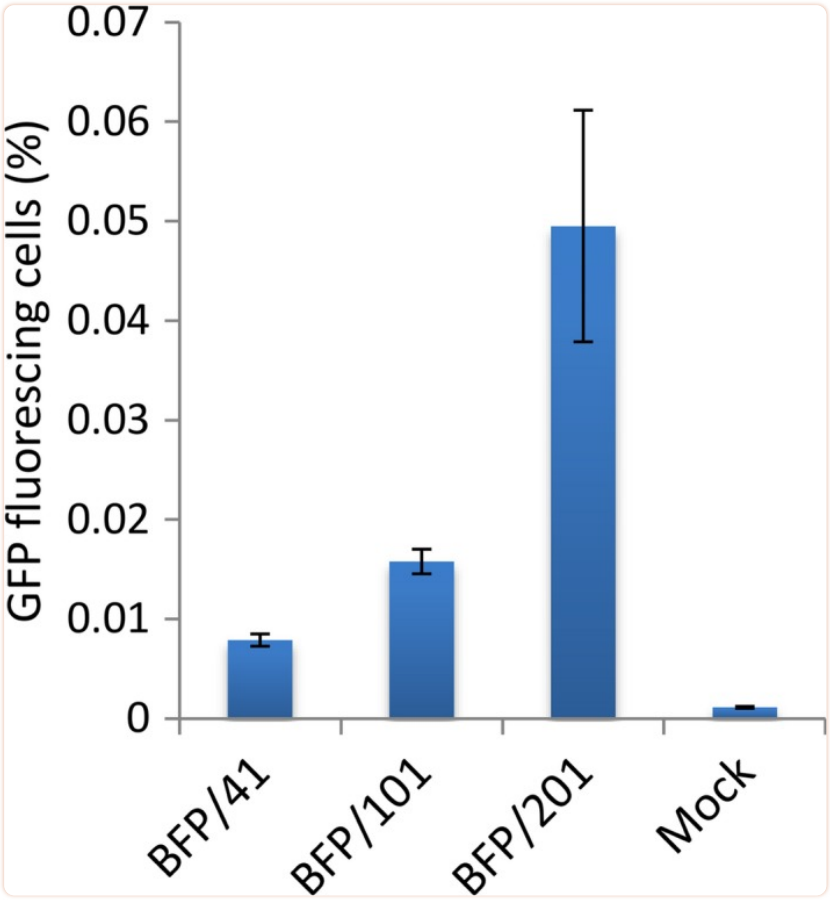

Figure 2

Frequency of transgene editing using ***RTDS*** technology in *Arabidopsis* protoplasts. Oligonucleotides of three different lengths (nb) each possessing the C→T edit in codon H66 (H66Y; CAC→TAC) required to convert blue fluorescent protein (BFP) to green fluorescent protein (GFP) were delivered into *Arabidopsis* protoplasts by the PEG method. Percentage of GFP fluorescing cells was measure 72 h after delivery by cytometry. Error bars are mean ± SEM (n = 3).

Regulatory view on ODM

With an ability to precisely change sequences in genomes, ODM is one of several new breeding techn that is leading to the commercialization of crop plants. In the United States, the Animal Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture indicated that a herbicide tolerant canola

developed by Cibus through ODM is not subject to their regulation and that is it is exempt from biotechnology regulation under 7 CFR Part 340. In Canada, the approval for release of a plant variety with a new trait does not take into account the method with which it was produced (Shearer, 2015). Therefore, crops developed through ODM are evaluated the same way as crops developed through other breeding methods. In 2007, the European Commission set up a New Plant Breeding Techniques working group with the task to assess whether eight new plant breeding techniques, among them ODM, would fall under the scope of European GMO legislation. Based on the final report to the European Commission (discussed in Schiemann and Hartung, 2015, p 202), a majority of the members of the working group deemed that ODM is a mutagenesis technique and, therefore, would fall outside the scope of the directives governing GMOs. Similar conclusions were reached in a commentary article by a group of scientists, in which regulatory aspects of ODM are discussed (Breyer *et al*., 2009). This decision will allow for the commercialization of crop plants with traits developed by ODM to occur in a timely manner.

Improving conversion efficiency and future prospects

While significant and practical gene editing frequency has been demonstrated with ODM techniques in many plant species, correction rates have been relatively low and editing has depended on large amounts of the introduced oligonucleotide.

Various treatments have been explored to enhance conversion efficiency. In mammalian systems, recognition by the mismatch repair machinery or components of the non-homologous end-joining (NHEJ) DNA repair pathway have had a negative effect on conversion frequency, and consequently conversion frequencies have increased when such components are knocked down or inactivated (Dekker *et al*., 2003, 2006; Morozov and Wawrousek, 2008). By comparison, chemicals or treatments that augment homology-based DNA repair also increased conversion efficiency (Morozov and Wawrousek, 2008; Olsen *et al*., 2005 Parekh-Olmedo *et al*., 2005).

Another such improvement has been achieved through the use of DNA double-strand breakers. When oligonucleotides were combined with chemicals or antibiotics that generate DNA double-strand breaks, significant enhancement in the frequency of gene targeting was observed (Ferrara *et al*., 2004; Parekh-Olmedo *et al*., 2005). Similarly, engineered nucleases have been shown to enhance the efficacy and precision of gene editing in combination with oligonucleotides, primarily in eukaryotic systems (Chen *et al*., 2011; Connelly *et al*., 2010; DiCarlo *et al*., 2013; Gratz *et al*., 2013; Strouse *et al*., 2014; Suzuki *et al*., 2003; Svitashev *et al*., 2015; Voytas, 2013; Wang *et al*., 2015; Wu *et al*., 2013; Zhao *et al*., 2014). These nucleases were engineered to cleave DNA in a target-specific manner. They include meganucleases, zinc finger nucleases (ZFN), TAL effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9 (CRISPR-Cas9) (Bortesi and Fischer, 2015; Carroll, 2014; Rousseau *et al*., 2011).

While numerous studies using the combination of engineered nucleases and oligonucleotides have been reported in fish and mammals (Ding *et al*., 2013; Hwang *et al*., 2013; Strouse *et al*., 2014; Wefers *et al*., 2013; Yang *et al*., 2013), the published data for plant systems is very limited. To date, we are aware of only five examples. In one study using rice protoplasts, Shan *et al*. (2015) introduced two restriction enzyme sites into the sequence of the *OsPDS* gene in a partial transgenic approach using an integrated CRISPR/Cas9 an stranded oligonucleotides. In this publication, the authors did not report successful regeneration of p from edited protoplasts. In another study, Wang *et al*. (2015) targeted conversion of the enolpyruvyls mate-3-phosphate synthase (EPSPS) locus in rice by employing TALENs transgenically integrated int genome combined with a chimeraplast. *EPSPS* encodes an enzyme in the shikimate pathway found in but not mammalian systems, which is a key step in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan. In plants, EPSPS is a target for the herbicide, glyphosate, where it acts as a

competitive inhibitor of the binding site for phosphoenolpyruvate (Schönbrunn *et al*., 2001). They reported detecting 1 of 25 transgenic lines with the intended EPSPS gene edit. However, this line, in addition to the single base edit, also contained an imprecise NHEJ event, sending the coding sequence with the intended edit out of frame (Wang *et al*., 2015).

In a recent preprint (Svitashev *et al*., 2015), CRISPR/Cas9 and a phosphinothricin acetyltransferase (PAT) selectable marker to confer resistance to bialaphos were delivered into immature corn embryos using bombardment, in combination with either a double-stranded PCR product (794 bp) or two different single-stranded 127mer oligonucleotides, which served as template DNA targeting the P197 locus in the AHAS gene. After selection with bialaphos and/or chlorsulfuron, an editing frequency of three or four events in 1000 was reported for oligonucleotides, compared with two events in 1000 for double-stranded DNA. In a fourth example, a double-strand DNA donor was also used as the repair template for a CRISPR/Cas9-induced DSB in the work of Li *et al*. (2013) to target a new restriction site in the PDS locus of *Nicotinia benthamiana*. The converted protoplasts in this work were not regenerated into whole plants. In our unpublished data (Woodward, M. and Narvaez-Vasquez, J.), a study was carried out targeting the two *EPSPS* loci in flax using a combination of TALEN and oligonucleotide. In this work, we detected 0.19% precise and scarless *EPSPS* edits in both loci in 7-day-old microcolonies. Collectively, these studies, as well as work performed by our group, demonstrate that while significant precise gene editing events in plants can be achieved using oligonucleotides alone, this effect can be enhanced by a variety of reagents that cause DNA double-strand breaks.

The promise of ODM and ***RTDS*** to precisely deliver predictable targeted edits to specific targets within the nuclear genome, as directed by exogenously supplied chemically synthesized oligonucleotides, remains strong. For more than a decade, Cibus has focused on improving this process and extending it from model systems to develop traits in commercial crops. As was the case in *E. coli*, by applying treatments that improve conversion efficiency, the range of possibilities will expand to allow multiple conversions in single gene targets as well as simultaneous conversion of multiple targets in a single cell. Together, improvements to this non-transgenic breeding technology will deliver traits more rapidly and offer the promise of accelerating and completely revolutionizing the breeding process.

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