
Non-Transgenic Trait Development in Crop Plants Using Oligo-Directed Mutagenesis: Cibus' Rapid Trait Development System

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A huge technological pivot is going on in crop breeding. If the end of the 20th century was the era of transgenic breeding or GMO technologies, the 21st century is silently turning to non-transgenic breeding or non-GMO technologies. Due to significant regulatory issues, cost of development and technology constraints, transgenic breeding technologies have been limited to a few crops grown on large acreages, *i.e.* about 10% of the available cultivated acres globally¹. The promise of non-transgenic breeding technologies is their extraordinary breadth. Crops bred using these technologies can be planted on all farming acreage worldwide. And these technologies can address better, more novel non-transgenic traits both in large- and in small-acreage crops. Small crops are accessible because the development cost is so much less than for transgenic crops.

This shift to non-transgenic technologies has been led by advances in precision gene-editing technologies that include molecular scissors such as CRISPRs (clustered regularly interspaced short palindromic repeats) and TALENs (transcription activator-like effector nucleases), bolstered by the explosion in genome-sequence information. Our understanding puts us at the threshold of a new era in non-transgenic crop breeding. With powerful

¹FAO statistical databases, United Nations.

advances in crop-breeding technologies at the core of this silent revolution in crop breeding, these new technologies will enable the development of new traits more rapidly and more precisely. Because of their promise, these powerful technologies are quickly becoming the most important tool to ensure global food security through sustainable agriculture in the foreseeable future.

Cibus is a non-transgenic-trait-development company at the apex of this revolution. With a proprietary technology built over more than a decade, Cibus has a large and broad patent portfolio in this space (over 170 issued or pending). Cibus has developed precision gene-editing platforms for a variety of crops that are accurate and predictable with reliable timelines. As opposed to younger companies just starting to work with these technologies, Cibus has a broad product line both in commercialization and in development. This revolution in agriculture, food and microbes promises both to address the growing needs for a safe and sustainable food future and to replace the need for the current transgenic technologies that, today, dominate the industry. Cibus is a leader in this revolution.

OVERVIEW

Technology and an understanding of genetics has been at the core of accelerating plant breeding (Figure 1). Articulated by Gregor Mendel, single traits were tracked as visible phenotypes from one generation to the next, with outcomes limited by the genetic diversity available within the parental lines. To increase this diversity, various classical mutagenesis techniques were employed, including the treatment of whole plants, seeds or tissue pieces with various DNA-damaging chemicals (*e.g.*, ethyl methanesulfonate [EMS]) or radiation (*e.g.*, gamma radiation). This approach helped to address one of the limitations of traditional breeding, the wait time for new mutations, thus accelerating the breeding process. That said, the mutations obtained are random, so influencing specific outcomes was not possible with this method. Currently more than 2,500 such products in 180 crops are sold in commerce without labeling or other restrictions applied (<http://www.fao.org>). Many characteristics of current crops, such as orange- and yellow-colored sweet peppers, are the result of this process, known as chemical mutagenesis or mutation breeding.

With advancements in the field of molecular biology, marker-assisted selection (MAS) enabled the development of complex traits leveraging both existing variation among all available lines of a crop and that induced using classical mutagenesis approaches. The marker is a small piece of DNA that always coexists in a plant with the desired trait or a proportion of that desired trait. By quickly and easily identifying plants containing the marker associated with the desired trait, the breeding process is faster and more efficient using MAS. MAS is, however, limited by the diversity that exists in the crop already. If the trait does not exist in the crop, there is no way to breed for it using MAS.

The development of transgenic techniques, inserting DNA foreign to the plant, beginning in the late 1970s, enabled movement of entire genes to generate single traits. Initial transgenic traits used genes from bacteria or other non-plant sources; however, transgenic traits can also be developed by inserting genes from wide crosses that could not occur in nature. Although extremely powerful, the location at which the transgene lands is ran-

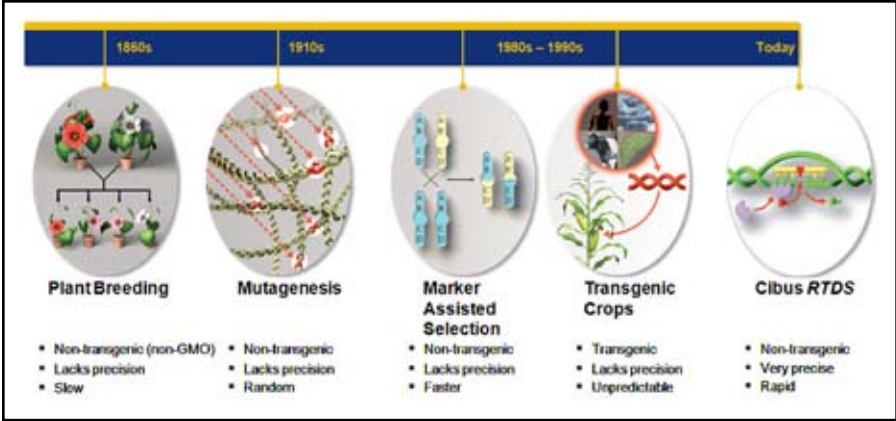


Figure 1. History of plant breeding from Mendel to today.

dom, occurring in well expressed and less well expressed regions of the genome alike. To ensure the appropriate level of transgene expression requires that many transgenic lines be assessed for the trait's performance. Random transgene insertion may inactivate an existing gene, one that is critical for some aspect of the crop's development, therefore agronomic performance of the crop must also be assessed. Even when this does not occur, because a completely new sequence (the transgene) is present on one arm of a chromosome pair, it may reduce recombination frequency at that point, making it difficult to reduce linkage drag associated to the new locus.

Gene-editing techniques, including Cibus' Rapid Trait Development System (*RTDS*TM), enable the development of non-transgenic traits with laser precision. These traits can be developed more rapidly, with extremely reduced cost and global consumer acceptance.

RTDS IN PLANTS

Gene editing has been a holy grail in biology with technology to obtain precisely targeted mutations being explored for almost 40 years. Initially, work to develop precise single-nucleotide polymorphisms (SNPs) focused on simpler and less-complex plasmid DNA targets (Hutchison *et al.*, 1978). A decade later, Fred Sherman used chemically synthesized oligonucleotides to achieve targeted mutations in yeast (*Saccharomyces cerevisiae*) (Moerschell *et al.*, 1988).

As early as 1998, oligonucleotides were used to achieve targeted mutations in the genome of the gut bacterium *Escherichia coli* (Zhang *et al.*, 1998). Then, with the advent of the lambda-Red system, precise targeting in *E. coli* became routine in 2001 and, by 2011, improvements in that technology enabled more than 10 SNPs to be targeted simultaneously in a single genome (Isaacs *et al.*, 2011). These were combined by breeding to obtain more than 300 targeted mutations within a single genome (Isaacs *et al.*, 2011).

In 1996, oligonucleotides were first used in mammalian cells, initially to correct an episome (Yoon *et al.* 1996), and then within the nuclear genome to correct the point

mutation in the human β -globin gene that causes sickle-cell anemia (Cole-Strauss *et al.*, 1996). At that time, since the oligonucleotide chemistry used was an RNA/DNA hybrid molecule, the first generation of the *RTDS* technology was termed chimera-plasty.

A few years later in the first application in plants, chimera-plasty was used to target the acetolactate synthase (ALS) gene in a tobacco cell line known as Nt-1 (Beetham *et al.*, 1999). The enzyme encoded by this gene target is responsible for the biosynthesis of branched-chain amino acids including leucine, isoleucine and valine. By targeting this gene the researchers were able to modify it, allowing the cells to become herbicide resistant. The herbicide in this example belonged to a class known as sulfonylureas. Included in this report was evidence that *RTDS* could also modify a transgene (a marker gene known as GFP—green fluorescent protein—introduced transgenically into either tobacco cells or whole plants). The researchers were able to reactivate an inactivated form of this gene.

This work was further supported by a complementary study by researchers at Pioneer Hi-Bred, Inc., who modified a similar gene in maize (Zhu *et al.*, 1999, 2000). They modified the tobacco ALS homolog in maize known as the acetohydroxyacid synthase (AHAS) gene. The converted cells were also herbicide resistant. Additionally, they modified a GFP transgene in maize. Modified cells were then cultured, and plants were regenerated and allowed to mature. The subsequent progeny of these plants confirmed that the gene mutations were heritable and stable.

Kochevenko and Willmitzer (2003) also confirmed the utility of *RTDS* to modify plant genes by repeating and extending the work of Beetham *et al.* (1999) with the ALS gene in tobacco with a more extensive study that included the regeneration of whole plants from cells that had undergone *RTDS* conversion. Sequence analysis and enzyme assays showed that the DNA had been converted at the target site and the ALS enzyme was resistant to the herbicide. Applying chlorsulfuron to the regenerated tobacco plants confirmed that the herbicide-tolerant phenotype exhibited Mendelian inheritance. This paper confirmed that the gene-repair oligonucleotide (GRON) -targeted mutations are distinct and precise. Further, Okuzaki and Toriyama (2004) also targeting the AHAS gene, successfully converted two independent sites in rice, selecting with chlorsulfuron and bispyribac.

TRAIT-DEVELOPMENT PROCESS—HERBICIDE TOLERANCE IN CANOLA

Cibus has used conversion of a visible marker gene to GFP in the model system *Arabidopsis*, canola and other crops, yielding improvements in conversion efficiency of up to three orders of magnitude.

One method of practicing *RTDS* involves regenerating protoplasts to whole plants. By applying the process described in Figure 2, mutations were obtained in canola AHAS homologs, one of which was key to developing Cibus' herbicide-tolerant *SU Canola*TM product, which is currently in the launch phase in the United States.

Despite over a decade of experience in continuously improving the canola cell-culture system, the latest technology advancements have made this process twice as fast and 16 times more efficient. This provides opportunities to greatly accelerate project timelines and has made non-selectable changes a reality.

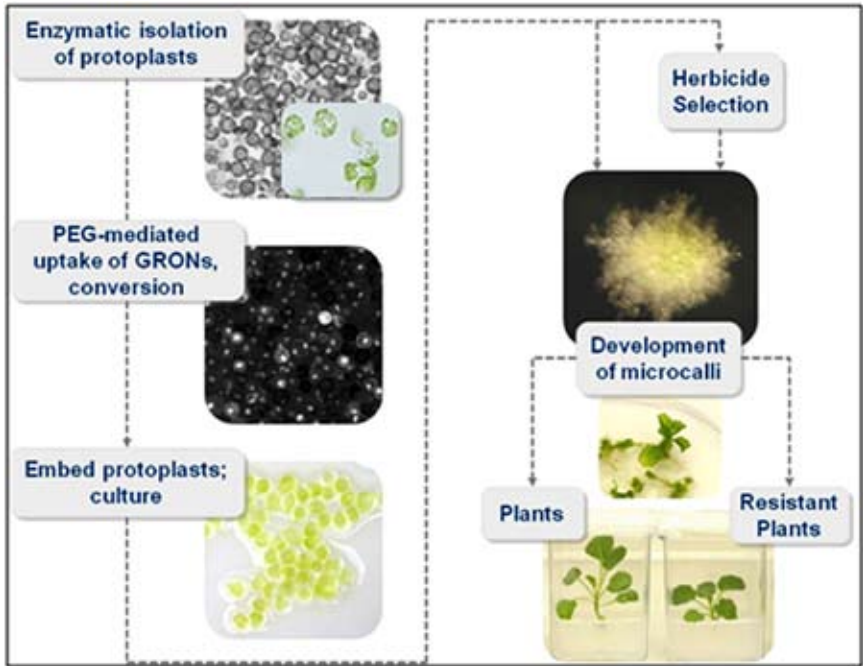


Figure 2. *RTDS* process to develop herbicide tolerance in canola.

***RTDS* CONVERSION PROCESS—GRONs ARE CHEMICALLY SYNTHESIZED DIRECTED MUTAGENS**

GRON structure and chemistry are designed purposefully. The GRON is designed with a mismatch in one or a few bases compared to the target gene’s sequence. GRONs are chemically synthesized structures consisting of both DNA and modified nucleotides or other end-protective chemistries. The GRON is blocked from undergoing recombination due to its chemical structure, with the GRON acting as a mutagen and *RTDS* being a targeted mutagenesis system and not a transgenic or GM process. Company scientists, through their understanding of the target gene’s sequence, design the GRON to effect (a) specific sequence change(s), the replacement, insertion or deletion of nucleotides. GRONs are produced with an automated chemical synthesizer and purified like any other chemical agent; they contain no biologically derived material. The GRON is formulated without the need for a delivery vector, which ensures that no foreign or extraneous DNA is inserted into the plant.

Over the years, scientists have continued to actively work on *RTDS*, focusing on understanding the mechanism to help optimize GRON design. Incrementally, alterations in design have been developed and tested. In our standard bacterial assay, GRONs are orders of magnitude more efficient, than our original designs. The efficiency in the

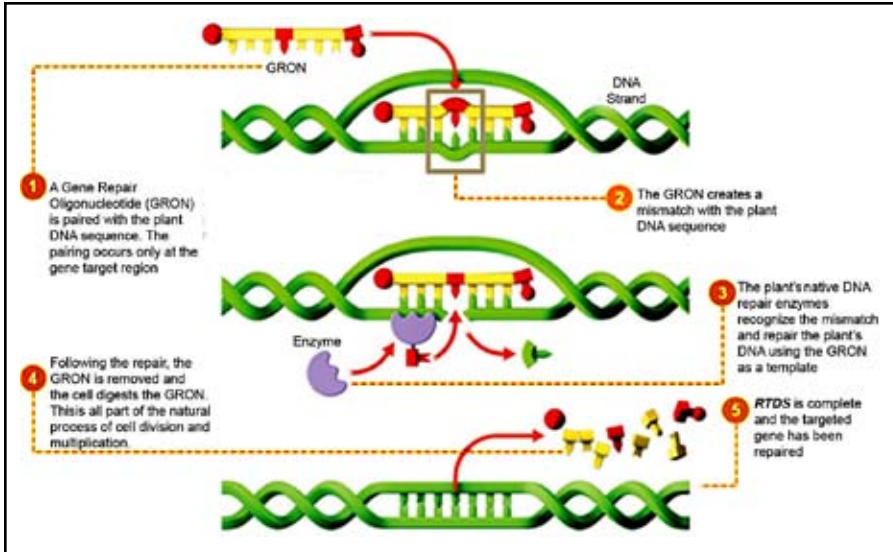


Figure 3. *RTDS* conversion process.

bacterial assay has also translated to higher efficiencies in eukaryotic cells, *i.e.*, plant, mammalian and yeast.

MECHANISM OF GRON ACTION

RTDS is a mutagenesis technology that uses the natural or inherent mismatch-repair system to effect a change. In eukaryotic cells, the GRON crosses the cell membrane, traverses the cytoplasm to the nucleus, locates and binds selectively and specifically to its target sequence and effects (a) specific sequence change(s) in its target gene. Nucleases and other degrading enzymes in the cells then break down the GRON (Figure 3).

A working model of the *RTDS* mechanism has emerged from studies in bacteria and mammalian cells. These studies suggest that the GRON will modify the nucleotide sequence in the genome via a process of genome/GRON “pairing” followed by GRON-directed mismatch repair (Figure 3). In *E. coli*, studies by Cibus scientists have clearly shown that the process is *mutS*- and *recA*-dependent. These two proteins are an integral part of the cell's own endogenous repair system. Rice *et al.* (2000) and Gamper *et al.* (2000) have shown that cell-free extracts from a variety of plants—including banana, corn and tobacco—were able to precisely modify a target-DNA sequence and that mutation efficiency varied with the chemistry of the supplied GRON. Among others, the effects of chemistry of single-strand GRONs have also been studied by Andrieu-Soler *et al.* (2005), Radecke *et al.* (2006), and de Piédoue *et al.* (2007), showing that some designs are toxic, leading to delayed progression through the cell cycle and, in some cases, selective apoptosis.

This mismatch signals the cell's "mismatch" repair system to change the gene's sequence such that the mismatched nucleotide is removed enzymatically, and the new sequence of the gene is resynthesized with the GRON acting as the template over the targeted portion of the gene. The working model that has emerged from studies in bacteria, yeast and mammalian cells indicates that the GRON will modify the nucleotide sequence in the genome via a process of genome/GRON "pairing" and then GRON-directed DNA repair.

Once inside the cell, the GRON is transported to the nucleus and hybridizes to the targeted gene sequence. Specific hybridization is a critical step for determining the efficiency of the gene-correction process. The GRON would then be used as the template for a DNA polymerase to correct the removed nucleotide or nucleotides, thereby producing a continuous sequence using the cell's own source of nucleotides. The ability of the GRON to specifically hybridize with great affinity to its target, and its resistance to degradation, allows the cellular gene-repair mechanism time to locate and replace, insert or delete the targeted DNA nucleotide(s) on both strands of the genomic DNA. When the DNA strands are corrected to the GRON's DNA sequence, the GRON is degraded and the gene then functions under its natural control mechanisms.

REGULATORY CONSIDERATIONS

Using *RTDS*, new traits can be added to a plant with only very minor changes to the genes and their resulting proteins. Results presented above show single-nucleotide substitutions in the AHAS gene lead to tolerance to three classes of AHAS inhibitors. The resulting plants are substantially equivalent to non-converted plants, with no risk of allergenicity or toxicity. Further, the GRON-induced mutation is made within the native pattern of expression for the target gene. There is no possibility that flanking genes may have expression patterns altered in unexpected ways.

In 2004, the USDA confirmed that *RTDS* is a modern form of mutagenesis and should not be regulated by state or federal agencies. In the United States, Cibus' commercial *SU Canola* product is now in the launch phase. In Canada, in late 2013, Cibus and its partner BASF received PNT (plant with novel trait) approvals for herbicide-tolerant canola. Work is underway to develop and register hybrids for the Canadian market as well as to achieve herbicide registration.

In Europe, *RTDS* is known as oligo-directed mutagenesis (ODM). Towards assessing ODM, in 2011, an Expert Working Group on Novel Plant Breeding Techniques, appointed by the European Commission, concluded that *RTDS* should be treated as mutagenesis and excluded from regulation under 2001/18/EC. This position has been endorsed by recent reports from a number of Member State advisory groups, including ZKBS in Germany and ACRE in the United Kingdom.

OPPORTUNITIES—BROAD APPLICABILITY ACROSS MULTIPLE ORGANISMS

This technology can be used in a multitude of applications including repair of gene defects and mutations that modify genes and, therefore, their protein functions. Extensive research, at Cibus and by academic scientists, has demonstrated the successful application of the GRON-directed gene repair or gene mutation in human, animal, yeast, plant, and

bacterial cells, both in culture (*in vitro*) and in live animals, plants, yeast and bacteria (*in vivo*). Therefore, a large number of genes in a wide spectrum of living organisms appear to be accessible to *RTDS* through a commercially viable process.

CONCLUSIONS

Cibus' technologies are the core of our Rapid Trait Development System (*RTDS*). They have been developed over the past decade, focused on the molecular and cellular aspects of obtaining targeted spelling changes in target genes. Molecular aspects have focused on characterizing gene targets, mutation discovery to achieve desired traits in these targets as well as developing and improving our single-nucleotide polymorphism (SNP) screening technology. Cell-biology aspects have focused on developing crop platforms that enable SNPs to be obtained efficiently in single cells and regenerating those cells into normal fertile plants—plants that are the same as the parent with one or a few precisely targeted SNPs. Speed to market for future products has been cut materially because of advances in Cibus' technologies and with our systematized approach. These advances mean:

- Precision in trait modification
- Faster product-development timelines
- A proven and reproducible methodology for trait development
- Clear regulatory path

Due to technology constraints, development costs and regulatory hurdles, the current transgenic technologies have fallen short and cannot meet the needs of the market. In order to meet what has been reported as a doubling in global food production by 2050, we need a reliable method. This is the promise of precision gene editing for non-transgenic crop breeding. This is the promise of Cibus.

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WITH AN extensive background in molecular biology, **Greg Gocal** has been central in developing the *RTDS* technology in plant and microbial systems. Currently, he leads the technology group at Cibus, and is part of the senior executive team tasked with exploring the many commercial opportunities to deliver on *RTDS* potential applications. In the past, he has focused primarily on developing the molecular aspects of the technology, including target character-

ization, mutation discovery, molecular screening and assay development. Also, he has played a key role in the development of Cibus' first commercial product, *SU Canola* (sulfonyleurea tolerant), and in helping achieve regulatory approval for this trait in Canada. Over the past decade, his research leadership has led to multiple commercial partnerships for the company. In addition, his experience has expanded to include the development of key intellectual property through to products moving to market.

DR. GOCAL has authored numerous peer-reviewed publications, several book chapters and is a co-inventor of many applications within Cibus' patent estate. He earned a BSc in biochemistry/botany and an MSc in plant physiology from the University of Calgary, and received his PhD in plant molecular biology from the Australian National University.