HUDSON RIVER

Bio technology

Technologies for Better Crops

TiGER

CRISPR editing with the TiGER workflow

What drives us

From our oxygen and vegetables, to our vaccine development, and to the rubber in our car tires and the biofuel heating our houses. **We cannot escape our need for plants.**

But with a growing world-population, the effects of climate change, declining soil quality, and competing demands on land, we are running into the limitations of our bioeconomy.

How can we utilize technology to solve these challenges?

Hudson River Biotechnology intends to deliver on the promise that crop improvement can address the demands of a resilient, sustainable bioeconomy.

We solve plant production challenges across the value chain. To do this, we deliver technologies that are needed to develop and grow crops of higher quality, more efficiently and sustainably.

We focus on two important aspects:

- Speeding up the development of plant varieties due to the targeted editing of traits and single-cell regeneration;
- Increasing the efficacy of field application to enable efficient use of crop input through nanotechnology.

By providing these technologies to our partners, we contribute to better plants and higher plant yields while reducing the inputs necessary. Ultimately decreasing the impact on our natural ecosystems. We can help you greatly reduce the time and cost of plant breeding, and accelerate crop improvement in terms of **adaptation**, **resilience**, and **end-use**.

Our proprietary **TiGER Workflow** helps to remove the hurdles which are usually encountered in CRISPR-based gene-editing in plants, leading to a faster process and higher quality product.

In this whitepaper we tell you more about our proven approach.

CRISPR at a glance

HRB employs CRISPR-based genome editing to improve traits of interest in plants (e.g. crops, ornamentals or cell lines). The essence of CRISPR is simple: it's a targeted way of finding and altering a specific bit of DNA inside a cell. CRISPR can be used to edit highly specific pieces of DNA while leaving the rest of the genome intact. This allows for fast introduction of desired (or even new) traits in already carefully bred crops.

A diverse range of traits can be targeted. Some examples:

- Disease resistance
- Drought tolerance
- Nutritional content
- Flowering time
- Improved shelf life

To change a specific DNA sequence, the CRISPR system relies on a DNA cutting enzyme (endonuclease) that is guided to a target sequence using a guide RNA (gRNA). By cutting (and later repairing) the DNA, the sequence can be changed in a desired way. HRB uses MAD7 as its in-house endonuclease. 3 main steps are necessary:

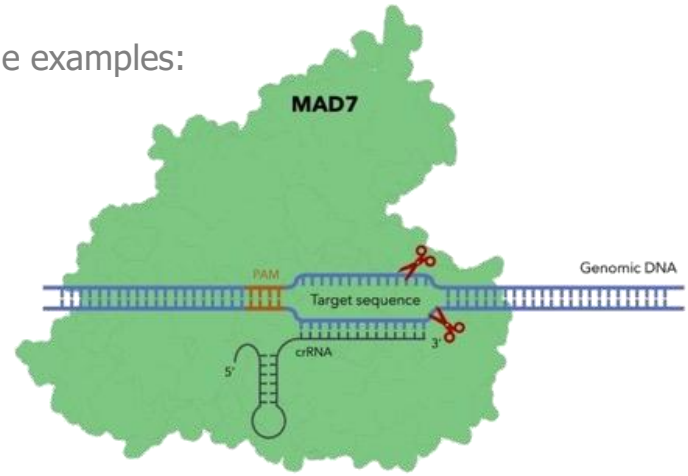
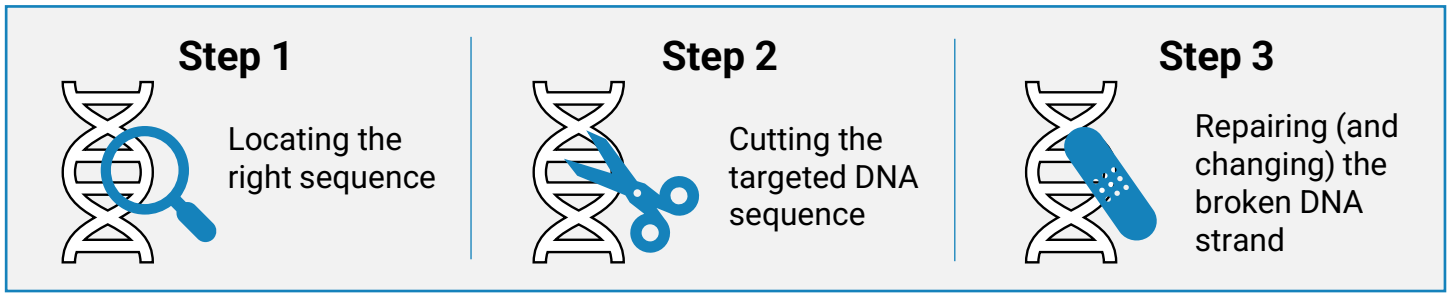


Figure 2. Elements of a CRISPR genome editing system.
In green the CRISPR enzyme MAD7 with its coupled guide RNA in black. Part of the guide RNA, the crRNA, is bound to a complementary sequence in the genome (the target). Upon binding MAD7 will cleave at a fixed distance from the protospacer adjacent motif (PAM).



Step 1: Locating the right sequence

The gRNA drives the first step of CRISPR: finding the right DNA sequence on the genome of a cell. One part of the gRNA's sequence can be designed to fit a complementary sequence in the DNA of the genome. This then guides the cutting enzyme to any location in the genome where this recognizable motif is present (Figure 1). If the chosen sequence is unique enough in the genome, this location will be the only site targeted.

Step 2: Cutting the targeted DNA sequence

Upon binding of the gRNA to the DNA of the genome, the associated endonuclease initiates the second step of the CRISPR system: cutting the targeted DNA motif. It cleaves both DNA strands so that open ends are created in the genetic material.

CRISPR at a glance

Step 3: Repairing & editing the broken DNA strand

In the final CRISPR step, the cell itself performs the actual genome editing. DNA breaks occur naturally all the time. As these are potentially damaging, the cell has its own repair systems in place. We can make use of these natural repair systems to fix and edit the DNA we intentionally cut.

At this point we can choose between two editing outcomes depending on the repair system we use:

1. **Inactivate the targeted gene** by creating a nucleotide deletion in the DNA.

2. **Insert a novel functionality into the genome** by repairing the DNA with a pre-designed template.

1

Inactivate the target gene

The predominant repair system, for natural as well as CRISPR-induced breaks, is the non-homologous end-joining (NHEJ) pathway. Although this system is fast and effective, often one or more nucleotides are lost in the repair process. This occurs because immediately after a break, some nucleotides are removed from the open DNA ends. In most cases, loss of a few nucleotides would not affect the integrity of the genome, but when the open reading frame of a gene (the part of a gene that encodes a functional protein) is repaired in such a manner, it can lead to a non-functional protein.

This type of editing can also be used to increase or reduce the number of certain proteins by targeting the gene promotor that drives gene expression.

2

Insert a novel functionality by using a repair template

A second, more elegant repair system is the homology directed repair (HDR) pathway. This pathway is only active during specific stages of cell division, when a repair template (normally another chromosome) is available for repair. Principally, it allows one strand of DNA to serve as a repair template for another single-stranded piece of DNA if both are complementary (homologous) to each other. Such repairs do not result in a loss of nucleotides. It is possible to provide a cell with a custom-made repair template. When the cell is provided with a designed repair template in which the desired changes to the gene sequence are present, the cell will repair the DNA break using the provided template, and insert the desired changes onto its own DNA.

How to address the challenges of targeted mutagenesis

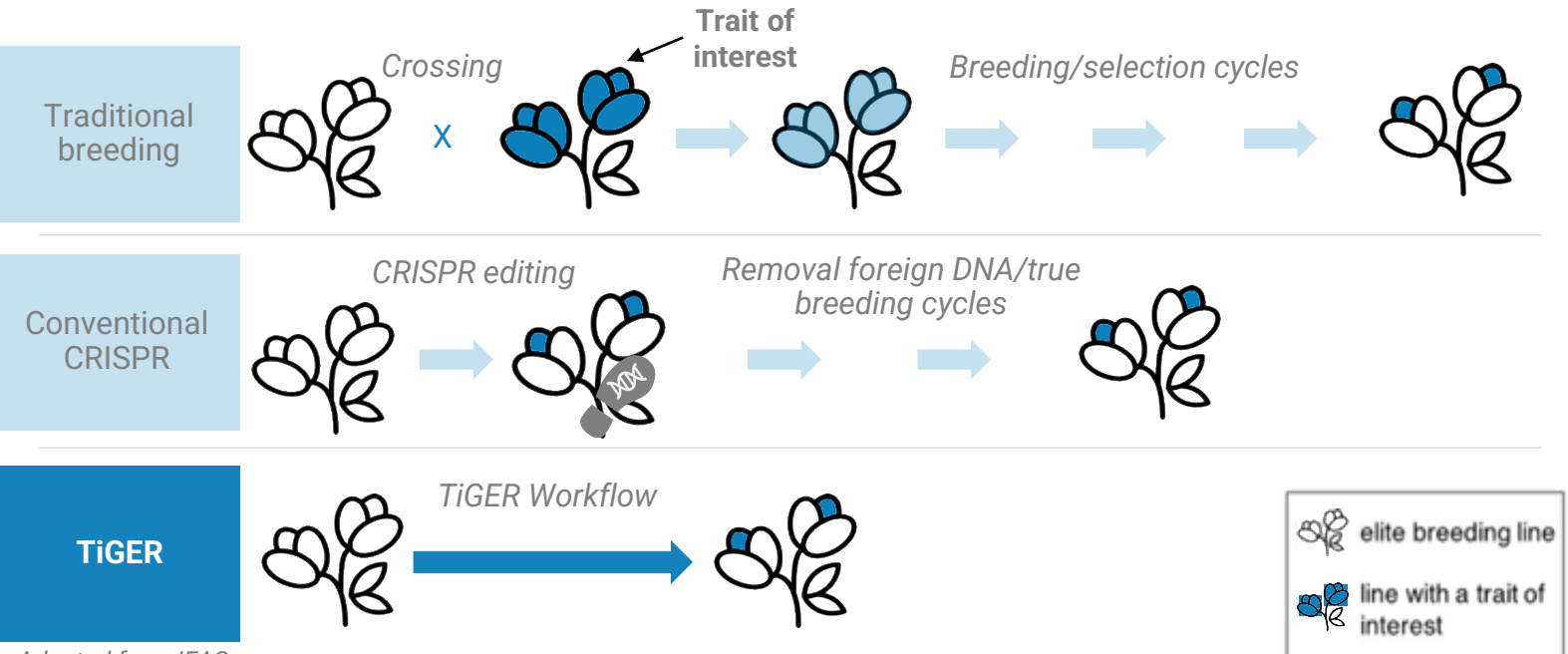
HRB is specialized in CRISPR genome editing. We offer superior gene editing services as compared to standard gene editing approaches. For a wide range of species, our TiGER workflow can rapidly deliver top-quality products that can be directly introduced into the market development pipeline. With our approach we overcome various challenges and annoyances that are commonly associated with CRISPR gene editing:

Regulatory issues	**High licensing Costs**	**Lengthly and costly projects**	**High complexity**

Our proprietary TiGER workflow yields a genetically uniform edited organism in 6-18 months by addressing these challenges through:

- **Transgene-free editing protocols** without the use of foreign DNA, which limits regulatory issues;
- **MAD7 nuclease**, a commercially more attractive option than Cas9 because there are no licensing costs, with high editing efficiencies (>70% in protoplasts) to minimize costs for edit screening;
- **Proprietary guide design software** to target single or multiple genes in one editing round while minimizing chances of off-target edits;
- **Single-cell protoplast regeneration** to avoid edited chimeras. Even for recalcitrant species difficult to handle *in vitro*, we have achieved CRISPR active delivery in intact cells using nanoparticle-based transfection methods.

By applying our proprietary workflow, you can speed up the development of plant breeding, even in comparison to other CRISPR approaches.



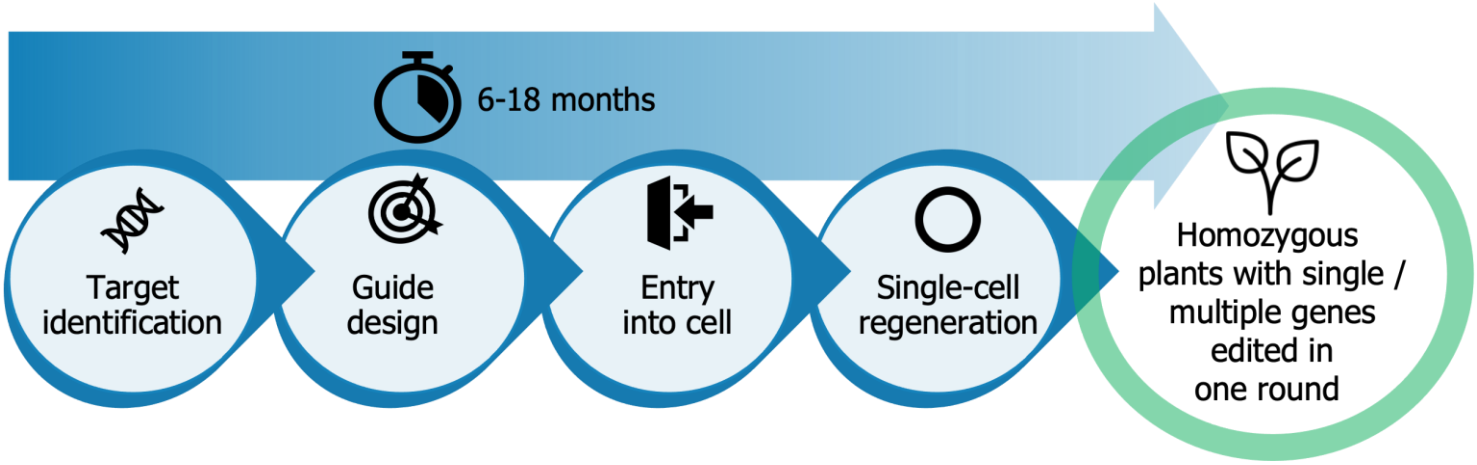
Adapted from IFAS

MAD TiGER Workflow

While CRISPR genome editing is straightforward in principle, the functional application is a considerable challenge, especially in green biotech. TiGER enables molecular breeding in an end-to-end fashion, providing innovative solutions to the typical barriers encountered in (plant) CRISPR breeding projects. In addition, we are actively developing specific aspects of the workflow further to continuously broaden its range and scope.

Our CRISPR enzyme of choice is MAD7 and HRB has in-house production capabilities for high-grade CRISPR proteins and guide RNAs that enable highly efficient genome editing.

Our TiGER Workflow follows 4 essential steps



MAD TiGER - Step by step:

1 TiGER

Target identification

In the first step of our TiGER workflow we work together with our customers and partners to identify the right genomic target for the intended trait adjustment. Targets can be specific genes or regulatory elements within the genome.

Hudson River Biotechnology (HRB) collaborates with partners to apply the SuRE platform technology from validated pharmaceutical applications to new agricultural applications.

SuRE, 'survey of regulatory elements', is a highly novel platform technology that can identify gene regulatory elements on a genome-wide scale. SuRE has valuable applications, such as the identification of novel, unique and sophisticated targets for molecular plant breeding through targeted mutagenesis approaches, as well as of novel, strong endogenous promoters to drive transgene expression.

2
TiGER

CRISPR guide design and validation

Step 2 of our TiGER workflow is CRISPR Guide selection. Guide efficiency is an important factor in overall genome editing efficiency. HRB has proprietary guide design software that allows for the design of efficient guides for multiple different CRISPR enzymes. We can produce all enzymes and their guide RNAs in house, achieving comparable performance to products from commercial suppliers. During this step we validate CRISPR guides by first measuring endonuclease efficiency on purified DNA *in vitro.* This is then followed by an *in vivo* assessment of editing efficiency in live cells, for which we often use protoplasts (Figure 5). This allows *in vitro* and *in vivo* validation in less than a month.

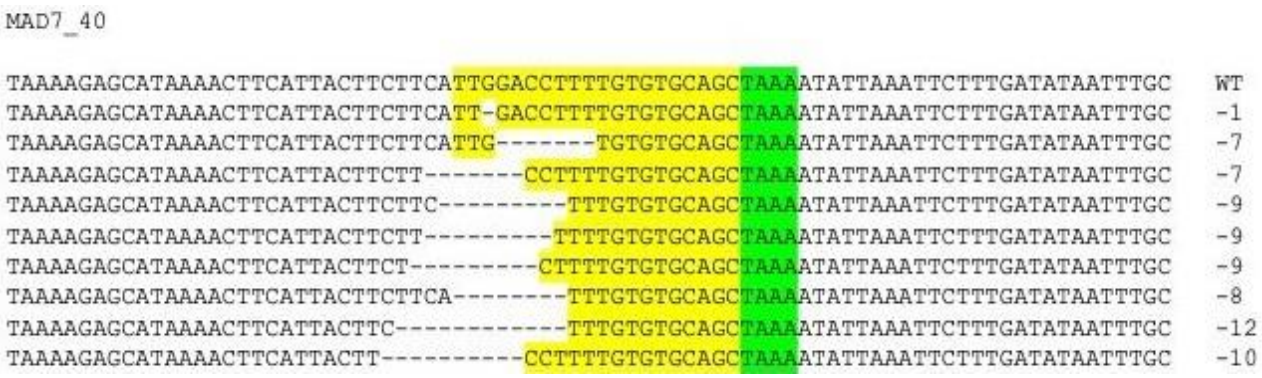


Figure 5. Output of an in vivo CRISPR activity assay.
Alignments of the target region of a wild-type (WT) genome (top sequence) and gene-edited genomes (all other sequences).

3
TiGER

Entry of CRISPR into the cell

Step 3 of our TiGER workflow is enabling entry of the CRISPR elements into the cells or tissues of interest. For this, we have access to common technologies such as PEG-based transfection and biolistics, which we apply without the need for transgenic selection markers. In addition, HRB has ongoing developments of nanoparticle-based platform technologies for encapsulation and functional delivery of CRISPR protein-guide complexes (RNPs) into different types of cells and (plant) materials in a highly efficient manner. These technologies include delivery to protoplasts, callus and tissues in planta, as well as solutions that circumvent the need for plant regeneration.

Of note, we take a **DNA-free approach** to CRISPR genome editing, whereas most others rely on the incorporation of transgenic DNA and use of antibiotic selection for the expression of the CRISPR enzyme and the guide RNA inside the plant cell. Our system has substantial benefits as it is:

- **Faster:** No need to outcross transgenes;
- **More efficient:** No need to express multiple components in sync in the plant cells;
- **Less prone to off-target effects:** CRISPR is only active for a short period of time;
- **More versatile:** Allows editing of plants where out-crossing of transgenes is not an option because this is biologically impossible or undesirable.

An added benefit is that the DNA-free method is likely to reduce the regulatory burden that accompanies the use of transgenes.

4
TiGER

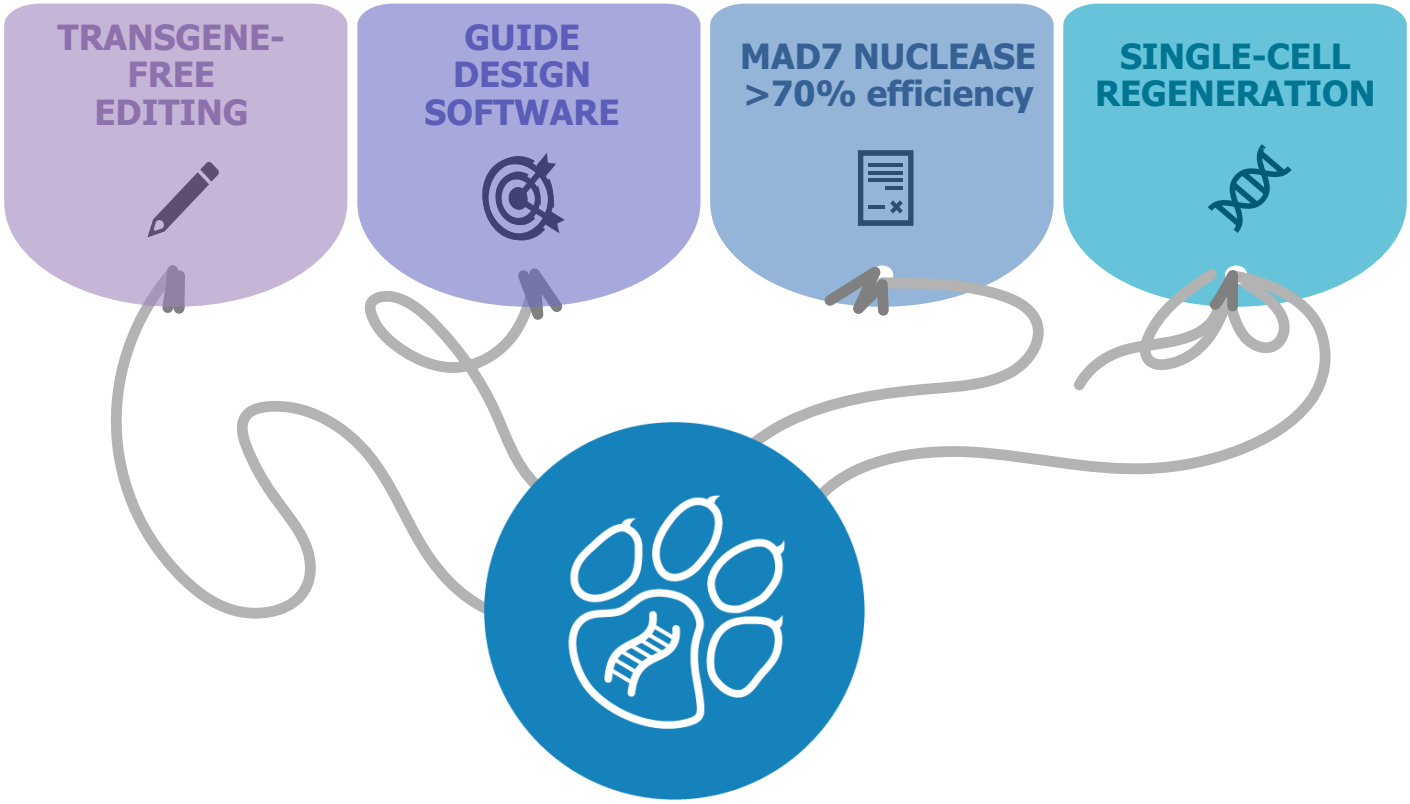
Plant regeneration

Step 4 of our TiGER workflow is regeneration. In many scenarios we work with *in vitro* plant tissues or cells. After successful genome editing, these need to be regenerated back into a plant, ready for generation of lines, scale-up and field or greenhouse testing. HRB has validated protocols for regeneration of multiple species. In addition, we are developing advanced materials that substantially shorten the timeline associated with the regeneration process, while improving efficiency. More on our approach to regeneration on the next page and in our *Regeneration Whitepaper.*

The benefits of our workflow

Our proprietary TiGER Workflow enables molecular breeding to reduce the time and cost of crop improvement significantly, by solving the typical barriers encountered in (plant) CRISPR breeding projects. Where releasing commercial varieties by conventional/molecular breeding would traditionally take between 7 and 10 years (if possible at all), they can now be done within 2 to 4 years.

- Proven application in multiple plant species
- No need for selection upon regeneration
- Active delivery and direct editing of intact plant cells
- >10% of transfected protoplasts form micro-callus at low cell densities

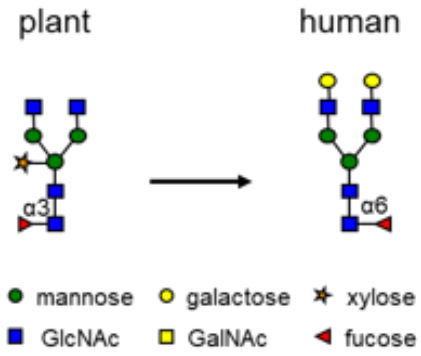


Client Case

In 2019, LenioBio approached us with a challenge for our CRISPR gene editing workflow. LenioBio provides a cell-free protein production platform based on a lysate from the tobacco BY-2 cell line: ALiCE®.

For LenioBio's cell line to produce proteins that are better suitable for pharmaceutical applications, plant-specific sugars should be removed to eliminate the risk of a human immune response to such plant-like proteins.

Their request to us was to knock-out in a single cell 28 genes responsible for plant-specific protein glycosylation with fucose and xylose: fucosyltransferases (FucTs) and xylosyltransferases (XylTs).



What we did

The tobacco BY-2 cell line is a tetraploid and has been cultured over the last 50 years. In this project, we made use of our proprietary TiGER (Target identification, Guide design, Entry into the cell, Regeneration) workflow, which overcomes CRISPR bottlenecks for a wide range of species.

During the first phase, **Target identification**, we identified 7 relevant targets responsible for plant-specific protein glycosylation (in total 28 alleles). Next, in the **Guide design** phase, we grouped gene targets based on homology and designed, tested and selected the best guides per gene group. In the next phase (**Entry into the cell**), we used PEG-based transfection of protoplasts and the MAD7 CRISPR endonuclease, which in HRB's hands can achieve high editing efficiencies of 40-60% in protoplasts (reducing costs and effort for edit screening). In the final phase, **Regeneration**, we applied our proprietary single-cell regeneration protocol and performed a PCR and antibody-based screening to identify and confirm the mutants we were looking for.

What we achieved

In a period of 18 months, we developed the required protocols and delivered Tobacco BY-2 cell lines lacking plant-specific glycosylation for LenioBio's ALiCE®. We achieved this by using our proprietary, transgene free CRISPR editing workflow. After further testing, LenioBio confirmed the multiple knock-out, which performs very well in culture and results in production of proteins lacking plant-specific Xyl or Fuc moieties for ALiCE®.

What they said

Dr. Ricarda Finnern – Chief Scientific Officer

It has been a journey developing the plant glycan knock-out cell line. The team at HRB, who are real experts of the TiGER technology, could translate it to our needs and cared about us as client. Their service helps us to achieve our goal to bring new pharmaceuticals to patients that are effective and affordable, wherever they are needed.

Dr. Mainak Das Gupta - Senior Scientist

The use of the MAD-Tiger technology to knock-out 28 alleles in a single cell is a technological milestone which we are all very impressed with. The journey with the team at HRB to achieve this breakthrough has been very exciting for us at the scientific level.

Highlight

Our approach to regeneration

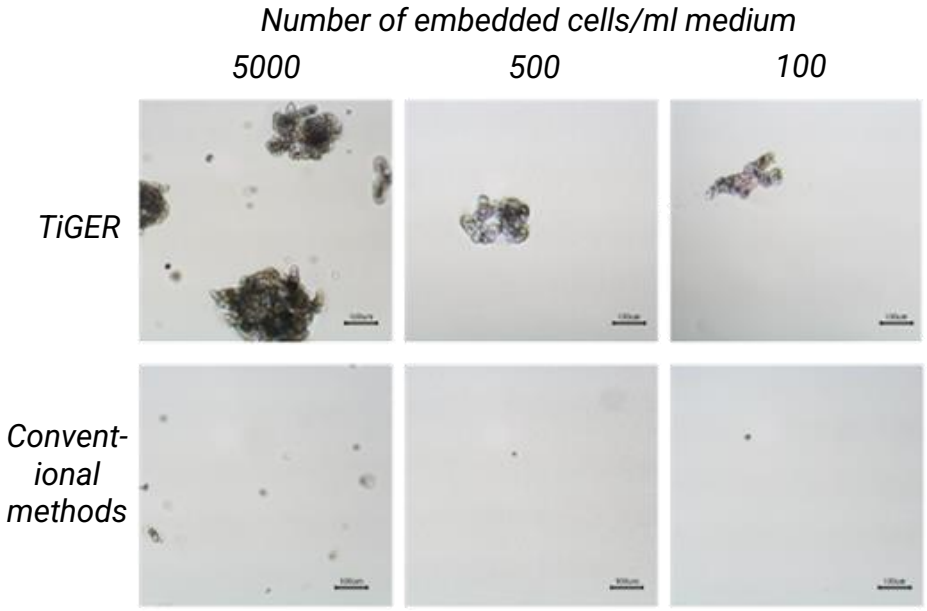
Application of transgene-free CRISPR technology to plants is associated with a number of technical barriers, including the difficulty to regenerate plant material from modified *in vitro* plant tissue. As part of its TiGER workflow, HRB optimizes solutions that can address these hurdles for various crops.

Single-cell regeneration

Traditional regeneration methods often lead to the formation of chimaeras (plants with sectors of edited and non-edited tissues) and thus genetically heterozygous end-results. Often, this results from the incorporation of multiple protoplasts into expanding microcalli. By regenerating plants from diluted single cells, HRB can **circumvent the risk of chimaeras** and **establish a clean knockout** in plants. For our validated single-cell regeneration approach, HRB has optimized media composition and regeneration protocols, adding several proprietary components. Patent application by HRB is under preparation.

PIC-gels

HRB continuously integrates new technologies to further innovate our single-cell regeneration. These include multiple patented or patent-pending technologies that enable or improve the efficiency of starting up protoplast cell divisions, callus regeneration and differentiation. A key example is our use of PIC (Polyisocyanopeptide) synthetic polymers which are crucial for protoplast embedding. PIC gels are proven, highly effective gelling agents in the field of mammalian organoid culture. Compared with classical alginate, PIC gels can **enable faster onset of cell divisions** in regenerating and de-differentiating protoplasts towards micro-calli formation.



Using the TiGER approach, protoplasts can be diluted down to ensure single-cell regeneration:

Microcalli derived from embedded cells (images taken after 3 weeks post transfection and embedding). At these dilutions, microcalli can only be seen with the TiGER approach. Using conventional methods, the number of embedded cells needs to be increased to at least 50,000 cells/ml medium to enable regeneration; at this concentration, expanding microcalli rapidly incorporate multiple protoplasts.

If you would like to learn more about our regeneration approach reach out to Gabino Sanchez at gabino.sanchez@hrb.bio

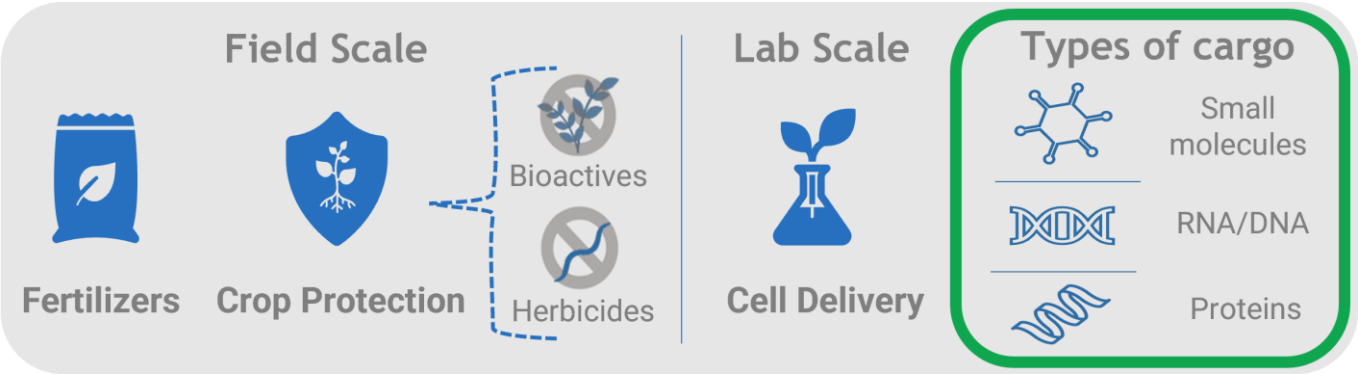
Highlight

Plentrance™

Nanotechnology for efficient delivery of biologicals and agrochemicals inside plants

New and better molecules for plant treatment are currently being brought on the market. But efficient delivery remains a challenge, especially for field application.

Building on the applications in our TiGER workflow, we have now crossed over to applying nanoparticles for delivery of agro-biologicals in plant tissue. This can be used in our TiGER workflow to simplify delivery of editing agents to plant cells but is also highly relevant for field practices by enabling efficient use of crop inputs.

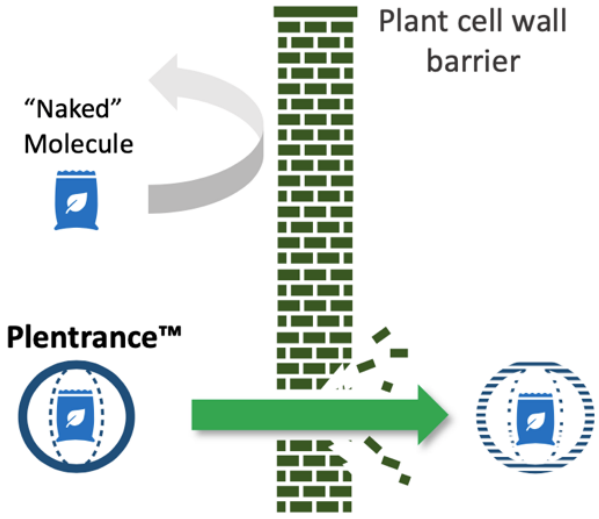


Plentrance™ nanoparticle formulations are **scalable**, **biodegradable** and provide **better control and efficiency**

How does it work?

Plentrance™ nanoparticles **penetrate the plant cell wall** and make active ingredients available in the right place at the right time.

Together with you, we develop **tailor-made nanoparticles** that increase the efficacy of the delivery, significantly lowering the dosage requirements. This lowers the associated costs for farmers and decreases the strain on our natural ecosystems. Our solutions include multiple patented formulations for delivery of various substances, such as herbicides, fertilisers and nucleotides.



The main benefits of our Plentrance™ nanoparticles:

- Optimization of field application to enable efficient use of active ingredients
- Customized and controlled release over time
- Biodegradable and compatible with ecological agriculture
- Increased bio-availability leads to less waste

About us

Hudson River Biotechnology is an independent technology provider, specialized in plant breeding, crop production, genetics, and biotechnology. Since HRB's founding in 2015, we have established a name in the market as a go-to player for disruptive innovations and cutting-edge technological developments. As our first portfolio technology, we have worked with a variety of companies to develop new traits and solve technological barriers in gene editing with CRISPR. In 2021 we have expanded our portfolio to offer delivery of biologicals and small molecules in field applications through nanotechnology.

Our customers range from large multinationals to family-owned businesses in, for example, the food, pharmaceuticals, plant breeding and biotech industries. Our goal is to make plant-focused technologies accessible, tailor-made to each customer and create mutual success. In short: HRB is perfectly positioned to become your strategic technology partner.

If you would like to learn more or discuss possibilities for collaboration reach out to Gabino Sanchez at gabino.sanchez@hrb.bio