Chapter 5
Oligo-Mediated Targeted Gene Editing

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Abstract Oligonucleotide-mediated targeted gene editing encompasses technology described by many names. These include Oligo-Directed Mutagenesis (ODM) and the commercial Rapid Trait Development System (***RTDS***™) from Cibus. ODM is a non-transgenic (non-GMO) base pair-specific oligonucleotide-directed gene editing platform that has been advanced at Cibus over the past decade and has achieved novel and commercially valuable traits in crops. This technology harnesses the cell's normal DNA repair system to correct and change specific targeted bases within the genome of a cell. The Gene Repair OligoNucleotide (GRON), a chemically synthesized oligonucleotide, is designed to create mismatched base pairs compared to the target sequence within the host organism's genome. The GRON hybridizes at the target region and the mismatched base pairs work to direct the cell's repair system at those sites to correct (replace, insert, or delete) the designated base(s). Once the correction process is complete the GRON is degraded and the now-modified or repaired gene retains its normal pattern of expression and stability within the genome. This technique has been successfully deployed in bacterial, fungal, mammalian, and plant systems. Our work in achieving herbicide tolerance traits in acetohydroxyacid synthase (AHAS) genes in oil seed rape (OSR) as well as work converting a blue fluorescent protein (BFP) transgene to green fluorescent protein (GFP) in an *Arabidopsis* model system will be discussed.

1 Introduction

Chemically synthesized and non-modified oligonucleotides were first used for gene editing in vivo in yeast (Moerschell et al. 1988). Almost 8 years later, oligonucleotides were used in mammalian cells initially to correct an episome (Yoon et al. 1996) and then to correct the point mutation within the nuclear genome in the human β-globin gene that causes sickle cell anemia (Cole-Strauss et al. 1996).

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At that time, since the oligonucleotide chemistry used was a RNA/DNA chimeric molecule, the first generation of the ODM technology was termed chimeraplasty.

This exciting result spawned a commercial foray into applying this technology for gene editing in a variety of organisms and systems demonstrating its applicability across plant, animal, human, fungal, and bacterial cells, and showing that this technique does not appear to be restricted by cell cycle, gene expression, transcription, or other known cellular or genetic activities (Cole-Strauss et al. 1996; Kren et al. 1997; Beetham et al. 1999; Zhu et al. 1999; Alexeev and Yoon 1998; Kochevenko and Willmitzer 2003; Okuzaki and Toriyama 2004; Dong et al. 2006). The ODM process produces non-transgenic, site-specific mutations. Traits produced using ODM are similar to the more than 2,500 products of classical mutagenesis in 180 crops including Clearfield canola, wheat, rice, and sunflower, which are already moving freely in world trade with no labeling or other restrictions applied (http://www.fao.org). In plants the initial proof of concept experiments were performed in tobacco and corn (Beetham et al. 1999; Zhu et al. 1999, 2000). In both cases point mutations in acetohydroxyacid synthase (AHAS) genes conferring tolerance to AHAS-inhibiting herbicides were targeted. AHAS-inhibiting herbicides are a modern class of herbicides that are used widely in broad acre agriculture. Similar to many modern herbicides the interaction between the plant's AHAS and the herbicide is well understood (Tan et al. 2005) and specific mutations in the gene(s) encoding AHAS are directly related to herbicide tolerance. Sulfonylurea herbicides are one of the five classes of AHAS-inhibiting herbicides that block the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (Tan et al. 2005). Tobacco is an allotetraploid species with two AHAS-encoding loci, SuRA and SuRB. In the experiments of Beetham et al. (1999), one of two AHAS genes/loci (SuRA) was targeted in a tobacco cell line known as Nt-1. At a cellular level, this work targeted an amino acid substitution at P196 to confer resistance to the herbicide chlorsulfuron (Glean, DuPont). This paper demonstrated specificity for SuRA and reported evidence that ***RTDS*** could also reactivate a mutant transgene encoding a green fluorescent protein (GFP) marker gene, introduced into either the tobacco cells or whole plants.

In complementary studies by scientists at Pioneer Hi-Bred International, Inc., Zhu et al. (1999, 2000) converted the AHAS homologs in maize, again to making the cells herbicide tolerant. Additionally, they converted and therefore re-activated a mutant GFP transgene in maize. Cells of both the AHAS and GFP conversions were then cultured on various media; plants were regenerated and allowed to mature. Progeny of these plants confirmed that the gene conversions were heritable and stable as is expected for Mendelian inheritance.

It is well documented that the molecular basis of many traits is due to small genetic differences, or single nucleotide polymorphisms (SNPs), within critical genes. For more than a decade, Cibus, its predecessors and others have been developing and applying this molecular "spell checking" technology to *direct conversions* (nucleotide changes; SNPs) to the desired location in the specified gene in nuclear-encoded genes to develop traits in crop plants. These changes can be site-specific nucleotide substitutions, as well as insertions and deletions in individual

genes of multigene families. Therefore the conversion process can be used to repair mutant genes, alter genes, or interrupt normal gene function. This technology is not limited to manipulating genes that code for proteins, but has demonstrated that any nucleotide sequence (regulatory, coding, and noncoding) can be converted to enhance and/or reduce the function and activity of a gene product. In all cases, the SNP changes are emulating what often occurs in nature as part of the continuing natural genetic diversity. We see this in exponentially increasing detail as we generate more genome sequence data from plant populations.

From a regulatory stand point, the USDA has classified this technology as a mutagenesis technique like chemical mutagenesis, only with a directed outcome. An assessment of the ***RTDS*** technology, in Europe known as oligonucleotide-directed mutagenesis (ODM), was published several years ago by a Belgian group (Breyer et al. 2009). Their conclusions are in line with the USDA analysis where the outcomes of the technology are similar to traditional mutagenesis. It is clear that ***RTDS***-derived products are produced from a more precise and targeted technology as compared to traditional mutagenesis. In theory these products are isogenic to the parental genetics except for the targeted SNP. Regulators are favorable to technologies that are more defined and measurable (Kuzma and Kokotovich 2011). One difficulty will be that SNPs will occur in nature and if by chance these are the same as ***RTDS-***derived SNPs, they will be indistinguishable from each other. Interestingly these SNPs work well with more advanced breeding techniques now deployed in agriculture. Many quantitative trait loci (QTLs) are followed using SNPs as molecular markers in advanced breeding programs are well accepted protocols.

1.1 Gene Repair Oligonucleotide Structure

At Cibus and in the public domain, many oligonucleotide designs have demonstrated conversion activity (Beetham et al. 1999; Metz et al. 2002; Dong et al. 2006; Wagner et al. 2010). Since there are cellular enzymatic activities which can destroy open-ended RNA and DNA mono and duplex molecules, the ends of these molecules can be protected by various chemical modifications including capping with "hairpins." Such hairpins were used in the original DNA and RNA chimeric oligonucleotides. Self-complementary chimeric oligonucleotides contain a DNA "mutator" region of 5-bp nucleotides complementary to the target site flanked by 2′-*O*-methyl RNA segments. The "mutator" region is synthesized with a mutation designed to be introduced into the endogenous target gene (Beetham et al. 1999; Zhu et al. 1999; Gamper et al. 2000; Oh and May 2001; Kochevenko and Willmitzer 2003; Ruiter et al. 2003; Okuzaki and Toriyama 2004). This design attempted to mimic the formation of a complement-stabilized D-loop to direct the targeted conversion.

Second generation GRON designs are linear molecules approximately 40 nucleotides in length with blocked ends. These molecules are designed to be complementary with either the coding or the noncoding strand of the target gene, with targeting GRONs generally having the targeted nucleotide change (mismatch) towards their

center. Non-targeting GRONs, which are used as controls, are completely homologous with the target sequence. Within the peer-reviewed literature, the most common design generally blocks the oligo's termini with three phosphorothioate linkages (Pierce et al. 2003). Another design blocks the 5′ terminus with the fluorescent label Cy3 and 3′ terminus with a reverse cytosine base (Dong et al. 2006; Wagner et al. 2010).

1.2 Delivery

In plant systems, various methods for GRON deliver have been used successfully to convert endogenous gene targets. For protoplast systems, these include electroporation and PEG-mediated delivery, whereas for monocot crops including corn, rice, and wheat, particle bombardment has been used as the preferred method (Zhu et al. 1999; Kipp et al. 2000; Okuzaki and Toriyama 2004; Dong et al. 2006). Many of the early delivery studies were focused on single cells or embryogenic cell suspensions using biolistics (Kipp et al. 2000). They focused on whether plant cells had the gene repair machinery to correct a nonfunctional plasmid carrying the GFP gene. These studies confirmed that both delivery of the plasmid and GRONs and subsequent successful repair was possible in many cells. These cells also continued to grow. Studies also showed that cell-free extracts from various tissues and plants where also competent for plasmid repair using GRONs (Kmiec et al. 2001). These studies have clearly shown that once the parameters for delivery are optimized for GRONs, plant cells have the competence do complete ODM.

Importantly, the next endeavors for delivery were to complete chromosomal conversion. The two gene targets where this work has been described are AHAS and GFP (Beetham et al. 1999; Zhu et al. 1999, 2000; Kochevenko and Willmitzer 2003; Okuzaki and Toriyama 2004; Dong et al. 2006). Here we also describe work done more recently using a Blue Fluorescent Protein (BFP) chromosomal target, where by changing one nucleotide the BFP becomes GFP.

1.3 RTDS Mechanism/Process

By (a) mechanism(s) that are becoming more clearly understood, the mismatched nucleotide directs a specific mutation in the target gene (see Fig. 5.1). We loosely designate this process as "*gene conversion*." Once conversion is complete, the GRON is degraded and the modified gene remains expressed under the cell's normal control mechanisms. In vitro assays using "cell-free" extracts and various pure enzymes demonstrate that the GRON is stable for sufficient time to direct gene conversion and then is quickly degraded.

Requirements for gene conversion vary considerably between bacterial, yeast, and mammalian systems. In *E. coli* both MutS and RecA activities are required (Metz et al. 2002). However, in yeast overexpression of a RecA homolog RAD51 or

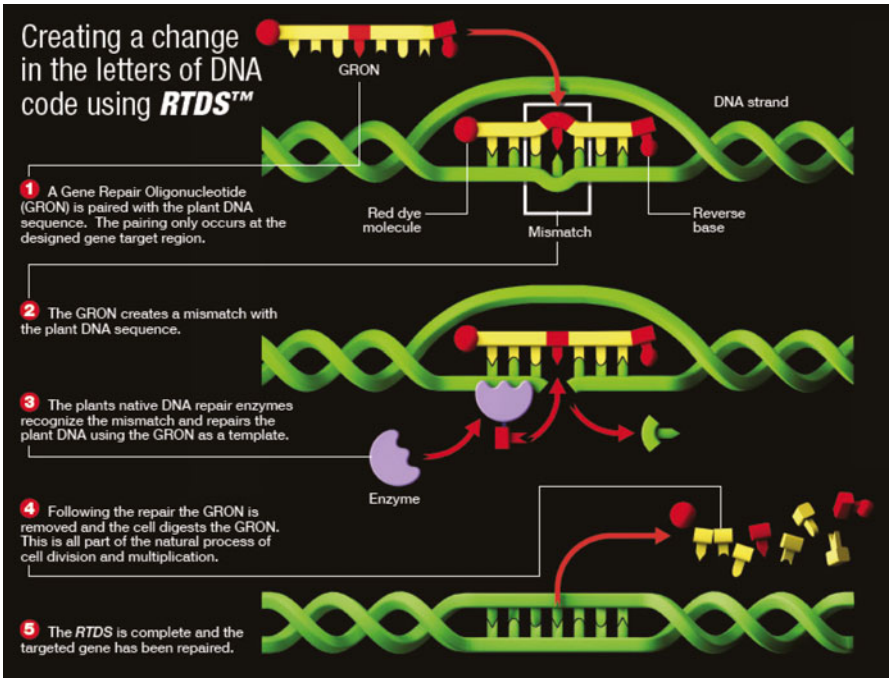

Fig. 5.1 The ***RTDS*** process. Included with permission from Cibus

the helicase RAD54 significantly improved conversion (Liu et al. 2002). In contrast, complete loss of expression of MSH2, a component of Mutα (MSH2 and MSH6), a eukaryotic homolog of MutS did not affect conversion efficiency (Rice et al. 2001) or might even reduce efficiency (Maguire and Rice 2007). Also in yeast, loss of function mutations in either of the two Mutβ components (MSH3 and MSH6) somewhat improved conversion efficiency (Rice et al. 2001). By comparison, in mouse embryonic stem cells, overexpression of RAD51 or RAD54 significantly improved conversion, whereas a loss of function mutant of msh2(−/−), a component of Mutα (MSH2 and MSH6) and a eukaryotic homolog of MutS, substantially improved conversion efficiency (Aarts et al. 2006; Morozov and Wawrousek 2008). Furthermore Morozov and Wawrousek (2008) significantly improved conversion efficiency by inhibiting Ku70/86, components of the nonhomologous end-joining pathway.

1.4 Application of RTDS

1.4.1 Targeting Acetohydroxyacid Synthase

As detailed above for tobacco and maize as well as in rice (Beetham et al. 1999; Zhu et al. 1999, 2000; Kochevenko and Willmitzer 2003; Okuzaki and Toriyama 2004), a common target used for conversion experiments of targets within plant nuclear

genomes is their acetohydroxyacid synthase (AHAS) gene. In OSR, the BnAHAS I and BnAHAS III genes both code for nearly identical (99 % homology) protein monomers. The BnAHAS I gene is located in the C genome, whereas BnAHAS III is located in the A genome of *Brassica napus*. The proteins encoded by these genes combine in all three permutations and combinations to form the active AHAS enzyme. Through chemical mutagenesis, a mutation was found in the AHAS I gene (Tan et al. 2005). This mutation is known as PM-1 (a mutation at an equivalent position known as 653 based in the AHAS amino acid sequence of *Arabidopsis*, a serine to asparagine amino acid change, respectively, encoded as AGT to AAT). Another mutation was found in the higher expressed gene AHAS III, known as PM-2 (a mutation at an equivalent position known as 574, a tryptophan to leucine amino acid change, respectively, encoded as TGG to TTG). These two mutations, PM-1 and PM-2, are combined in a commercial variety of Canola known as Clearfield Canola (Tan et al. 2005). Although the level of conferred tolerance to each herbicide may vary between the PM1 and PM2 mutations, each mutation provides tolerance to the plant by disrupting the herbicide's ability to interact with the AHAS. The introduction of a single base change in the nucleotide sequence at a specific location in each of the AHAS I and III genes produces enzymes, each with a change in a single amino acid that reduces the ability of the herbicide to block the active binding site of that enzyme, allowing branched chain amino acid synthesis to occur even in the presence of the herbicide.

1.4.2 Targeting Green Fluorescent Protein Transgenics

We utilized the well-known green fluorescent protein (GFP) gene for monitoring conversion resulting from ODM. In the peer reviewed literature, the single amino acid substitution at amino acid 66 of GFP from Y66 (Tyrosine encoded by the codon TAC) to H66 (Histidine encoded by the codon CAC) causes the protein to appear blue rather than green (Sommer et al. 2006). By transforming plants with this modified GFP at this position, we engineered a system where with one SNP we could switch its fluorescence from blue to green.

In this chapter we will discuss conversion experiments of two targets (1) the AHAS loci in oil seed rape (OSR; aka canola) and (2) blue fluorescent protein (BFP) in transgenic *Arabidopsis* lines.

2 Materials and Methods

2.1 Oil Seed Rape AHAS

In its simplest form, the ODM process involves introducing into cells a GRON designed to target a specific mutation within a gene target, the GRON pairing by homology with its target and causing the intended mutation followed by

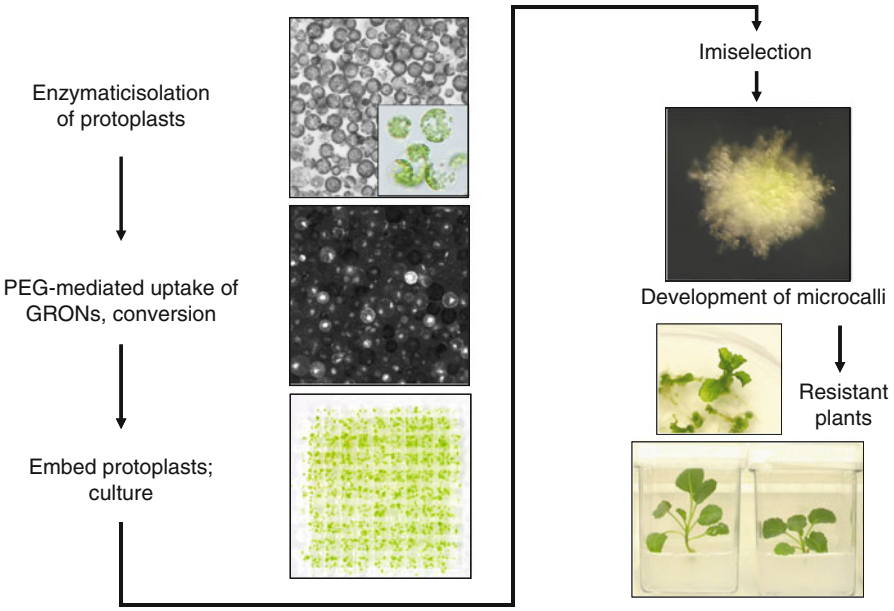

Fig. 5.2 The operating system for ODM in OSR. Leaf-derived protoplasts are isolated, GRONs introduced and the protoplasts are embedded in calcium alginate grids where they are cultured to form microcalli. After continuous selection, imazethapyr-tolerant calli develop. Tolerant calli are transferred to shoot induction media and the developing shoots rooted in vitro

regeneration of the converted cells into normal fertile plants. The cell biology process illustrating these steps is presented in Fig. 5.2. In brief, shoots derived both from seeds and from microspore-derived embryos were propagated under sterile conditions in vitro. Cuttings were subcultured every 2–4 weeks and cultured in Petri dishes (25×90 mm) in a volume of 40–45 mL RS medium (Dovzhenko 2001) and young leaves used for protoplast isolation.

As detailed in patent application WO 2009 046334, leaves of 2–3-week-old in vitro shoots were cut into small strips with a scalpel in a Petri dish with 5 mL medium B (Pelletier et al. 1983), and the pH adjusted to 5.8. After approximately 1 h, medium B was replaced with enzyme solution, consisting of medium B in which 0.5 % (w/v) Cellulase YC and 0.75 % (w/v) Macerozyme R10 (both from Karlan Research Products, Cottonwood, Arizona), 1 g/L bovine serum albumin, and 1 g/L 2-morpholinoethanesulfonic acid were dissolved. The enzyme solution was vacuum-infiltrated into the leaf tissue, and the dish with leaf pieces in enzyme solution was incubated for at 25 °C in darkness. Protoplast purification was performed using an iodixanol density gradient (adapted from Optiprep Application Sheet C18; Purification of Intact Plant Protoplasts; Axis-Shield USA, 10 Commerce Way, Norton, MA 02776). After the density gradient centrifugation, the band with purified

protoplasts was removed together with about 5 mL W5 medium (Negrutiu et al. 1987). The protoplast yield was determined with a hemocytometer, and the protoplasts were stored for 2 h at 4 °C.

The protoplast suspension was mixed with an equal volume of W5 medium, transferred to a 50 mL centrifuge tube, and centrifuged for 5 min at the lowest setting of a clinical centrifuge (about 50 × *g*). The supernatant was removed and replaced with TM medium (Klaus 2003), adjusting the protoplast density to 5×10^6/mL. Aliquots of 100 μL containing 5×10^6 protoplasts each were distributed into 12 mL round bottom centrifuge tubes. In each experiment a variety of control treatments were performed including no treatment, PEG treatment alone, and non-targeting GRONs (whose sequences were identical to the target sequences). GRONs targeting a mutation in one or both AHAS genes were then introduced into the protoplasts using a PEG treatment. To introduce the GRONs into the protoplasts, 12.5 μg GRON dissolved in 25 μL purified water and 125 μL of a polyethylene glycol solution (5 g PEG MW 1500, 638 mg mannitol, 207 mg $CaNO_3 \times 4H_2O$, and 8.75 mL purified water; pH adjusted to about 9.0) was added. After a 30 min incubation on ice, the protoplast-PEG suspension was washed with W5 medium and resuspended in medium B. The suspension was kept overnight in a refrigerator at about 4 °C.

One day after the GRON introduction, protoplasts were embedded in calcium alginate. The embedding of protoplasts in gel substrates (e.g., agarose, alginate) has been shown to enhance protoplast survival and to increase division frequencies of protoplast-derived cells. The method applied was based on that described in Dovzhenko (2001).

The selection of imazethapyr-resistant calli was carried out using sequential subcultures of the alginates in media according to Pelletier et al. (1983). Selection was started 1 week after the PEG/GRON treatment at a concentration of 0.5 μM imazethapyr. Cells and colonies were released by treating the alginate for 30–45 min with culture medium containing 50 mM sodium citrate. Two to three weeks after the transfer to solidified selection medium (occasionally earlier), actively growing calli appeared among a background of brownish cells and microcalli.

Over time, various SNP screening methods have been used to identify converted callus lines with targeted mutations in either BnAHAS I or BnAHAS III, and sequence confirmation was obtained for these mutations in the relevant locus. Plants were regenerated from protoplast-derived, herbicide-tolerant calli with a confirmed mutation in an AHAS gene. Imazethapyr-tolerant calli that had developed on solidified selection medium and whose DNA upon analysis had shown the presence of a mutation were transferred to herbicide-free medium E (Pelletier et al. 1983) to accelerate development. Individual callus lines varied in their growth rates and morphologies. In general, the development towards shoot regeneration followed these steps: Undifferentiated, green callus → callus with dark green areas → development of roots → development of shoot initials → development of stunted shoots with hyperhydric (vitrified) leaves.

Once shoots with three to four leaves had formed on medium E, they were transferred to RS medium (Dovzhenko 2001). On this medium, over time shoot and leaf tissue developed that was morphologically "normal" (i.e., non-hyperhydric). After in vitro plantlets had produced roots, standard protocols were used for the adaptation to greenhouse conditions.

2.2 BFP to GFP Conversion in Arabidopsis

A. thaliana ecotype Col-0 lines containing single or multiple BFP copies were produced through *Agrobacterium*-mediated transformation (Clough and Bent 1998). As described in Sect. 1.4, the BFP gene used for transformation was based on an eGFP gene into which a mutation was introduced at amino acid position 66 (Tyr66 encoded by codon TAC to His66 encoded by codon CAC). For expression in plant cells, the BFP coding sequence was driven by the mannopine synthase promoter (Guevara-García et al. 1999) and was used in combination with the pea rbcS-E9 terminator (Genbank Accession number M21375). Protoplast isolation, PEG-mediated DNA delivery, and protoplast culture were carried out essentially as described in Mathur et al. (1995).

The sequences of the targeting coding (C) and noncoding (NC) GRONs are as per Wagner et al. (2010). The non-targeting versions of these GRONs are identical to the BFP target in sequence (His66 encoded by codon CAC). The phosphorothioate version of these GRONs lack the Cy3 label and reverse base at their 5′ and 3′ termini, respectively, and have three phosphorothioate linkages at both ends.

For the determination of conversion efficiency, the percentage of green-fluorescing protoplasts was measured one day after GRON delivery using flow cytometry (Guava EasyCyte; Millipore). In other experiments protoplast were cultured in liquid medium in 24-well dishes, and the development of green-fluorescing (=converted) cells was followed over time. Images were acquired with an ImageXpress Micro System (Molecular Devices) equipped with a black-and-white CCD camera. Black-and-white images of green-fluorescing cells were colorized green using the imaging program MetaXpress (Molecular Devices).

3 Results

3.1 Chromosomal Conversion

3.1.1 Oil Seed Rape AHAS

Over the years, Cibus has successfully targeted three independent mutations in two AHAS loci in canola (BnAHAS I and BnAHAS III). The dataset shown in Fig. 5.3 consists of experiments targeting the mutations (SNPs) S653N (A**G**T to A**A**T) and

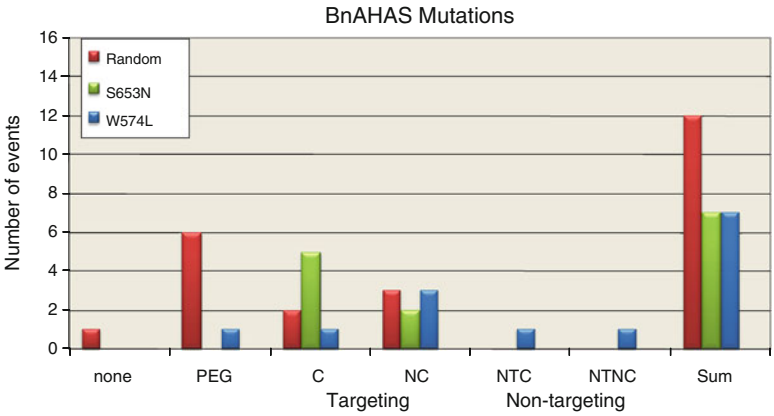

Fig. 5.3 Frequency of events with mutations in either BnAHAS I or BnAHAS III obtained in ten experiments with 8 targeting S653N and 2 targeting W574L. Callus forming efficiency is approximately tenfold higher in the control treatments without GRON (none and PEG) than in its presence (targeting or non-targeting GRON). *Green bars* indicate the presence of S653N (AAT), *blue bars* indicate the presence of W574L (TTG), and *red bars* indicate the presence of other mutations (not S653N or W574L) in either BnAHAS I or BnAHAS III

W574L (T**G**G to T**T**G) in both BnAHAS I and BnAHAS III. Of 10 experiments, 8 targeted S653N and 2 targeted W574L using the targeting GRONs detailed in Table 5.1. The target regions were identical to those in the reference sequences for BnAHAS I (Genbank accession# Z11524) and BnAHAS III (Genbank accession# Z11526; Rutledge et al. 1991). In each experiment, similar numbers of protoplasts were used in control treatments (no treatment, PEG alone, and/or non-targeting GRON—also detailed in Table 5.1). Under optimal conditions, up to 2 % of the PEG treated protoplasts develop into microcalli (see Fig. 5.2). In the absence of herbicide selection, from GRON introductions using up to 60 million protoplasts, as many as 1.2 million calli can form. As expected, somatic mutations were obtained in control treatments for nearly every experiment, the targeted S653N mutation in either BnAHAS I or III was *only* obtained in treatments employing either a coding or a noncoding targeting GRON. In fact the S653N mutation in BnAHAS III in the OSR lines we were able to obtain in our experiments was not obtained in chemical mutagenesis experiments performed over several decades in OSR (Tan et al. 2005).

The Cy3 label enables visualization of delivery efficiency with >80 % of the treated protoplasts receiving GRON in these experiments.

By contrast, in experiments targeting the W574L mutation, a similar number of targeted mutations (one line obtained with the coding GRON and 2 obtained using the noncoding GRON) were obtained in treatments with targeting GRONs compared with events obtained in control treatments. The dataset for the experiments targeting W574L is, however, significantly smaller than that for the S653N experiments. Regenerated plants and seed was obtained from more than 80 % of OSR calli with confirmed AHAS mutations with the level of Imi tolerance conferred by each targeted mutation being indistinguishable across multiple lines with each mutation.

Table 5.1 GRON sequences

	GRON	Sequence
Targeting	BnALS1653/C/41/5′Cy3/3′idC	VTGTGTTACCGATGATCCCAA**A**TGGTGGCACTTTCAAAGATGH
	BnALS1653/NC/41/5′Cy3/3′idC	VCATCTTTGAAAGTGCCACCA**T**TTGGGATCATCGGTAACACAH
	BnALS1574/C/41/5′Cy3/3′idC	VCTTGGGATGGTCATGCAATTGGAAGATCGGTTCTACAAAGCH
	BnALS1574/NC/41/5′Cy3/3′idC	VGCTTTGTAGAACCGATCTTCCAATTGCATGACCATCCCAAGH
Non-targeting	BnALS0653/C/41/5′Cy3/3′idC	VTGTGTTACCGATGATCCCAAGTGGTGGCACTTTCAAAGATGH
	BnALS0653/NC/41/5′Cy3/3′idC	VCATCTTTGAAAGTGCCACCACTTGGGATCATCGGTAACACAH
	BnALS0574/C/41/5′Cy3/3′idC	VCTTGGGATGGTCATGCAATGGGAAGATCGGTTCTACAAAGCH
	BnALS0574/NC/41/5′Cy3/3′idC	VGCTTTGTAGAACCGATCTTCCCATTGCATGACCATCCCAAGH

The converting base is shown in bold
V = Cy3; *H* = 3′DMT dC CPG

3.1.2 BFP to GFP Conversion in *Arabidopsis*

The first BFP-transgenic *Arabidopsis* lines obtained had multiple copies of the BFP gene. Experiments with these lines aimed at comparing the effect of coding and noncoding GRONs and showed that the coding GRON resulted in conversion barely above background, while the noncoding GRON achieved conversion rates in the range of 0.05–0.1 % (Fig. 5.4a). Conversion showed dependence on the concentration of the noncoding GRON. Increasing the GRON concentration from 40 to 80 μM led to a threefold increase, and at 160 μM the percentage of converted protoplasts was six times higher compared with the 40 μM treatment (Fig. 5.4b). Subsequent experiments with a line containing a single copy of the BFP transgene resulted in similar conversion frequencies.

Protoplast derived from BFP/GFP conversion experiments were cultured in 24-well dishes in liquid medium MSAR1 (Mathur et al. 1995). The first division of protoplast-derived cells was observed after about 1 week. Interestingly, the majority of dividing converted cells followed an unusual pattern: the division resulted in two daughter cells that were separate from each other (Fig. 5.5). This interpretation is based on the fact that in a given well there were only a few fluorescing units, most of them single cells, and some consisting in clusters of cells that were in close proximity, but not physically connected. In Fig. 5.5a the development of such a cluster composed of three cells, one of which is dividing, is shown over the course of 3 days.

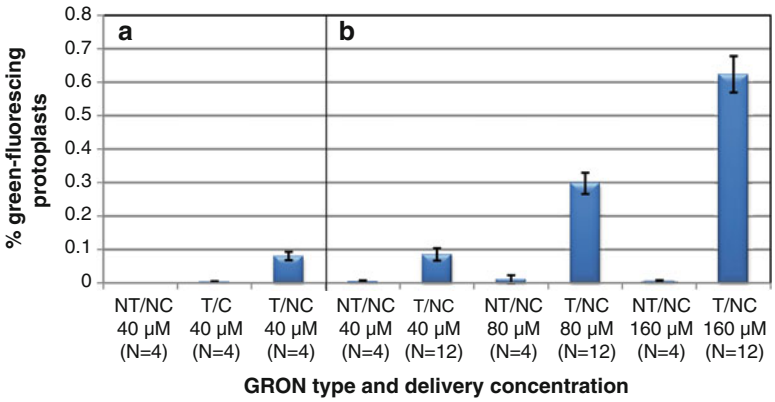

Fig. 5.4 Effect of GRON type and concentration on BFP to GFP conversion in *Arabidopsis* protoplasts containing eight copies of the BFP gene. The conversion frequency is the number of green-fluorescing protoplasts expressed as percentage of the protoplasts that were analyzed by flow cytometry 24 h after GRON introduction. (**a**) Targeting coding (T/C) and targeting noncoding (T/NC) were delivered into protoplasts. The non-targeting, noncoding GRON NC/NT was used as negative control. (**b**) Protoplasts were transfected with different concentrations of noncoding (NT) GRONs, either with the non-targeting (NT) or the targeting (T) version. The GRON concentration given in μM refers to the protoplast suspension after adding DNA and PEG

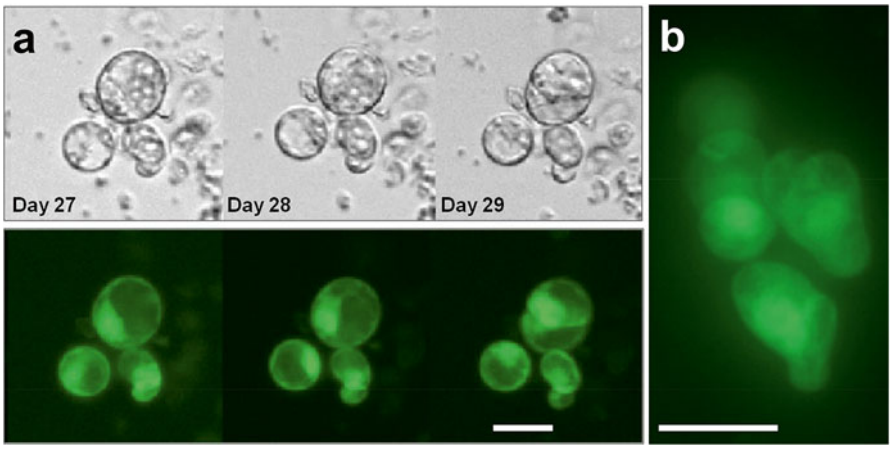

Fig. 5.5 Development of converted *Arabidopsis* cells. Protoplasts from a transgenic line containing eight copies of the BFP gene were treated with a GRON targeted at converting the BFP gene into a GFP gene. (**a**) Time lapse images of cells derived from a converted protoplast showing the division of a converted cell on day 29 after GRON delivery. *Upper panel*: images acquired in brightfield; *lower panel*: images acquired with a GFP filterset. (**b**) Colony of about six converted cells, 4 weeks after GRON delivery. Scale bars 10 μm

4 Discussion

Just like site-directed mutagenesis of plasmid DNA in vitro changed the course of molecular biology, in vivo gene targeting has been a goal that will forever change the course of biological research and product development. Concerted efforts at Cibus for more than a decade have advanced this technique in *Arabidopsis* as a model system and in OSR to develop the first commercial product using this technique. To our knowledge this is the first report of gene editing in OSR. Required elements to achieve this success were an understanding of the endogenous loci being targeted, conversion of these loci at a cellular level and regeneration of the converted cells into normal fertile plants.

Many peer reviewed publications have demonstrated conversion at a cellular level including some with show transient GRON-mediated conversion of plasmid templates in vitro and in plant cells (Rice et al. 2000; Kmiec et al. 2001; Dong et al. 2006).

A distinct advantage of traits developed using ***RTDS***, a directed mutagenesis technique, over those developed as transgenics is that the performance the trait is indistinguishable across lines (events) since the level and pattern of gene expression for the endogenous gene is retained in the convertant lines. The level of herbicide resistance of plants regenerated from calli with a specific targeted mutations detailed

in Fig. 5.3 was similar between lines with that mutation. As would be expected, the targeted mutations in these lines show stable Mendelian inheritance with that for our earliest product, to date demonstrating stability for more than 12 generations. To our knowledge, some of the specific mutations we obtained in the BnAHAS I and III targets in OSR were not previously obtained in chemical mutagenesis campaigns conducted by others over several decades. Together, we believe, the targeted and somatic mutations obtained in these experiments represent the largest collection of AHAS mutations in a single crop and parental background.

In tobacco a single clone and in rice two clones with the W574 (tryptophan) equivalent position successfully mutated to leucine were obtained (Kochevenko and Willmitzer 2003). Although the S653 (serine) homologous position (S627) was targeted in rice by Okuzaki and Toriyama (2004), no clones were obtained. By comparison, Zhu et al. (1999) obtained 13 independent events targeting the S653 homologous position (S621) in corn calli. The large number of control (no treatment, PEG alone, and/or non-targeting GRON) treatments as well as the depth of the 8 experiments targeting S653N (serine to asparagine) demonstrate the efficacy of ***RTDS***. This is further endorsed by our significant progress in improving conversion efficiency in our *Arabidopsis* model system, which has experienced improvements in excess of two orders of magnitude.

In the mammalian literature, transgenic cell lines containing one or multiple copies of mutated versions of the GFP gene are employed for the analysis of gene editing experiments because the phenotype of converted cells can be quantified by flow cytometry. The BFP gene in our transgenic *Arabidopsis* lines can be seen as a mutated GFP gene that has the advantage of potentially providing a phenotype for non-converted cells (Sommer et al. 2006). However, the background fluorescence in transgenic *Arabidopsis* protoplasts was too strong to permit the visualization of BFP fluorescence.

It is often difficult to compare BFP or mutated GFP conversion frequencies obtained by different authors. The efficiency of introduction of targeted changes through oligonucleotides measured within the first 2 days after transfection can depend on cell type, oligonucleotide concentration and chemistry, method of delivery, cell cycle status, copy number of the targeted gene, and many other factors. When flow cytometry is used for the quantification of converted cells, the type of flow cytometer (sensitivity) and the gating to distinguish green fluorescing cells from the background fluorescence can have an influence on the detectable number of converted cells.

There are few publications on the use of a BFP transgene to assess oligo-mediated gene targeting. Andrieu-Soler et al. (2005) compared the effect of different oligo chemistries and lengths on conversion frequency. Depending on the oligo used for transfection, chromosomal conversion rates varied from 0.007×10^{-3} to 2×10^{-3}. Sommer et al. (2006) describe the conversion of a chromosomal BFP gene to GFP. They evaluated conversion qualitatively by microscopy, but do not give information about the conversion efficiency per number of oligo-treated cells.

Wagner et al. (2010) used GRONs provided by Cibus (described in Sect. 2.2). The same GRON was used in our conversion experiments targeting BFP in transgenic *Arabidopsis* protoplasts as to convert their single copy BFP gene in a transgenic HEK cell line. In those experiments, Wagner et al. (2010) reported that neither the noncoding nor the coding version of the GRON resulted in conversion rates above background. However, in those experiments, no attempt was made to optimize the GRON concentration.

In the cited publications and through what is presented in this chapter, the successful application of the technology has been demonstrated in *Arabidopsis*, oil seed rape, corn, rice, tobacco, and wheat. To our knowledge, this is the first published report of gene targeting being performed in OSR and leading to a commercial outcome.

References

Aarts M, Dekker M, de Vries S, van der Wal A, te Riele H (2006) Generation of a mouse mutant by oligonucleotide-mediated gene modification in ES cells. Nucleic Acids Res 34:e147

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

Andrieu-Soler C, Casas M, Faussat AM, Gandolphe C, Doat M, Tempe D, Giovannangeli C, Behar-Cohen F, Concordet JP (2005) Stable transmission of targeted gene modification using single-stranded oligonucleotides with flanking LNAs. Nucleic Acids Res 33:3733–3742

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. Proc Natl Acad Sci U S A 96:8774–8778

Breyer D, Herman P, Brandenburger A, Gheysen G, Remaut E, Soumillion P, Van Doorsselaere J, Custers R, Pauwels K, Sneyers M, Reheul D (2009) Genetic modification through oligonucleotide-mediated mutagenesis. A GMO regulatory challenge? Environ Biosafety Res 8:57–64

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

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

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

Dovzhenko A (2001) Towards plastid transformation in rapeseed (*Brassica napus* L.) and sugarbeet (*Beta vulgaris* L.). Ph.D. dissertation, LMU Munich, Faculty of Biology

Gamper HB, Parekh H, Rice MC, 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 Res 28:4332–4339

Guevara-García A, López-Bucio J, Herrera-Estrella L (1999) The mannopine synthase promoter contains vectorial cis-regulatory elements that act as enhancers and silencers. Mol Gen Genet 262:608–617

Kipp PB, Van Eck J, Beetham PR, May GD (2000) Gene targeting in plants via site-directed mutagenesis. In: Kmiec E (ed) Methods of molecular biology—gene targeting protocols. Humana, Totowa

Klaus S (2003) Markerfreie transplastome Tabakpflanzen (Marker-free transplastomic tobacco plants). Ph.D. dissertation, LMU Munich, Faculty of Biology

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

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

Kren BT, Cole-Strauss A, Kmiec EB, Steer CJ (1997) Targeted nucleotide exchange in the alkaline phosphatase gene of HuH-7 cells mediated by chimeric RNA/DNA oligonucleotide. Hepatology 25:1462–1468

Kuzma J, Kokotovich A (2011) Renegotiating GM crop regulation. Targeted gene-modification technology raises new issues for the oversight of genetically modified crops. EMBO Rep 1:883–888

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

Maguire K, Kmiec EB (2007) Multiple roles for MSH2 in the repair of a deletion mutation directed by modified single-stranded oligonucleotides. Gene 15:107–114

Mathur J, Szabados L, Koncz C (1995) A simple method for isolation, liquid culture, transformation and regeneration of *Arabidopsis thaliana* protoplasts. Plant Cell Rep 14:221–226

Metz R, DiCola M, Kurihara T, Bailey A, Frank B, Roecklein B, Blaese M (2002) Mode of action of RNA/DNA oligonucleotides: progress in the development of gene repair as a therapy for alpha(1)-antitrypsin deficiency. Chest 121:91S–97S

Moerschell RP, Tsunasawa S, Sherman F (1988) Transformation of yeast with synthetic oligonucleotides. Proc Natl Acad Sci U S A 85:524–528

Morozov V, Wawrousek EF (2008) Single-strand DNA-mediated targeted mutagenesis of genomic DNA in early mouse embryos is stimulated by Rad51/54 and by Ku70/86 inhibition. Gene Ther 15:468–472

Negrutiu I, Shillito R, Potrykus I, Biasini G, Sala F (1987) Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Mol Biol 8:363–373

Oh TJ, May GD (2001) Oligonucleotide-directed plant gene targeting. Curr Opin Biotechnol 12:169–172

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

Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Rouselle P, Renard M (1983) Intergeneric cytoplasm hybridization in *Cruciferae* by protoplast fusion. Mol Gen Genet 191:244–250

Pierce EA, Liu Q, Igoucheva O, Omarrudin R, Ma H, Diamond SL, Yoon K (2003) Oligonucleotide-directed single-base DNA alterations in mouse embryonic stem cells. Gene Ther 10:24–33

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 Physiol 123:427–438

Rice MC, Bruner M, Czymmek K, Kmiec EB (2001) *In vitro* and *in vivo* nucleotide exchange directed by chimeric RNA/DNA oligonucleotides in *Saccharomyces cerevisiae*. Mol Microbiol 40:857–868

Ruiter R, van den Brande I, Stals E, Delauré S, Cornelissen M, D'Halluin K (2003) Spontaneous mutation frequency in plants obscures the effect of chimeraplasty. Plant Mol Biol 53:675–689

Rutledge RG, Quellet T, Hattori J, Miki BL (1991) Molecular characterization and genetic origin of the *Brassica napus* acetohydroxyacid synthase multigene family. Mol Gen Genet 229: 31–40

Sommer JR, Alderson J, Laible G, Petters RM (2006) Reporter system for the detection of in vivo gene conversion. Mol Biotechnol 33:115–121

Tan S, Evans RR, Dahmer ML, Singh BK, Shaner DL (2005) Imidazolinone-tolerant crops: history, current status and future. Pest Manag Sci 61:246–257

Wagner S, McCracken J, Cole S, Laible G (2010) DNA oligonucleotides and plasmids perform equally as donors for targeted gene conversion. Biochem Genet 48:897–908

Yoon K, Cole-Strauss A, Kmiec EB (1996) Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA.DNA oligonucleotide. Proc Natl Acad Sci U S A 93:2071–2076

Zhu T, Peterson DJ, Tagliani L, St. Clair G, Baszczynski CL, Bowen B (1999) Targeted manipulation of maize *in vivo* using chimeric RNA/DNA oligonucleotides. Proc Natl Acad Sci U S A 96:8768–8773

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