Isolation and transformation of perennial ryegrass protoplasts for CRISPR/Cas9 applications

Master's thesis

Student: Erki Eelmets

Supervisor: Maria Cecilia Sarmiento Guerin, Department of Chemistry and Biotechnology, Senior Researcher

Co-supervisor: Ferenz Josef Sustek Sánchez, Department of Chemistry and Biotechnology, Early-Stage Researcher

Study program: YASM

Tallinn 2024

Karjamaa-raiheina protoplastide isolatsioon ja transformatsioon CRISPR/Cas9 rakendusteks

Magistritöö

Õpilane: Erki Eelmets

Juhendaja: Maria Cecilia Sarmiento Guerin, Keemia ja biotehnoloogia instituut, Vanemteadur

Kaasjuhendaja: Ferenz Josef Sustek Sánchez, Keemia ja biotehnoloogia instituut, Doktorant-nooremteadur

Õppekava: YASM

Tallinn 2024

Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not previously been presented for grading.

Author: Erki Eelmets
(Digitally signed)

The paper conforms to requirements in force.
Supervisor: Maria Cecilia Sarmiento Guerin
(Digitally signed)

Co-supervisor: Ferenz Josef Sustek Sánchez
(Digitally signed)

Permitted to the defence.
Chairman of the Defence Committee: Ott Scheler
(Digitally signed)

Table of contents

Abstract

Climate change brings with it an ever-growing necessity for perennial ryegrass (*Lolium perenne* L.*)*, as a widely used ruminant feedstock to better adapt to changing environmental conditions. As sexual reproduction in *Lolium perenne* is difficult due to a high level of self-incompatibility, classical breeding methods cannot be applied to this grass species. Genome editing technologies can however be used to overcome this short-coming, enabling the creation of plants more tolerant to both freezing and drought conditions. To test the results of genome editing, plants must be regenerated beforehand, which is a time-consuming endeavour, thus a time-efficient method of transformation, the PEG-mediated DNA transfer of protoplasts can be applied to test the efficacy of CRISPR/Cas9 components used for gene editing.

The goals of this thesis were to establish a high-yield protocol for isolating mesophyll protoplasts, as well as a high-efficiency protocol for PEG-mediated transformation and to use these protocols to validate the efficiency of guide RNAs of the CRISPR/Cas9 complex to edit the genome of *Lolium perenne*. The research was performed as part of the EEA-project "Improving adaptability and resilience of perennial ryegrass for safe and sustainable food systems through CRISPR/Cas9 technology".

Through the combination of various methods used to optimize the enzymatic isolation of perennial ryegrass protoplasts, a high yield protocol was established for *Lolium perenne*. The isolated protoplasts were successfully transformed using PEG with sequences transcribing the components of the CRISPR/Cas9 system. Moreover, the applicability of this system was confirmed for testing the efficacy of guide RNAs used for CRISPR/Cas9 gene editing. The methodology developed in this study can be applied to future CRISPR-Cas9 research in *Lolium perenne*.

Abbreviations and terms

bp – basepair

BSA – Bovine serum albumin

Cas – CRISPR associated protein

CAT – Chloramphenicol acetyl transferase

CBP20 – Cap-Binding Protein 20

CRISPR – Clustered regularly interspaced short palindromic repeats

DEAE – Diethylaminoethyl

DMSO – Dimethyl sulfoxide

EB – Evans blue

FDA – Fluorescein diacetate

GFP – Green fluorescent protein

gRNA – guide RNA

GUS – β-glucuronidase

LacZ – β-galactosidase gene of *Escherichia coli*

Luc – Firefly luciferase

MES – 2-(N-morpholino)ethanesulfonic acid

PEG – Polyethylene glycol

rcf – Relative centrifugal force

rpm – revolutions per minute

TALEN – Transcription activator-like effector; Transcription activator-like effector nuclease

TB – Trypan blue

ZF; ZFN – Zinc-finger; Zinc-finger nuclease

Introduction

The research of this thesis was performed as part of the EEA-project "Improving adaptability and resilience of perennial ryegrass for safe and sustainable food systems through CRISPR/Cas9 technology". The aim of this project is to increase the drought and cold tolerance of *Lolium perenne* L. using CRISPR/Cas9 technology, as this species is not well adapted to the climates of northern Europe, especially now that climate change is also affecting this area. Reduced precipitation and warmer temperatures brought on by global warming may coincide with lower levels of snow protecting perennial plants from winter freeze. The goal of this thesis is to develop a time-efficient method for testing guide RNAs (gRNAs) for CRISPR-Cas9 applications.

A plant protoplast is a plant cell that has had its cell wall removed. The first ever successful attempt to isolate plant protoplasts was recorded in 1892 by J.E.F. af Klercker [1]. This method based on mechanical means remained the only one in use until 1960, when E. Cocking first described the enzymatic digestion for isolating protoplasts [2]. After that, the enzymatic method for protoplasts isolation has been modified and improved. Since the discovery of *Agrobacterium tumefaciens* and its application for gene transfer, the use of protoplasts has fallen out of the scientific limelight. Over the last decade however, with CRISPR/Cas9 gene editing becoming a widely researched topic, protoplast-based methods have gained a new relevance.

There are various delivery systems that can be used in plants for applying CRISPR/Cas9. The PEG-mediated plasmid transfer to protoplasts is widely used. It has been theorized to rely on either passive diffusion or caveolae-mediated endocytosis for transformation. Other systems such as the *Agrobacterium*-mediated transformation require callus culturing and regeneration to produce a plant with an edited genome and are thus work intensive and time consuming. To time-efficiently test whether specific gRNAs can be used for CRISPR/Cas9 based gene editing in *Lolium perenne*, a protoplasts isolation protocol was optimized. The isolated protoplasts were then transformed with plasmids containing different gRNAs and the results were validated for their efficiency.

The literature review of this thesis provides an overview of *Lolium perenne*, protoplast isolation methodology, gene transfer methods in plants and genome editing technologies. The materials and methods chapter describes the plants and growing conditions, used molecular cloning methods, solution preparation used for protoplasts isolation, methodologies for protoplasts isolation and PEG-mediated transformation of protoplasts, principles used for DNA sequencing, gRNA efficiency and statistical analyses as well as methods used for imaging and image editing.

A high yield protoplast isolation protocol was established, successful PEG-mediated transformation was performed, and the suitable application of the system for testing gRNAs editing efficiency was confirmed.

1. Literature review

1.1. Perennial ryegrass

Perennial ryegrass (*Lolium perenne* L.) is a cool-season perennial bunchgrass indigenous to temperate areas of Eurasia and northern Africa [3]. It is a monocot plant, classified in the *Poaceae* family and the *Pooidae* sub-family along with cereals such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and the model grass *Brachypodium distachyon* (L.) P.Beauv. [4]. *Lolium perenne* can reproduce both sexually by seeds, a single inflorescence yielding anywhere from 45 to 333 seeds, and vegetatively from short rhizomes and tillers [5]. Sexual reproduction in *Lolium perenne* is wind-pollinated but outcrossing and highly self-incompatible [6], due to which it's capacity for adaptation to environmental changes is relatively low [7], [8]. Due to its importance as a forage grass for ruminant livestock, Perennial ryegrass has widely been distributed throughout the rest of the world [3]. High palatability and digestibility make Perennial ryegrass a greatly valued feedstock for cattle and sheep forage systems. Due to this it is often the preferred forage grass in temperate regions worldwide [9]. Perennial ryegrass natively has a diploid genome, but tetraploids have also been created via the application of colchicine during pollination, splitting the original two sets of 7 chromosomes into four [10], [11]. The latest chromosome-scale assembly of diploid Perennial ryegrass genome has a total length of 2.55 Gb [12]. Diploid ryegrass usually has a 2% higher dry matter content than its tetraploid counterpart due to having smaller cells and thus are more nutritious. Tetraploid ryegrass has larger and heavier seeds, germinates quicker enabling them to compete for resources more successfully. However, tetraploids also present larger leaves, which are more upright, and a more open growth habit resulting in them having a higher risk of poaching in heavier soils. It has also been noted that livestock tends to prefer to grazing on tetraploids rather than diploids [11].

Perennial ryegrass adapts best to moist and cool climates where winter kill does not occur [3]. Poor survivability in cold climates can be the result of a variety of winter stresses like low temperatures and related diseases i.e., snow mold, suffocation due to ice cover, crown hydration and desiccation [13]. It grows best on fertile and well-drained soils, but also has a wide range of soil adaptability. Moreover, it can tolerate flooding for periods up to 25 days unless temperatures rise beyond 27 °C. Perennial ryegrass tolerates a soil pH range of 5.1-8.4 but grows optimally ranging 5.5-7.5. Optimal growth occurs during spring and autumn. During summer months Perennial ryegrass becomes dormant. The optimal growth temperature for Perennial ryegrass is 20-25 °C. Even in high-moisture conditions, the growth of Perennial ryegrass is inhibited by daily temperatures exceeding 31 °C or nightly temperatures exceeding 25 °C [3]. It should be noted that optimal growth conditions of *Lolium perenne* are specific to each cultivar [14].

1.2. Plant protoplasts

The outer layer of plant cells are structures called cell walls. There are 2 types of cell walls: the primary and the secondary cell wall. Primary cell walls consist mainly of cellulose, hemicellulose and pectin while secondary cell walls consist of cellulose, hemicellulose and lignin [15]. Cell walls can be removed both mechanically and enzymatically. The living cytoplasm of wall-less cells

surrounded only by the plasma membrane, are called protoplasts. Once the cell walls are removed, the isolated protoplasts take on a spherical shape and become osmotically fragile [16], [17] but otherwise retain other features and activities common to plant cells. Due to being easily accessible models for plant cells, protoplasts make for an excellent subject for researching cellular events like transformation and recombinant DNA expression [18].

Protoplasts are theoretically capable of regenerating into whole plants. Viable protoplasts are therefore potentially totipotent, having the ability to regrow their cell walls, dedifferentiate, enter the cell cycle once more and after multiple mitotic divisions, proliferate or regenerate into different organs. Via tissue culture and the use of certain chemical and physical stimuli, whole plants can be regenerated from protoplasts. Whole plant regeneration has been described for a multitude of plant species and there is extensive literature relating to scientific applications of protoplasts. [16], [17]

1.3. Protoplast isolation

Protoplast isolation is a scientific method hailing from the closing decade of the 19th century and has historically been achieved via mechanical means from plasmolyzed cells. The original method was first recorded in 1892 by J.E.F. af Klercker [1]. The application of increased external osmotic pressure plasmolyzes plant cells – the cell membrane retracts and the protoplast inside takes on a different shape which varies depending on the shape of the surrounding cell walls [19]. Plasmolysis of isodiametric leaf mesophyll, callus and suspension culture cells in hypertonic solutions usually causes overall shrinkage of the protoplasts, while elongated cells can separate into multiple subprotoplasts without rupturing the plasma membrane. In this case only one of the subprotoplasts contains the nucleus [20]. During plasmolysis the plasmodesmata of protoplasts seal up [21]. When plant tissue is cut, the protoplasts of which have been plasmolyzed via the use of calcium chloride or calcium nitrate solutions, it is possible that some of the protoplasts survive and exit the tissue intact. This is the original method for protoplast isolation [1]. Since then, sucrose solutions have also been used to induce plasmolysis [22]. The largest drawback of this method is the low yield of viable protoplasts. Researchers have only been able to isolate small numbers of protoplasts through the application of extensive plasmolysis and none of meristematic origin [21], [23]. This method also requires large vacuolated cells for isolation and produces protoplasts of low viability due to harmful substances released by damaged cells [23].

The somewhat more modern method of protoplast isolation via enzymatic digestion was first recorded by Edward C. Cocking [2] and has since become a rather common procedure for the observation of cell division, embryogenesis, photosynthesis and many other plant processes [24] in a variety of different plant species. Even though plant protoplasts are well studied, the general principles of protoplast isolation have not changed much over the years [16], [17]. Although the general process of protoplast isolation is much the same for differing plant species, the specifics of isolation protocols for each species play a large role in achieving high protoplast yields. Minor changes to enzyme concentrations and the chemicals used in enzymatic solutions, varying incubation times and conditions and differing forms of mechanical agitation or handling can have drastic effects on the viable protoplast yields of the isolation process and the following transfection efficiency [25]. Along with the capacity to isolate meristematic protoplasts, the enzymatic method

is also able to isolate polynucleate protoplasts formed via spontaneous fusion during enzymatic treatment [26]. In this case, spontaneous fusion of plant protoplasts does not require a specific chemical treatment and it has been shown that fusion occurs through the expansion of intercellular plasmodesmata [27].

The use of protoplast-based techniques has strayed from scientific limelight over the past couple of decades, as *Agrobacterium* and Biolistics mediated gene transfer techniques have risen to the forefront of plant research. The isolation and culturing of protoplasts however remains fundamental to gene transfer via cell fusion and for some aspects of transformation studies. Protoplast fusion techniques allow for wider possibilities of genetic combinations at the nuclear or organelle level when compared to conventional sexual hybridization methods. [28]

1.3.1. Source tissue for protoplast isolation

Protoplasts can be isolated from virtually any plant tissue of varying age such as leaves, stems, roots, callus, cell suspension cultures etc. [23]. As cell walls thicken with age [29], it might be more efficient to extract protoplasts from young tissues. The physiological condition of the source material does influence the viability and yield of the isolated protoplasts, so the plants used for experiments should be grown under controlled conditions. Isolation from callus tissue and cell suspension cultures benefit from being in the early log phase of growth. Better results can generally be obtained from tissues with low starch content. The most widely used source tissue is the mesophyll of fully expanded leaves of young plants or new shoots. The popularity of said source tissue derives from the capacity of isolating large numbers of relatively uniform cells without killing the plant itself. Mesophyll cells are also loosely arranged allowing for an easier access to the cell wall [23].

The foremost issue in the isolation of protoplasts is an adequate penetration of the wall-digesting enzymes into the source tissue. Prior physical separation of the source tissue facilitates the efficiency of cell wall degradation due to the wall being water insoluble [21]. Depending on the plant species and the specific structure of the source tissue used for protoplast isolation, methods for accessing the protoplasts can vary. For *Arabidopsis* mesophyll protoplasts, the most efficient way is to strip the epidermis via the use of tape [30] This method does not work with the leaves of grasses, as the epidermis is more tightly secured to the mesophyll cells. For grasses the most used method is the cutting of leaves into thin slices prior to enzymatic treatment [23], [31], [32]. Enzymatic penetration of the source tissue can be further enhanced using vacuum for infiltration [33].

1.3.2. Osmotic pressure as an important factor during protoplast isolation

Protoplasts which are released to isotonic solutions will burst. This happens because in vivo the outward pressure of protoplasts is counterbalanced by the mechanical support of the cell wall. During protoplast isolation the mechanical support is replaced by an appropriate osmotic pressure of a hypertonic solution. Osmotic stress is however harmful to the metabolism of cells as it causes condensation of the nuclear DNA and inhibits protein synthesis. Both effects are reversible. Lower osmotic potentials are usually achieved via addition of either ionic or non-ionic solutes. Among the

ionic solutes used are substances such as potassium chloride, calcium chloride and magnesium sulphate among others. The non-ionic solutes are carbohydrates such as mannitol, sorbitol, glucose, fructose, galactose or sucrose. Mannitol and sorbitol are the most frequently used carbohydrates, the former of which is the preferred solute for leaf mesophyll protoplast isolation. Mannitol is also more metabolically inert, infusing slowly into the protoplasts. Carbohydrate solutions of molarity ranging 0.3-0.7 M can generate a suitable osmotic potential for isolation. Metabolically active carbohydrates are preferable if isolated protoplasts are later used for regeneration. The lowering of the osmotic potential is counteracted by the synthesis of new cell walls [23].

Even if non-ionic solutes are used for plasmolysis, the use of calcium chloride can still help achieve a large yield as calcium ions may help stabilize the cell membrane but also have been shown to induce cell aggregation [34]. Moreover, the presence of potassium ions has been linked to stress tolerance in plants, including responding to changes in osmotic pressure and pH, like the hyperpolarization/depolarization of the cell membrane [35]. Therefore, adding potassium chloride in the enzymatic solution may increase viable protoplast yield.

A plasmolytic treatment of an hour in a hypertonic solution prior to enzymatic digestion has been shown to improve viable protoplast yield [28], [36]. It is recommended that this treatment is conducted in a solution isotonic to the enzymatic solution [28]. This treatment also reduces the amount of protoplast fusion during the enzymatic treatment [36].

1.3.3. Enzymatic digestion for protoplast isolation

Cell walls consist in large part of cellulose, hemicellulose and pectin. Cellulose and hemicellulose are the main components of both the primary and secondary cell wall. Pectin is the main component of the middle lamella, which joins the cells together. There are three enzymatic activities necessary for efficient release of protoplasts: those of cellulase, hemicellulase and pectinase respectively [21], [23]. Besides the three main enzymes, there are also a variety of auxiliary enzymes such as helicase, colonase, and zymolyase, which can be applied for tissues which do not easily release protoplasts [23]. The macromolecular crystalline form of cellulose vastly inhibits the enzymatic activity of cellulases to hydrolyze the glucosidic bonds of cellulose. Due to the limited access, amorphous cellulose is more easily dissolved than crystalline cellulose [21]. For example, when the aleurone cells of barley are treated with cellulase, the cell walls become cellulase resistant. The cell walls of such cells can however be digested with glusulase [23].

Enzymatic digestion can be carried out either simultaneously or sequentially. Simultaneous digestion is performed by carrying out the digestion of both the primary and secondary cell walls along with the middle lamella in a single reaction. Sequential digestion is performed in separate enzyme solutions, with one focusing on the degradation of the middle lamella and the other one on the digestion of primary and secondary cell walls [21], [23]. Simultaneous digestion is less work intensive and unlike sequential digestion, can produce fusion protoplasts [21].

The timeframe for enzymatic digestion can vary greatly depending on the species of plant, tissue type, enzymes used and both the incubation temperature and the pH of the solution, therefore enzymatic treatment can range anywhere from half an hour to 20 hours [23]. Application of vacuum

can speed up the enzymatic infiltration of tissues and speed up the whole treatment process, however the fast release of vacuum can damage protoplasts [33] Even though the activity of enzymes is temperature and pH dependent, in practice temperatures ranging 25-30 °C and pH values ranging 4.7-6.0 are used generally [23]. Incubation temperature should not be raised above 35 °C, and sensitive systems may yield better results from long-term isolations using low temperatures such as 7-12 °C. Difficult systems might also be incubated at sequences of high and low temperatures [33].

The recommended leaf mass to enzyme solution ratio is 1g to 10mL. Gentle shaking may assist the release of protoplasts from source tissue. Enzymatic digestion is recommended to be carried out in a container with a large surface to depth ratio [33], as a high aeration rate can enhance enzymatic activity of certain enzymes, one of which being cellulase [37]. Cellulase activity is also increased by the addition of bovine serum albumin (BSA) into the enzymatic solution [38]. Moreover, the enzymatic digestion is recommended to be carried out in darkness as both blue and white light can cause the hyperpolarization of the cell membrane [39] and produce free radicals inside cells containing chloroplasts [36].

1.3.4. Harvest and purification of protoplasts

The resulting solution after the enzymatic treatment has finished will contain healthy protoplasts, but also substantial amounts of cellular debris, undigested tissue, and broken protoplasts. This mixture is then further purified via a combination of filtration, centrifugation and washing. Filtering is done using either stainless steel or nylon sieves with sizes ranging 50-100 µm. Multiple sieves can be used sequentially depending on the estimated size of protoplast isolated, which can vary depending on the plant species, age and growth conditions. This helps remove larger pieces of undigested tissue and cell aggregates [23], [28]

A filtered enzyme solution of protoplast isolation will still contain lots of debris. The purification of the protoplast solution can be achieved via the use of a sucrose cushion or a Ficoll/Percoll gradient [23]. Additionally, pelleting via centrifugation followed by the removal of the supernatant and resuspension in a medium isotonic to the enzymatic solution can also be used [28]. The sucrose cushion is usually performed by layering the enzymatic solution on top of a 20-21% sucrose solution and then centrifuging it at relatively low speeds (75-100 rcf) for 7-10 min [23], [40]. After centrifugation the debris pellets or remains suspended in the denser sucrose layer while an intact protoplast layer will form at the junction of the sucrose and the protoplast suspension medium or the enzymatic solution, depending on whether the protoplasts were pelleted beforehand. Both the Ficoll and Percoll gradient work via the same principle as the sucrose cushion but use differing solutions, amounts of layers and centrifugation conditions to achieve protoplast flotation [23]. The floating protoplast layer is then collected and resuspended in a washing solution [23], [28].

1.3.5. Viability and density of protoplasts

Protoplast viability is usually estimated and not determined because the only true method of determining viability is regenerating plants. There are many methods for estimating the viability: positive staining methods such as Fluorescein diacetate (FDA) staining [23], triphenyltetrazolium

chloride (TTC) staining [41] and Calcofluor white (CFW) staining. There are also multiple counterstaining methods used for protoplast viability estimation such as phenosafranine stain and azo dye counterstaining techniques like methylene blue (MB), Evans Blue (EB) or Trypan Blue (TB). Furthermore, there are other methods of viability estimation which do not include staining [23]. There are advantages and disadvantages to each estimation technique and the counting of cells is usually performed using a haemocytometer [23].

Manual cell counting via the use of a haemocytometer is the oldest form of quantifying individual cells, which is a technique over a century old. In principle, the haemocytometer is a counting chamber, into which a certain volume of liquid is dispensed [42]. The counting areas of haemocytometers usually contain grids of measured dimensions. When a cover glass is fixed onto the haemocytometer, the counting areas will also gain a measure of depth, making it possible to calculate the number of cells counted in a measured volume of liquid. Cell counting using haemocytometers is performed under a microscope.

One of the most common staining methods to assess viable protoplasts is the FDA stain. When FDA passes the cell membrane, thereby entering the cell, it is cleaved by intracellular esterase activity into fluorescein and acetate [43]. While FDA itself is not fluorescent under a fluorescence microscope, fluorescein is. As the membranes of intact protoplasts accumulate fluorescein, they attain a green fluorescence when exposed to UV radiation [23]. In order not to also dye the background, the solution wherein protoplasts are immersed must have low to no esterase activity of its own. This excludes the use of FDA staining on solutions which contain commercial cellulase enzymes produced by species of *Trichoderma* and *Aspergillus*, because alongside cellulase they secrete a variety of esterases which remain as contaminants after purification [44]. FDA staining is however a relatively quick procedure in which cells are stained in 5-10 min but must also be examined in 15 min after which fluorescein starts to dissociate from the membranes [23].

TTC staining is another staining method which can be used for dyeing protoplasts. As the natively white TTC enters a living cell, it is reduced to a red 1,3,5-triphenylformazan by dehydrogenase activity present in living cells. This technique, however, cannot be applied to chloroplast containing cells such as leaf tissue as the endogenous pigments interfere with the reading. [43]

While the previous two staining techniques relied on intracellular enzymatic activity, the CFW binds to 1-3β and 1-4β polysaccharides such as cellulose and pectin. When bound, CFW attains a blue fluorescence under UV radiation. As living protoplasts have the capacity to regenerate cell walls, this method can be used to estimate viable cells [23], however cell wall synthesis is a time-consuming process [45], which is counter-productive to downstream applications which rely on the accessibility of protoplasts.

Counterstaining methods stain dead tissue, leaving viable protoplasts unstained. Even though all these methods stain dead tissue, differences occur in ways in which they affect live cells. TB cannot penetrate intact cell membranes as both are negatively charged but binds to intracellular proteins staining them blue [46]. Both MB and EB also stain tissue blue but are also able to enter intact cells where they are reduced to colourless forms, thus not staining living tissue [47], [48]. Like TB, phenosafranine cannot penetrate intact cell membranes, but stains dead tissue red instead [49]. All forementioned counterstaining techniques stain the background alongside dead tissue, making

the distinction of live tissue troublesome. Moreover, TB, MB and EB stains exhibit various levels of toxicity to plant protoplasts [48], [50], thus it is preferable to perform staining in a limited timeframe not to induce additional cell death.

Estimating the viability of protoplasts without staining can be performed in multiple ways. One of these methods is the observation of active streaming of the cytoplasm within protoplasts [23]. This a very time-consuming process as each individual cell must be closely examined. Another observation technique for viability estimation would be the determination of whether osmotic changes affect the size of protoplasts [23]. Viable protoplasts should react to changes in osmolarity by either growing or shrinking, but these changes might also kill the protoplasts, thus reducing viability in the estimating process. Finally, it is possible to estimate protoplast viability by measuring oxygen uptake from a solution by an oxygen electrode, which indicates a respiratory metabolism [23]. As this method does not include the counting of protoplasts, the output data does not reflect the amount individual cells but rather the collective metabolic capacity of all cells in a solution, which makes it a somewhat crude estimation method.

1.4. Gene transfer in plants

Genetic transformation is the directed transfer of foreign DNA into the cells of a target organism making use of a vector, often a plasmid. It results in the integration and expression of the foreign DNA [23]. While transferring plants specific genes into other plants tends to be generally successful, the species of origin can belong to a different taxon.

Gene expression after transformation can be either transient, in which case the DNA is only expressed for a limited period, or stable, in which case the DNA is expressed continuously. The vast majority of successfully transformed genes are expressed transiently, meaning that they are not fully integrated and will be therefore lost over time and cell divisions. Transient expression however provides a rapid use tool for monitoring the success rate of transformation, as it does not require the regeneration of the host plant. Stable transformation, on the other hand, happens when exogenous DNA gets integrated into the plant genome, and gene expression can be assessed in regenerated plants or their subsequent generations [23].

1.4.1. Plant marker genes

The detection and quantification of gene expression is performed by using auxiliary marker genes in transformed gene constructs. The use of marker genes allows for the detection of transformants without any additional downstream processing to determine integration. There are two categories of marker genes: selection markers and reporter genes. Some marker genes can function both as reporters and selection markers [23].

The selection marker genes either provide the host organism with the ability to tolerate toxic amounts of certain substances [23] or make them more susceptible to certain toxic substances. Gain-of-function marker genes, such as those providing resistance, are called positive markers and loss-of-function markers, such as those inducing susceptibility, are called negative markers. Some selection markers can act as both positive and negative markers [51]. Selection markers used in plants are antibiotic resistance markers, herbicide resistance markers and antimetabolite markers

[23]. The use of selection markers in protoplasts requires culturing and thus is more time consuming and labour intensive than using reporters.

Reporter genes can be screened for in cells or tissues due to a quantifiable phenotype. The principle of using reporter gene systems lies in introducing synthetic modifications into natural genes, thereby detecting gene products or distinguishing them from similar ones. The reporter genes most used in plants code for chloramphenicol acetyltransferase (CAT), octopine synthase (Ocs), nopaline synthase (Nos), β-glucuronidase, bacterial luciferases, firefly luciferase (Luc), anthocyanin and fluorescent proteins like GFP [23]. Other reporter genes can be applied to certain plant systems such as the *LacZ* of *Escherichia coli*, which transcribes β-galactosidase but is not widely used in plants as plant cells tend to contain endogenous β-galactosidases that can interfere with readings [52].

The first ever bacterial gene to be transformed into plants, *cat* [53], has nowadays become a commonly used reporter gene. There are two types of *cat* and the more widely used originates from the transposable element Tn9 [54]. The chloramphenicol acetyltransferase (CAT), the gene product of *cat*, as the name suggests, acetylates chloramphenicol. Via an enzymatic assay, it is possible to detect acetyl chloramphenicol from a crude extract of plant tissue using autoradiography [23]. The enzymatic assay for CAT is procedurally simple and highly sensitive however its use in plants can be problematic as some plants contain endogenous non-specific acetylases which can interfere with readings. Moreover, certain plant species contain agents which inhibit CAT [55].

Opine synthase transcribing genes, the two most common of which being *ocs* and *nos*, originate from the T-DNA of tumor inducing or root inducing plasmids of *Agrobacterium rhizogenes*. As plants naturally do not produce opines, their presence in tissues indicates it being transformed. There are two ways to determine opine presence in plant tissue. The first one involves measuring opine synthase activities by extracting proteins from the plant sample, incubating it in a solution containing specific precursors required for opine synthesis, and separating the reaction products via paper chromatography. The second method involves detecting opine presence directly in the plant tissue by extracting plant metabolites, isolating opines by paper electrophoresis and detecting them via staining [23]. Opine detection in tissue is a relatively simple process [23], [56], as their synthases are stable, and the enzymatic assay is not expensive nor procedurally complex [23]. Drawbacks to this method include the fact that certain strains of *A. rhizogenes* contain two T-DNAs, only one of which includes genes related to opine synthesis. Due to this only some of transformant plant cells can be detected by the presence of opines. The genes involved in opine synthesis, when integrated into a plant genome, can also become methylated, inactivating their expression, and thus interfering with readings. Lastly, reading interference can occur in certain plant species as they contain compounds which react with the same staining agents used in opine detection [55].

The bacterium derived gene *uidA*, which encodes β-glucuronidase (GUS), is another gene which can be used for analysing gene expression in plants [23]. GUS is a hydrolase which cleaves multiple different glucuronidases [55], giving a coloured reaction which can then be measured [23]. The enzyme has a molecular mass of 68 kDa and a pH optimum of 7-8 [23], [55]. This reporter does not rely on DNA extraction, electrophoresis, or autoradiography, thus making it a relatively simple tool

for assessing gene expression. The *uidA* gene of *E. coli* can also be used as a selection marker with the use of the selective agent benzyladenine [23].

The luciferase reporter systems function via the detection of bioluminescence to measure gene expression. Both luciferase systems can be used in cellular extracts as well as *in planta* [55]. The bacterial luciferases (Lux F2), originating from either *Vibrio harveyi* or *V. fischerii*, are heterodimeric flavin monooxygenases, which upon assembly catalyse a light emitting reaction [23]. The constituent monomers of Lux F2 are transcribed by *luxA* and *luxB* [57], [58]. The use of Lux F2 system is however hampered by relatively low thermostability of constituent monomers and even lower thermostability of the dimeric enzyme [59]. Moreover, *in planta* light emissions of Lux F2 are relatively low [55]. The firefly luciferase (Luc) transcribed by *luc* is a reporter system originating from *Photinus pyralis*, and catalyses oxidative decarboxylation of luciferin to oxyluciferin in the presence of ATP, O_2 and Mg^{2+}, thereby emitting light [23], [55]. While luciferase activity can be detected by simple observation, quantification measurements do require a special camera or a luminometer [55].

Anthocyanins are flowering plant pigments of the red and purple hue. There are at least ten genes which encode regulatory or structural proteins associated with the pathway of anthocyanin synthesis. Genes *C1* and *B/R* regulate structural gene activity. Chimeric gene constructs of these genes, when introduced into the plant cells, induce colouration of the cells. These pigments can then be detected either visually or under a microscope. The advantages of using anthocyanins as reporters are that they can be detected in live cells, expression can be located within the cell and detection does not require any additional substrates. [23]

The green fluorescent protein (GFP) encoded by *gfp* is a bioluminescent protein isolated from *Aequorea victoria* [60]. The fluorescence of GFP is produced by a β-hydroxy benzylidene imidazolinone chromophore composing of amino acid complex Ser-Tyr-Gly [61]. GFP is also a highly stable protein of a relatively small size of 27 kDa [55], that can be detected *in vivo* using a fluorescence microscope without the addition of any additional cofactors [23], [55]. Moreover, GFP does not adversely disrupt plant cell metabolism [23]. Due to these reasons GFP has become a widely used reporter not just in plants, but also in other taxa of life [55]. There are also multiple kinds of GFP used as a reporter such as the enhanced green fluorescent protein (EGFP). EGFP is a form of GFP which due to two amino acid changes in its sequence, is more photostable and emits a 35 times stronger fluorescence than GFP at 488 nm wavelength, which is also the excitation maximum of EGFP. The emission maximum of EGFP is 507 nm [62].

1.4.2. Chimeric gene vectors

Plant nuclear genes consist of differing regions, each of which have different functions related to transcription and translation of mRNA. The promoter region starting at 5’ end, initiates transcription and alongside enhancer or silencer regions regulate expression. The transcriptional start or cap site helps bind RNA polymerase. Transcribed regions can also contain one or multiple introns which are removed before translation. The translated region ends with a stop codon followed by a terminator with a polyadenylation signal at the 3’ end. [23]

Transferred genes are usually modified prior to transformation by placing them under the control of different promoters, terminators, and enhancer sequences. Such modified genes are called chimeric or transgene constructs as they include parts of varying origin. Promoters can be either constitutive or inducible. Bacterial promoters used in plants are *lac*, *trp* and *tac* and phage promoters are *T3*, *T7* and *SP6*. The 35S RNA promoter of cauliflower mosaic virus (*CaMV*) is also often used as plant viruses depend on transcription and translation factors present in plants. There are also some promoters which are specifically used in monocot plants like maize ubiquitin I promoter (*Ubi1*), ribulose bisphosphate carboxylase small subunit (*rbcS*), alcohol dehydrogenase (*Adh1*), nopaline synthase and the rice actin promoter (*Act1*). [23]

Regulating gene expression has an important role in plant development so care should be given to choosing constituent parts of artificial gene constructs. More complex gene constructs can include combinations of multiple enhancers, silencers, promoters, terminators, open reading frames, organelle specific targeting sequences, selection markers, reporter genes and vector sequences. Chimeric constructs in plants can be transferred as linear DNA sequences, plasmids or via a specialized vector for *Agrobacterium* mediated transfer. [23]

1.4.3. Plant gene delivery systems

There are a multitude of transfer methods which are used in plant science. These fall in the two broader categories of biological or vector mediated methods and non-biological or vector-less methods [23], [63]. The vector-less methods can also be categorized into subcategories of *in planta* methods and protoplast-based methods [63].

Plant gene vectors in biological delivery systems can indicate both gene transfer between plants and the transfer of genes from other taxa of life such as bacteria, fungi, or animals. Gene vectors used in plant transformation are plasmids of *Agrobacterium*, viruses, and transposable elements. There are two methods for *Agrobacterium* mediated transformation: co-cultivation with explant tissue and *in planta* transformation [23]. There are not any published papers on successful *in planta Agrobacterium* mediated transformation in *Lolium perenne* and both viral and *Agrobacterium* mediated transformation methods in explants require tissue culturing [63], which makes them more time-consuming than protoplast-based approaches.

The *in planta* methods of vector-less delivery systems include those of particle bombardment or biolistics, silicon carbide fiber mediated gene transfer and laser microbeam mediated gene transfer. Silicon carbide fiber and laser microbeam mediated gene transfer are also dependent on tissue culturing [63], making them time-inefficient methods. While particle bombardment does not necessarily require tissue culturing [63], the intracellular targets of this method are not specific and non-targeted DNA is not protected from damage. Due to this, complex integration patterns and duplicated insertions are relatively common side-effects of particle bombardment, which can induce downstream gene silencing as well as variations in the expression patterns of transgenes [64], [65].

Physical methods of vector-less gene delivery in plants include methods such as electroporation, sonication, microinjection and lipofection. Electroporation is a physical gene delivery system which harnesses electrical impulses to temporarily permeabilize cell membranes. This allows the cells to

take in large molecules, which usually would not penetrate cell membranes. Electroporation is conducted using an electroporator. Electroporation is a procedurally simple and relatively time-efficient method of transformation [23], however there is not literature on its successful application in *Lolium perenne*. The sonication method functions similarly to electroporation in principle, however the permeabilization of membranes is achieved by ultrasound rather than electric pulses [23]. The sonication method nonetheless has a relatively low transformation efficiency [63]. Microinjection is a method which includes the direct mechanical injection of DNA with a thin needle under a microscope. As foreign DNA is physically introduced to cells, no further treatment is required to permeabilize the membranes. However, this is a very slow and expensive method which requires highly skilled staff to perform [23]. Lipofection is like microinjection in the sense that foreign DNA is physically introduced to the cell. Instead of a needle, exogenous DNA is packed into artificial vesicles of lipids which fuse with plant cell membranes, thereby delivering the DNA into the cells [23].

Chemical methods of vector-less gene delivery in plants include systems such as calcium phosphate co-precipitation, polycation dimethyl sulfoxide (DMSO) technique, diethyl amino ethyl (DEAE) dextran procedure and the polyethylene glycol (PEG) mediated transformation [23]. In the calcium phosphate co-precipitation method, DNA is mixed with a solution of calcium chloride and an isotonic phosphate buffer, thus precipitating the formed DNA-$CaPO_4$ complex. The precipitate is then left to react with dividing cells [23], which internalizes the exogenous DNA to the cells [66]. After this, the cells are washed and incubated in a new culture medium. The success rate of this technique is largely dependent on a high exogenous DNA concentration [23]. The polycation DMSO technique functions by using polybrene to adsorb DNA to the cell surface. The cells are then treated with DMSO to increase membrane permeability and facilitate DNA uptake [23], [67]. High transformation efficiency with this method does not require large quantities of plasmid DNA [23]. In the DEAE dextran procedure, DNA forms a complex with the DEAE dextran polymer [23]. The exact mechanism of entry to the cell is not entirely understood, but it has been theorized that the positively charged DEAE polymer binds to both negatively charged DNA and the cell surface [68], [69]. After DEAE-DNA complexes have entered the cell via endocytosis [70], [71], the DNA is transported into the cell through an unknown mechanism from increasingly acidic endosomes [72]. The PEG method in plants includes using PEG and either calcium or magnesium ions to carry DNA into the recipient cell [23]. The underlying molecular mechanisms of PEG mediated transformation are not fully understood either. It has been theorized that PEGs enter cells by either passive diffusion and/or caveolae-mediated endocytosis, with low molecular weight PEGs entering via passive diffusion and high molecular weight PEGs through a combination of both passive diffusion and caveolae-mediated endocytosis [73]. It has also been noted that higher levels of expression and genomic integration can be achieved using linearized DNA plasmids in contrast to supercoiled forms of DNA. Genomic integration has also been recorded to occur at random sites. The PEG method allows for transformation of DNA without using a biological vector for mediation. It is however a protoplast-based method and thus requires a functional protoplast system for successful integration in plant studies [23].

1.5. Genome editing technologies

Genome editing tools enable site specific cleavage and rejoining of DNA. This is accomplished using specialized enzymes such as restriction endonucleases and ligases. Genome editing is relatively

simple in small genomes such as those of viruses and bacteria but becomes much more complicated when applied to higher organisms such as plants. Target sites for restriction endonucleases are relatively short sequences of DNA, making their use difficult in complex genomes containing many repeated sequences [74].

Original efforts for editing complex genomes were focused on developing artificial enzymes, which could selectively bind to specific nucleotide sequences and cleave them [75]. Complex proteins composing of one or two structural units were designed, one of which would cleave DNA, and the other would have the capacity to direct cleavage via site-specific binding [76], [77]. These chimeric nucleases would be synthesized *in vivo* if appropriate vectors encoding said enzymes were previously transformed. Vectors coding chimeric nucleases were also supplied with a nuclear localization signal to facilitate access to the nucleus of the cell [74].

Genome editing technologies can be used for a variety of different genomic modifications such as creating point mutations, directed insertions of new genes or deletion of nucleotides and nucleotide sequences. Moreover, these technologies can be utilized for substituting individual genetic elements or even fragments of DNA [78]–[84].

1.5.1. ZFN

Zinc-finger nucleases (ZFN) are the first generation of genome editing tools. The discovery of the Cys2-His2 zinc finger (ZF) domain and its principles of operation led to the development of first chimeric nucleases [76], [78], [85], [86]. Every Cys2-His2 ZF domain is made up of 30 amino acid residues, which are folded into ββα configuration [86]–[88]. Cys2-His2 ZF proteins insert an α-helix into the major groove of DNA double helix thereby binding to DNA [89]. A ZF protein can recognize 3 tandem nucleotides of DNA. Generally, ZFN monomers are made up of two functional domains. The first of these is the artificial ZF Cys2-His2 domain located in the N-terminus. The second domain is the non-specific FokI endonuclease domain located in the C-terminus. ZFN activity is dependent on the dimerization of the FokI domain [85]. The 3 tandem nucleotide recognition capacity of individual ZF domains is interchangeable, and changing the order of these domains allows for engineering ZFNs to adhere to unique binding sites in DNA [74].

The interchangeability of ZFs allows for modular design and assembly of ZFNs, thereby enabling the linking of multiple ZFs to target longer sequences. Ever since they were first discovered, many ZF domains have been generated which can recognize a variety of different nucleotide triplets. The varying recognition capacity of generated ZF domains and the changeability of their order allows for precise targeting in DNA [74].

1.5.2. TALEN

Research towards efficient and selective means of manipulating DNA in plants led to the discovery of transcription activator-like effector (TALE) proteins in 2007 [90]. In the following year two separate groups also described the recognition sequence of TALE proteins [91]. TALE proteins can recognize and activate certain plant promoters via specific tandem repeats in DNA. Based on the functionality of TALE proteins, a new genome editing system was developed which consists of chimeric nucleases called transcription activator-like effector nucleases (TALENs) [77].

TALE proteins are made up of a central domain which binds to DNA, a nuclear localization signal and a domain responsible for transcription activation of a targeted gene [92]. The central DNA-binding domain further includes the central repeat domain (CRD), which directs host specificity and DNA binding. The CRD is made up of tandem repeats containing 34 amino acid residues, each repeat of which binds to a single nucleotide in the targeted sequence. Amino acid residues located at positions 12 and 13 of each repeat are highly variable and are called the repeat variable diresidue (RVD). RVDs confer site specific recognition ability. The final tandem repeat of CRD binds to a nucleotide at the 3' end of the recognition site and is made up of only 20 amino acid residues. While generally, TALE proteins can be engineered to bind any DNA sequences, it should be noted that the last nucleotide in the 5' end of a sequence targeted by TALE should always be a thymidine. This is required for the optimal functionality of TALE transcription factors, TALE recombinases and TALENs. [93]

Following the deciphering of the TALE code recognition, scientific effort was channelled into developing chimeric TALENs [94]. This was achieved by inserting the sequence which encodes the DNA-binding domain of TALE proteins into a plasmid vector which was previously used for ZFNs [95]. The resulting chimeric sequence-specific nuclease construct contained the DNA-binding domain of TALEs alongside the catalytic domain of FokI. This construct was then used to design synthetic nucleases with varying RVDs enabling targeting almost any DNA sequence [78], [96].

1.5.3. CRISPR/Cas9

The third genome editing technique, which has recently garnered much attention, is the clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein (Cas) system. Although there are multiple variants of this system, CRISPR/Cas9 is the most used. The work principle of this method derives from a form of adaptive immunity commonly found in bacteria and archaea, functioning due to the presence of certain genomic sites called CRISPR loci. These loci are made up of a Cas9 coding region alongside an array of repeated sequences called repeat spacer sequences. The repeat spacer sequences are short DNA sequences of exogenous origin which have integrated into the genome of the host due to recombination [97], [98]. The CRISPR/Cas9 system differs from the ZFN and TALEN systems in multiple ways, however one of the most important ones is the method of genomic targeting. Site recognition for CRISPR/Cas9 systems is dependent on complementary sequence-based interaction of the guide RNA (gRNA) and targeted DNA. When complexed, gRNA and Cas protein direct nuclease activity of the Cas9 endonuclease, thereby cleaving DNA [99]–[101].

The CRISPR/Cas9 system provides a relatively cheap and methodologically simple tool [102] for creating genetically modified cells *in vitro* and *in vivo* [103]. Plasmids or viral vectors are used for *in vitro* applications as they confer a high and stable synthesis of CRISPR/Cas9 system constituents. Plant based *in vivo* applications of CRISPR/Cas9 are either performed in protoplasts using plasmids for mediation of CRISPR/Cas9 constituent coding sequences [104] or make use of *Agrobacterium* for gene delivery [79], [97].

2. Aims of the study

The goal of this thesis was to develop a time-efficient method for testing the efficacy of gRNAs for CRISPR/Cas9 applications in *Lolium perenne*.

Subgoals of the study:

- To establish a high-yield protocol for *Lolium perenne* mesophyll protoplast isolation.
- To establish a high-efficiency PEG-mediated transformation protocol for *Lolium perenne* protoplasts.
- To validate the efficiency of gRNAs for editing the genome of *Lolium perenne* in protoplasts.

3. Materials and methods

3.1. Plants and growth conditions

Seedlings of *Lolium perenne*, cultivar Veja, were grown in growth chambers at 21 °C, 60% relative humidity and 26 μmol m^{-2} s^{-1} of light intensity. Protoplasts were isolated from mesophyll cells of three-week-old seedling leaves.

3.1.1. Growth media

Seedlings were grown in liquid half strength Murashige & Skoog media with 1% (w/v) sucrose. The pH of the media was adjusted to 5.7 with KOH before autoclaving at 120 °C for 15 min.

3.1.2. Seed sterilization and plating

Seeds sterilization was performed under a flow hood. All seed handling was performed using tweezers heat sterilized at 200 °C for 3 min with a glass bead sterilizer (Duchefa Biochemie). Firstly, approximately 100 seeds were added into 15 mL tubes. Then 10 mL of 70% ethanol was added into each tube of seeds sterilized and the tubes were left to soak for 1 minute. After soaking, the ethanol was removed as much as possible. 10 mL of commercial bleach (ACE; containing 5% of sodium hypochlorite) containing 0.1% Tween-20 was added to each of the tubes, which were then further sealed with parafilm and put on a tube roller (Starlab) for 1 h at 30 rpm. Following sterilization, the bleach solution was removed, and the seeds were washed three times with autoclaved water, soaking for 1 minute each time. After the last wash, the seeds were first passed onto autoclaved tissue paper in Petri dishes for preliminary drying of approximately 5-10 min. Following preliminary drying the seeds were passed onto Petri dishes containing autoclaved filter paper for final drying of approximately 15-30 min. After the final drying, the seeds were passed into 6-well plates, where each well contained 2 mL of growth media. Five sterilized seeds were placed into each well. The plates were then sealed with two layers of microfiber tape. Unused seeds were passed into sterile 1.5 mL tubes for later use.

3.2. Molecular cloning

3.2.1. Plasmids

The plasmid pHSE401/EGFP is an 18678 base pair long plant binary vector, that can be used for CRISPR/Cas9-based gene editing in plants. This vector contains 2 antibiotic marker genes allowing for selection in both bacteria and plants and the reporter gene *EGFP* under CaMV 35S promoter allowing for *in vivo* detection in plants [105]. The vector map can be found in Appendix 1.

Three vectors (pHSE401-CBP20_196; pHSE401-CBP20_220; pHSE401-CBP20_229) were created targeting regions near the start codon of the gene *CBP20*, which acts as a repressor of cuticular waxes biosynthesis and transportation in some plants including grasses such as barley [106]. Each of the three vectors had a different gRNA, which was designed using the web tool CRISPOR [107],

considering the latest genome assembly of *Lolium perenne* [12] as a query for specificity and calculation of off-targets.

For simplicity, plasmids pHSE401/EGFP, pHSE401-CBP20_196, pHSE401-CBP20_220 and pHSE401-CBP20_229 will be called from here on PEGFP, P196, P220 and P229, respectively. The gRNA-containing vectors were generated using the Golden Gate cloning method. This technique relies in the use of a type II restriction enzyme that excises a fragment of the plasmid generating over-hangs and a ligase that attaches the gRNAs into the plasmid thanks to the over-hangs left by the restrictase. Before performing the Golden Gate assembly, the sense and antisense oligos of the different gRNAs were annealed (The sequence of the oligos can be found in Appendix 2). For this, 1 µL of each oligo (100 mM) were mixed with 2 µL of T4 ligase buffer (10x) and nuclease free water up to 20 µL in a 1.5 mL tube, incubated in a heat-block at 85 °C for 10 min and cooled down to room temperature for 1h. For the Golden Gate reaction, T4 DNA ligase (1 Unit) and EcoR31I (Bsa) were used together with the annealed oligos and the empty pHSE401/EGFP plasmid (100 ng). A table with the Golden Gate reaction mix and program can be found in Appendix 3 and 4 respectively.

3.2.2. Heat-shock transformation

Chemically competent cells of *E. coli* DH5α strain were used for molecular cloning and transformed following the heat-shock method [108]. Frozen *E. coli* suspension was thawed on ice for 30 min. 10 ng of plasmid DNA was added to the tube containing *E. coli* and the tube was incubated on ice for further 30 min. Then a heat shock treatment was performed on a thermal shaker pre-heated to 42 °C for 45 s. The tube was then incubated on ice for 3 min, after which 500 µL of LB medium was added. The tube was then incubated at 37 °C for 1 h at 180 rpm in an orbital shaker-incubator (CERTOMAT® BS-1, Sartorius). Following the final incubation either 50 µL or 100 µL of transformed bacteria suspension were plated on Lysogeny Agar (LA) containing 50 µg/mL of kanamycin (Kan) and grown overnight at 37 °C.

3.2.3. Colony PCR

To confirm whether the transformed bacteria contained the desired vectors, colony PCR was performed. Through this method, colonies that contain the inserted gRNAs into the vectors can be identified. The empty plasmid was used as a negative control to compare the bands from the analysed colonies against it. The empty vector was expected to show a band of approximately 1625 bp, while the colonies with the correct inserts should present shorter bands (~420 bp) when visualizing the PCR products on a gel (the primers used were U6-26p-FW and U6-26p-RV, the sequences can be found in Appendix 2). Additionally, the antisense gRNA oligos were used together with the PCR forward primer, to generate a PCR product only visible if the gRNAs were indeed inserted into the plasmid Single colonies of transformed *E. coli* were selected from LA plates, touched with sterile pipette tips, and immersed in 20 µL tubes containing 5 µL of sterile nuclease free water. 3 µL of nuclease free water, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM) and 10 µL of DreamTaq PCR Master Mix (2x) was added to each tube tested, and PCR was performed on a thermal cycler. The PCR composition and program can be found at Appendix 5 and 6 respectively.

Following the end of the PCR program, the resulting products were loaded into an 2% agarose gel (stained with GelRed® (Biotium) before casting) for gel electrophoresis. GeneRuler™ 1kb (Thermo Scientific) was used as the DNA ladder. Gel electrophoresis was performed at 80 V for 1 h. Visualization of the gel post-electrophoresis was performed using a transilluminator. The colonies that showed the expected bands (~420 bp) were miniprepped to isolate plasmid DNA. The extracted DNA was externally Sanger sequenced with the same primers used for the Colony PCR (U6-26p-FW and U6-26p-RV) to check the correct insertion of the gRNAs into the plasmids.

3.2.4. Plasmid DNA miniprep by alkaline lysis

A modified version of the miniprep protocol described in the "Molecular Cloning: A Laboratory Manual" book was used [109]. For this, 3 mL Lysogeny Broth (LB) containing 50 µg/mL of Kan was inoculated with transformed bacteria and grown overnight in a shaking incubator at 180 rpm and 37 °C. The culture was cooled on ice for 5 min. Following this, 1.5 mL of bacterial culture was poured into a 2 mL tube, centrifuged for 2 min at 4500 rcf at 4 °C. The supernatant was discarded, and the pelleted bacteria was resuspended in 300 µL of previously chilled E1 and mixed on a vortex shaker. 300 µL of E2 was then added and the tube contents were mixed by gentle inverting and the tube was incubated on ice for 3 min. 310 µL of E3 was then added and the contents were once more mixed by gentle inverting and incubated on ice for 5 min, after which it was centrifuged for 10 min at 16100 rcf at 4 °C. 910 µL of the supernatant was passed into a new 1.5 mL tube and 635 µL of isopropanol was added. The tube was mixed on a vortex and incubated at room temperature for 5 min. Following incubation, the tube was centrifuged for 10 min at 16100 rcf at room temperature. Supernatant was removed and 900 µL of pre-chilled 70% ethanol was added. The tube was centrifuged again for 5 min at 16100 rcf at room temperature. The supernatant was then carefully removed, and the pellet was dried. When dry, the pellet was resuspended in 50 µL of sterile Milli-Q water and the DNA concentration was measured with a NanoDrop™ (Thermo Scientific™ ND-2000C) spectrophotometer.

3.2.5. Plasmid DNA midiprep

Transformed bacteria, suspended in 3 mL of LB containing 50 µg/mL of Kan was grown overnight in a shaking incubator at 180 rpm and 37 °C. The following day 2 mL of the liquid culture was transferred into a conical flask containing 18 mL of LB containing 50 µg/mL of Kan which was again grown overnight at 180 rpm and 37 °C. The resulting culture was passed into a 50 mL tube, which was then centrifuged at 4500 rcf for 15 min at 4 °C. DNA was then extracted with a PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen) following the manufacturer's instructions. DNA concentration was measured as previously described.

3.3. Solutions used for protoplast isolation and transformation

The solutions described below were either filter sterilized using a 22 µm syringe filter (Minisart® High Flow) or autoclaved (120 °C for 15 min) depending on if they were used immediately or stored for future use respectively.

3.3.1. Enzymatic solution

The enzymatic solution was prepared by adding 1.5%, 2%, 2.5% or 3% (w/v) of Cellulase Onozuka R-10 (Duchefa Biochemie), depending on which was used for the specific experiment, 0.75% (w/v) of Macerozyme R-10 (Duchefa Biochemie) and 0.1% (w/v) of Pectolyase Y-23 (Duchefa Biochemie) into distilled water. Then 2-(N-morpholino)ethanesulfonic acid (MES), previously adjusted to pH 5.7 with KOH (MES, pH 5.7), was added to a final molar concentration of 10 mM along with mannitol (0.6 M). The solution was then heat treated in a water bath at 55 °C for 10 min. After heat treatment, KCl and $CaCl_2$ were added to final molar concentrations of 20 mM and 10 mM respectively along with 0.1% (w/v) of BSA. The solution was cooled down to room temperature and the pH was adjusted to 5.7 with KOH. Finally, the solution was filter sterilized. All enzymatic solutions were prepared right before enzymatic treatment for each experiment.

3.3.2. Solutions used for protoplasts purification and transformation

A comparative table containing the exact composition of each of the solutions mentioned below can be found in Appendix 7.

W5 solution was adapted from previous studies [110], [111] and used as a washing solution after enzymatic treatment and protoplast PEG-mediated transformation. The pH of the solution was adjusted to 5.7 with NaOH, sterilized and stored at 5 °C if not immediately used.

W5A solution was adapted from a previous study [111] and used to resuspend pelleted protoplasts after washing them with W5. This solution contains glucose (5 mM) which allows it to be used when creating a sucrose cushion to separate alive protoplasts from cellular debris and dead cells. The pH of the solution was adjusted to 5.7 with NaOH, sterilized and stored at 5 °C if not immediately used.

WI solution was adapted from a previous study [111] It was used to resuspend the interphase of alive protoplasts obtained after performing the sucrose cushion purification and to wash transformed protoplasts with PEG solution. This solution contains mannitol (500 mM) and KCl (5 mM) which and appropiate environment for protoplasts survivability. The pH of the solution was adjusted to 5.7 with NaOH, sterilized and stored at 5 °C if not immediately used.

MMg solution was adapted from a previous study [111] and used to perform the PEG-mediated transformation of protoplasts. This solution contains $MgCl_2$ (15 mM) that has been shown to improve the transformation efficiency of protoplasts when using PEG. The pH of the solution was adjusted to 5.7 with NaOH, sterilized and stored at 5 °C if not immediately used.

21% sucrose solution was adapted from a previous study [111] and used to perform the sucrose cushion purification. It contains 21% (w/v) sucrose and allows the separation of alive protoplasts from cellular debris and dead cells by density gradient. The solution was sterilized and stored at 5 °C if not immediately used.

The PEG solution used for transformation was adapted from a previous study [111] and prepared by dissolving 0.4 g/mL PEG 4000 alongside mannitol (0.2 M) and $CaCl_2$ (0.1 M) in sterile Milli-Q water. To help dissolve the PEG, the solution was heated at 50 °C for 10 min or until fully dissolved.

The PEG solution was then cooled down to room temperature. This solution was prepared immediately before use.

3.4. Protoplast isolation experiments

The development of the protoplast isolation procedure was carried out in two series of experiments (Primary and secondary experiment series from here on in). The method used for protoplast isolation is a modified variant of a method described in a previous study [111]. In the primary experiment series, viable cell counts were attained directly from the enzyme solution at a single or multiple timepoints, depending on which variables were tested, using a haemocytometer under a microscope and TB for staining. Multiple variables (cellulase concentration of the enzyme solution and treatment time) were tested in the first set of the primary experiment series, while in the rest of the experiments of the primary series, only a single variable was tested (Table 1.). In the secondary experiment series, viable cell counts were attained following the purification of protoplasts from the enzymatic solution, counted using a haemocytometer under a fluorescence microscope, using FDA for staining. In both cases, the number of stained and non-stained protoplasts were used to perform viability assays estimations in which the percentage of alive protoplasts (stained) was calculated.

Multiple variable tests					
a.	Cellulase concentrations	1.5%	2%	2.5%	3%
	Enzymatic treatment time	8 h	12 h	16 h	20 h

Individual variable tests					
b.	Enzymatic treatment time	6 h	8 h	10 h	12 h
c.	Mannitol pretreatment concentrations	0.2 M	0.3 M	0.5 M	0.6 M
d.	Vacuum pressures	0 kPa	71 kPa		

Table 1. Primary experiment series. a) Protoplast counts were measured from enzyme solutions containing 1.5%, 2%, 2.5% and 3% cellulase at 8 h, 12 h, 16 h and 20 h timepoints. b) Protoplast counts were measured from an enzyme solution at 6 h, 8 h, 10 h and 12 h timepoints. c) Protoplast counts were measured from enzyme solutions, which had been subjected to a plasmolytic pretreatment at 0.2 M, 0.3 M, 0.5 M and 0.6 M mannitol concentrations. d) Protoplast counts were measured from two enzyme solutions, one of which had previously been vacuum infiltrated at 71 kPa.

3.4.1. Primary experiment series of protoplast isolation

Leaves of three-week-old seedlings were separated from the roots and weighed. Plasmolytic pretreatment was then performed by submerging the separated leaves in a mannitol solution (0.5 M if not otherwise stated in Table 1.) and put on a rotatory shaker (Heidolph Unimax 2010) for 1 h at 75 rpm at room temperature. Following plasmolytic pretreatment, the leaves were cut into approximately 1-2 mm pieces in a petri dish containing 3 mL of enzymatic solution, with cutting being performed immersed in enzymatic solution (containing 2% cellulase if not otherwise stated in Table 1.). After the leaves were cut into pieces, additional enzymatic solution was added to a leaf mass to enzymatic solution ratio of 1:10 (g/mL) with a minimum of 2 g of leaf material to 20 mL of enzymatic solution per petri dish. From this point on, the petri dishes were kept in the dark until the end of enzymatic treatment. Vacuum infiltration was then performed on the plates in a vacuum chamber (using vacuum pressure of 71 kPa if not otherwise stated in Table 1.) in three consecutive

5 min treatments. The petri dishes were then incubated at 30 °C for 1 h, after which they were put on a rotatory shaker (for 8h if not otherwise stated in Table 1.) at 75 rpm at room temperature. Following the enzymatic treatment, the solutions were gently mixed with a pipette tip to homogenise them, and aliquots were passed into separate tubes for staining. In experiments where multiple timepoints were tested, enzyme solutions were put back on the shaker, kept in the dark for further aliquoting at later times.

Staining was performed on the aliquoted solutions using 0.4% TB for 5 min and protoplasts were counted in a haemocytometer (Neubauer Pattern, BLAUBRAND) under a microscope. TB was prepared from 60% powder (Thermo Fisher). Protoplasts were considered viable if they retained an undisrupted round shape, the innards of which remained unstained by TB.

3.4.2. Secondary experiment series of protoplast isolation

For these experiments, a similar method to the one described at the beginning of Chapter 3.4.1 was followed. Only one parameter was used for the mannitol pretreatment, the enzymatic cell wall degradation, and the vacuum infiltration steps. The plasmolytic pretreatment of the tissue was done using 0.5 M mannitol, the enzymatic treatment was performed using an enzyme solution with 2% of cellulase for 8 h and the vacuum infiltration was done under 71 kPa. The incubation in enzyme solution was done similarly as to previously described, except for the agitation used during the 8 h of treatment. In these set of experiments two different speeds were used, 75 and 35 rpm. The lower speed was adopted to improve the number of alive protoplasts obtained after the purification step.

Purification was then performed by filtering the solutions through a 100 µm cell strainers, pre-wetted with W5 solution, using cut pipette tips into 50 mL tubes. Following filtering of the enzyme solution, 2 mL of W5 solution was added to the cell strainer to wash any remaining protoplasts from it. Later, 2 mL of W5 was added to the petri dishes which used to contain the enzymatic solutions, gently shaken to dislodge any remaining protoplasts, and passed through cell strainers into 50 mL tubes. The tubes were then centrifuged at 100 rcf at 11 °C for 5 min, with both the acceleration and deceleration set to minimum (Eppendorf® Centrifuge 5804 R). The supernatant was then discarded, and the pelleted plant material was resuspended in 2 mL of W5A solution. Separate 15 mL tubes containing 4 mL of 21% sucrose solution were then prepared for the sucrose cushion. The plant material suspended in 2 mL of W5A was then carefully layered on top of the 4 mL of 21% sucrose solution, making sure to keep the layers as separated as possible by pipetting slowly and continuously through the side of the tube. The 15 mL tubes were then centrifuged at 100 rcf at 11 °C for 10 min, with both the acceleration and deceleration set to minimum. This resulted in green layers forming at the interfaces between the W5A and the 21% sucrose solutions. The protoplasts layer was then collected and passed into separate 10 mL round-bottom tubes. The amount of liquid transferred was usually 2-3 mL to which WI solution was added at a 1:1 ratio. Aliquots were then taken for counting (method described in the following paragraph). The rest of the solutions were incubated at 4 °C overnight which resulted in the protoplasts settling at the bottom of the round-bottom tubes. The supernatant was removed, and the protoplasts were resuspended in half the previous volume of WI solution. Further aliquots were taken for counting and the rest of the solutions were used for transformation.

Aliquots taken during the secondary experiment series were gently resuspended by turning and swirling the containing tubes. The aliquots were then diluted with WI in 1:1 ratio and stained using 1.25 µL of FDA stock solution (5 mg/mL) per 50 µL of solution. The protoplasts were counted under a fluorescence microscope with the help of a haemocytometer. Protoplasts were considered viable when they fluoresced green under UV light.

3.5. PEG-mediated transformation of protoplasts

Transformation was performed with protoplasts, which were suspended in either WI or MMg solution. When MMg solution was used, the suspension containing isolated protoplasts in WI was first passed into a 2 mL tube and then centrifuged at 100 rcf at room temperature for 5 min. The supernatant was removed and replaced with the same volume of MMg solution. The next steps were the same for both WI and MMg protoplasts suspensions, except for the post PEG-transformation washing. A volume of plasmid (corresponding to 10 µg of PEGFP or 20 µg of P196, P220 and P229) was added to a 2 mL tube, followed by a volume of protoplast solution (suspended in either WI or MMg) corresponding to the necessary cellular concentration ($3x10^5$ protoplasts per mL of suspension). Plasmid DNA and protoplasts were then mixed by gently flicking the tube. After mixing, PEG solution was added to a volume ratio of 1:1. The resulting solution was further mixed by gently inverting the tube and then incubated in the dark for 15-20 min, leaving the tubes horizontally. Following incubation, a wash was performed either WI (for WI suspension) or W5 (for MMg suspension) was added to the tube depending on the experiment in 1:1 volume ratio, and the tube was then mixed again by gently inverting. After mixing, the tube was centrifuged at 100 rcf at room temperature for 5 min, with the acceleration and deceleration set to minimum, the supernatant was discarded, and the resulting pellet was resuspended in 1 mL of WI. The suspension was then passed into a 24-well plate, the wells of which were precoated with 5% BSA. The plate was then incubated for 24 h and 48 h in dark. At each timepoint, the solution was resuspended by gentle shaking and an aliquot was taken for counting. The aliquoted solution was then used for cell counting in a haemocytometer under a fluorescence microscope. Both fluorescent protoplasts and non-fluorescent protoplasts were counted to estimate how efficient the transformation experiments were. The efficiency of the transformation was calculated as the percentage of fluorescent protoplast detected. Non-fluorescent objects were considered protoplasts when they retained an undisrupted round shape.

3.5.1. Genomic DNA extraction and purification from transformed protoplasts

Genomic DNA extraction and purification was performed following a modified variant of a protocol from a previous study [112]. 1 mL of solution containing transformed protoplasts was centrifuged at 200 rcf for 10 min, followed by discarding of the supernatant. The protoplasts were then resuspended in 500 µL of extraction buffer (100 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 10 mM β-mercaptoethanol), which was transferred to a 2 mL tube. 2 glass beads with an approximately 5 mm diameter were added, and the tube was put into a TissueLyser homogenisator (QIAGEN) and run for 1 minute at 30 Hz. 35 µL of 20% SDS was then added, and the tube was mixed on a vortex shaker. Following this, the tube was incubated at 65 °C for 10 min. 130 µL of 3 M potassium acetate (pH 5.5) was then added to the tube and the contents were mixed on a vortex shaker again. Following this, the tube was incubated on ice for 5 min, centrifuged at room temperature at 16100 rcf for 10 min. The supernatant was then transferred to a clean tube, 60 µL

of sodium acetate (pH 5.2) and 640 µL of isopropanol was added and the tube was mixed by gently inverting for 5 times. The tube was then further incubated at -20 °C for 15 min and centrifuged at 16100 rcf for 10 min at 4 °C. The supernatant was discarded, 100 µL of 70% ethanol was added and the tube was further centrifuged at 16100 rcf for 5 min at room temperature. The supernatant was then carefully removed, and the tube was dried until there was no liquid remaining. The pellet was then redissolved in 30 µL of TE buffer (1M Tris, pH 8.0; 0.5 M EDTA, pH 8.0), mixed on a vortex shaker and 2 µL of RNase was added. The tube was then incubated at room temperature for 5 min after which it was incubated at 65 °C for 10 min. DNA concentration was then measured as previously described (Chapter 3.2.5.).

3.6. DNA sequencing and gRNA efficiency analysis

Samples from the different transformation experiments were externally processed using Sanger sequencing. For each transformation, four samples were sequenced. One sample from non-transformed protoplasts (WT) was sequenced together with samples from protoplasts transformed with the vectors containing gRNAs targeting the *CBP20* gene (P196, P220 and P229). For each sample, the same forward and reverse primers were used (gRNA_Sang_FW and gRNA_Sang_RV) (the primer sequences can be found at Appendix 1) both in a PCR reaction and for Sanger sequencing. These primers bind to a region ~150 bp upstream/downstream from the recognition site of the gRNAs.

The PCR reaction was done in 50 µL volumes, using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific). This polymerase has a lower error rate than other polymerases like *Taq,* which makes it suitable for detecting indels generated by the CRISPR/Cas9 system and not from the polymerase activity itself. For each reaction, 50 ng of DNA were used as template. The PCR composition and program can be found in Appendix 8 and 9 respectively.

For Sanger sequencing, 80 ng were prepared per sample and sent for external processing. Once the sequencing was done, the chromatogram data of each sample was quality checked using Benchling (Biology Software, 2023). Furtherly, the gRNA efficiency was calculated using a webtool called TIDE [113], which allows to identify genome edited sequences from a sample that contains a population of both WT and edited sequences. This tool is very useful when analysing edited protoplasts since the DNA that is isolated belongs to a population of both WT and transformed cells. Without this tool, a simple multiple alignment of the sequences is not capable of discerning the edited sequences present in the analysed data and will consider that the samples belonging to the transformed group are identical to those from the WT group. TIDE provides data representing the percentage of edited and non-edited sequences present in the analysed population, and how significant these values are by providing P Values for each percentage of edited and non-edited samples. It also provides an R^2 value that shows how well the generated data fits to the performed analytical model. Samples with R^2 values lower than 0.8 were excluded from further analysis.

3.7. Statistical analyses

Statistical analysis was performed using Graphpad Prism 10.1.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Two-way ANOVA using Geisser-Greenhouse

correction and Tukey's multiple comparison test was performed to calculate statistical significance of variability between viable protoplasts counts at different timepoints using varying cellulase concentrations. Two tailed unpaired t test was used for calculating statistical significance of variability between viable protoplast counts in vacuum pressure experiments. Repeated measures one-way ANOVA using Geisser-Greenhouse correction was performed on the rest of the data sets.P values were calculated for t tests and adjusted P values were calculated for all ANOVA tests.

3.8. Imaging and image editing

Protoplast imaging was performed with Zeiss AxioVert 200M, and the images were edited and processed with ImageJ [114] and Adobe Photoshop 24.1.0. SnapGene®Viewer (Dotmatics) was used to generate the plasmid map.

4. Results

4.1. Protoplast isolation experiments

4.1.1. Enzymatic treatment duration and cellulase concentration

Multiple statistically significant differences in viable protoplast densities were found in the different variable experiments of the primary experiment series. At 8 h, 12 h and 16 h timepoints, the enzymatic solution containing 2% cellulase yielded significantly more protoplasts than all the other solutions tested (P Values ≤ 0.01 in all comparisons except in 2% vs 1.5% and 2% vs 2.5% for the 16 h timepoint which had P Values ≤ 0.05) (Figure 1. a and b). At the 20 h mark, 2% cellulase solution was recorded to have a higher yield of statistical significance only over the 3% cellulase solution (P Value = 0.0193) (Figure 1. b). Moreover, statistically significantly higher cell densities were also recorded between 8 h timepoints and all the other timepoints recorded for each tested enzymatic solution except the 3% cellulase solution, for which statistically higher cell densities were recorded between 8 h timepoint and 16 h timepoint as well as between 8 h timepoint and 20 h timepoint (P Values ≤ 0.01 in both comparisons) (Figure 1. c-f). In summary, the 8 h treatment using an enzymatic solution with 2% cellulase gave the best results in terms of counted viable protoplasts. Therefore, it was decided to use said conditions for the next protoplast isolation experiments.

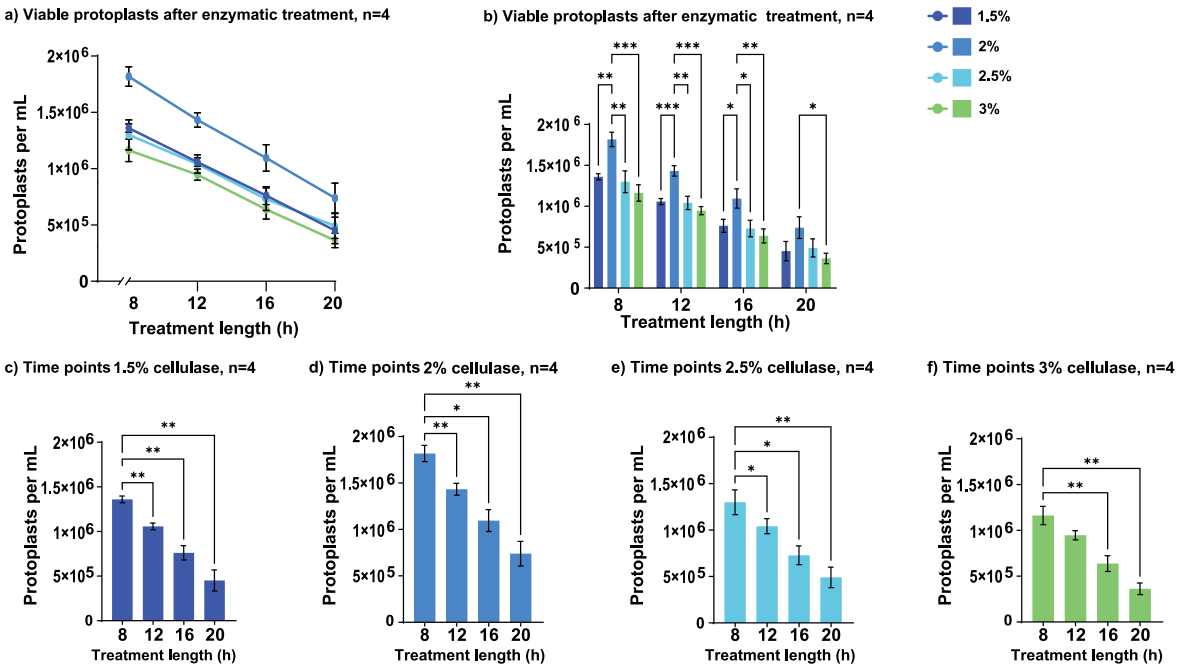


Figure 1. Enzymatic treatment duration and cellulase concentration experiments. The color-coded legends correspond to each tested cellulase concentration (w/v) in the enzyme solution. Viable protoplast counts are depicted for each of the timepoint measured. Error bars depict standard deviation, "n" equals to the number of repeated experiments and asterisks depict differences of statistical significance (* meaning P ≤ 0.05, ** meaning P ≤ 0.01 and *** meaning P ≤ 0.001). a-b) Line graph of all cellulase concentrations tested. b) Column diagram of all cellulase concentrations tested. c-f) Bar graphs corresponding to each individual cellulase concentration tested.

4.1.2. Enzymatic treatment duration

To decide if the length of the enzymatic treatment could be shortened, four experiments using 6-, 8-, 10- and 12-hour incubations were done. The results from these individual variable tests using the same cellulase concentration (2%) can be seen in Figure 2.a. Even though the 8 h treatment had a higher number of cells than the rest of timepoints, a statistically significant difference in viable protoplast count was only noted between the 8 h and 12 h marks (P Value= 0.0360) (Figure 2. a). Therefore, it was concluded to use an 8 h incubation length for the rest of the thesis.

4.1.3. Mannitol pretreatment

In the mannitol pretreatment experiments, statistically significant higher counts of viable protoplasts were recorded between the 0.5 M mannitol treatment and all the other tested concentrations of mannitol in plasmolytic pretreatment solutions (P Values ≤ 0.05) (Figure 2. b). These experiments allowed to establish the 0.5 M mannitol condition as the preplasmolysis treatment used in the subsequent tests.

4.1.4. Vacuum infiltration

During the vacuum infiltration experiments, a statistically significantly higher count of viable protoplasts was recorded from the samples treated at 71 kPa when compared to those that were not subjected to vacuum (P Value ≤ 0.0001) (Figure 2. c). In summary, vacuum use positively impacted the number of cells counted after the enzymatic treatment. This led to the decision to use 71 kPa in all the following experiments.

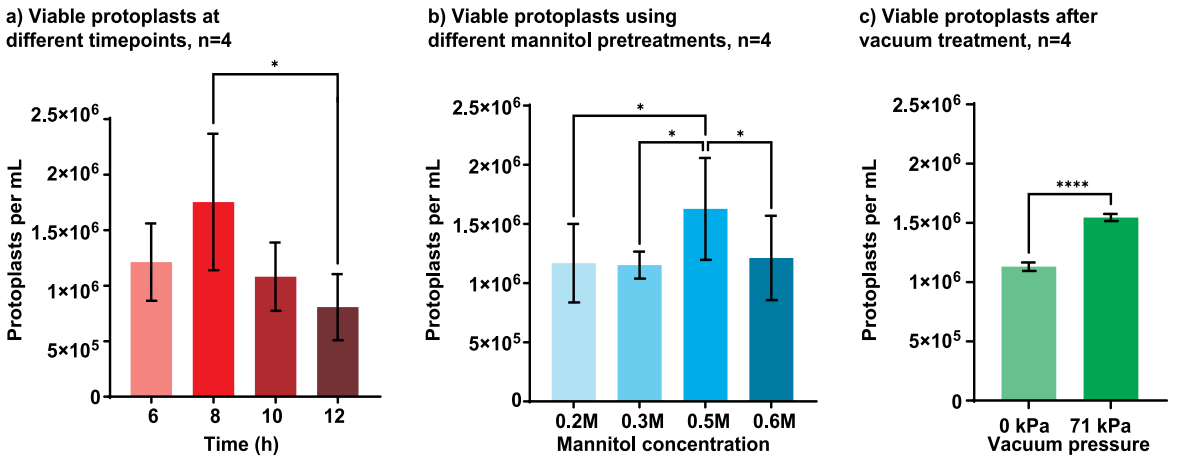


Figure 2. Single variable experiments of the primary experiment series. The error bars depict standard deviation, "n" equals to the number of repeated experiments and asterisks depict differences of statistical significance (* meaning P ≤ 0.05 and **** P ≤ 0.0001). Viable protoplast counts are depicted for varying conditions affecting protoplast yield. a) Column diagram depicting the effect of treatment duration. b) Column diagram depicting the effect of mannitol concentrations used for plasmolytic pretreatment. c) Column diagram depicting the effect of vacuum treatment.

4.2. Protoplast transformation experiments

The presence of EGFP was confirmed by fluorescence microscopy for each of the plasmids used for transformation, meaning plasmids were efficiently transformed using PEG (Figure 3.). EGFP was localized, as expected, all over the cytoplasm (first row) or mostly in one specific part of the cell, probably the nucleus (second row).

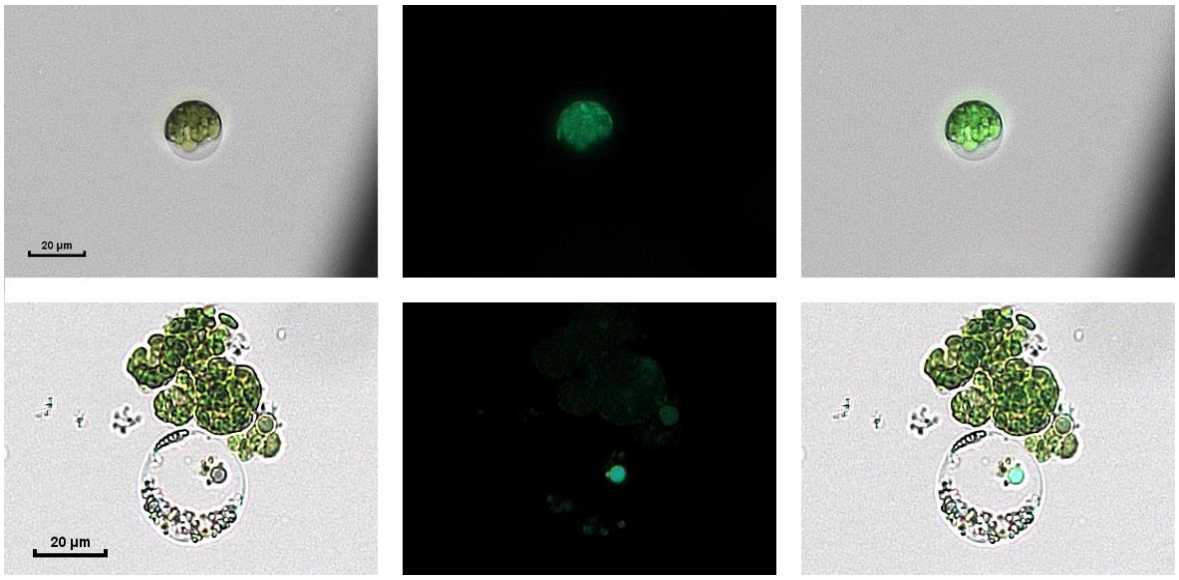


Figure 3. EGFP expression 48 h after transformation. Brightfield images depicted in the first column, fluorescence images depicted in the second column and overlayed images depicted in the third column. Images in the first row depict protoplasts transformed with PEGFP and images in the second row depict protoplasts transformed with a gRNA plasmid (P220).

The only statistically significant difference in percentage of fluorescent cells recorded from protoplast transformation experiments was found between the protoplasts transformed with PEGFP and P220 plasmids, when MMg was used and when the protoplasts were washed afterwards with W5 solution (P Value= 0.0407) (Figure 4.).

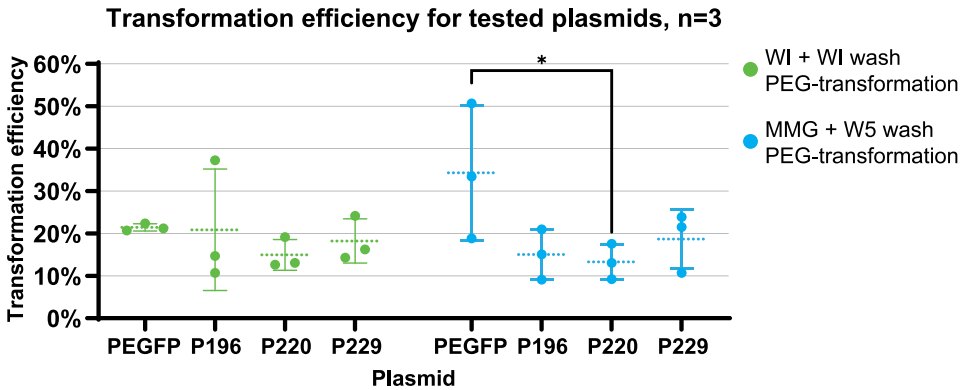


Figure 4. Scattered plot of individual values of protoplast transformation experiments. Transformation efficiency recorded at 48 h after transformation with PEG is displayed for each plasmid used, with the dotted line depicting the mean value and error bars depicting standard deviation. The asterisk depicts a difference of statistical significance (* meaning P ≤ 0.05) and "n" equals to the number of repeated experiments. WI + WI wash PEG-transformation was performed using protoplasts suspended in WI and washed with WI and MMg + W5 wash PEG-transformation was performed using protoplasts suspended in MMg and washed with W5.

4.3. Evaluation of gRNAs efficiency

To assess the editing ability of the different gRNAs, DNA extracted from transformed protoplast samples were externally sequenced and the resulting data was analyzed using TIDE (see chapter 3.6.). Of the 15 samples sent for sequencing, 2 were excluded from further analysis due to having R^2 values below 0.8 (TIDE output graphics of results plotted in Figure 5. can be seen in Appendices 11-16). During the evaluation of gRNAs efficiencies, no statistically significant differences were found between transformation methods or gRNAs used (Figure 5.).

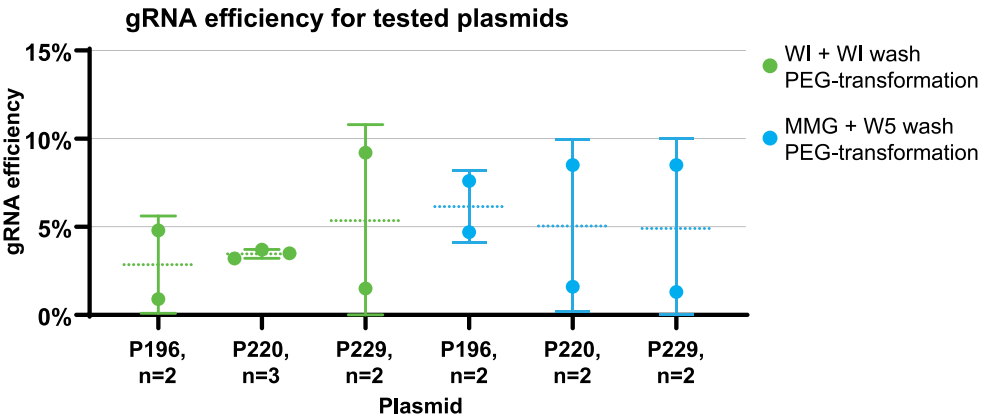


Figure 5. gRNA efficiency. Scattered plot of individual values of all recorded gRNA efficiencies per plasmid used. WI + WI wash PEG-transformation was performed using protoplasts suspended in WI and washed with WI and MMg + W5 wash PEG-transformation was performed using protoplasts suspended in MMg and washed with W5. The dotted line depicts the mean value. Error bars depict standard deviation. “n” equals the number of repeated experiments.

5. Discussion

5.1. Protoplast isolation

There is relatively little research published on protoplast isolation from *Lolium perenne* specifically, making it necessary to derive information from research done on similar species. The results of cellulase concentration experiments indicated that the optimal cellulase concentration for protoplast isolation in *Lolium perenne* from the tested concentrations (w/v of enzyme solution) was 2%, as using this concentration, the highest densities of viable protoplast were isolated. Both higher and lower cellulase concentrations resulted in lower densities of viable protoplasts being isolated at all recorded timepoints. Moreover, comparisons of extracted viable protoplast counts between 2% cellulase and every other cellulase concentration tested, carried statistical significance at every timepoint except the last one (20 h). The lack of statistically significant differences between 2% and 1.5% or 2% and 2,5% of cellulase protoplast yield at the final timepoint, might derive from the overall decline in viable protoplast counts at later timepoints combined with a higher variation between protoplast counts recorded in different experiments. Suffice to say, the results of this study indicate that the optimal cellulase concentration for isolating protoplasts from *Lolium perenne* might be approximately 2%. This however contrasts with previously published protocols on *Lolium perenne* [110] and on *Triticum aestivum* [111], which recommended using 1.5% and 1% of cellulase, respectively. Neither of these protocols use pectolyase in the enzymatic solution and the one on *Triticum aestivum* also uses lower concentration of hemicellulase, which may contribute to the differences in results.

Both previously mentioned protocols [110], [111] use different timescales for the enzymatic treatment (18-20 h in *Lolium perenne* and 3 h for *Triticum aestivum*), which might be another contributing factor to variation in the results. One of the subgoals of this study was however the time-efficient isolation of protoplasts, therefore, an optimal enzymatic concentration for a longer treatment carries less relevance. The timepoint resulting in the highest counts of viable protoplasts recorded in this study was 8 h, with differences of statistical significance for 2% cellulase solution being recorded when compared to 12 h, 16 h and 20 h timepoints. These results indicate that the optimal timeframe for enzymatic treatment using previously described conditions is 8 h.

The use of 0.5 M mannitol solution for pretreatment of leaves also seems to be optimal, however plasmolytic pretreatments have been previously recommended to be performed in solutions isotonic to the one used for enzymatic treatment [28], so these results might be connected to the exact enzymatic solution used. Conversely however, 0.5 M mannitol pretreatment resulted significantly more viable protoplasts than 0.6 M pretreatment, which is the same concentration used in the enzymatic solution. If the rest of the solutes are considered, the true tonicity of the enzymatic solution is probably even higher than that of the 0.6 M mannitol solution.

Finally, vacuum infiltration, as expected, did significantly increase viable protoplast counts. A major contributor to the statistical significance between the results from the vacuum pressure experiments, is the fact that all repeated experiments were performed in parallel. Vacuum infiltration is also another factor, which might have contributed to the optimal timeframe of protoplast isolation being recorded at the 8 h mark.

Previously published protocols used varying rotation speeds, with one published for *Lolium perenne* using 35 rpm [110] and another published for *Triticum aestivum* using 100 rpm [111], thus 75 rpm was chosen as an approximate middle value at the beginning. The preliminary experiments of the secondary experiment series (data not shown) performed using 75 rpm shaking for enzymatic treatment did not result in any viable protoplasts being isolated. This became evident when the sucrose cushion failed to produce a viable protoplast layer (Appendix 10. Figure 7.c.). The further experiments were performed using 35 rpm, which did result in a successful sucrose cushion (Appendix 10. b) and in viable protoplasts being isolated, possibly confirming the results of the previous protocol on *Lolium perenne* [110]. These results are difficult to compare however because the centrifugal force exerted by rotation is in correlation with the diameter of the rotor used and neither of the protocols [110], [111] referenced the exact model of the rotatory shaker used.

Results of the primary protoplast isolation experiments were counted using TB for staining and the secondary experiments were counted using FDA for staining. TB was picked for the primary experiment series because of the esterase presence in the cellulase used for isolation. The use of TB seemed to be more error prone than FDA because judging colour with the background also being stained made it difficult to distinguish unstained protoplasts. Another disturbing factor for counting with TB was the narrow timeframe needed for its use (3-5 min), as TB can be lethal to cells when exposed for too long.

5.2. Protoplast transformation

The EGFP was detected for each of the plasmids used for transformation, however the expression patterns were not always the same. These differences in expression patterns were not specific to any transformed plasmid. In protoplasts transformed by each of the plasmids, EGFP expression could be seen all over the cytoplasm (Figure 3. top row), or mostly expressed as aggregated in a single point, possibly in the nucleus (Figure 3. bottom row). This is possible, since even without a specific localization signal EGFP can localize to the nucleus [115]. This however wouldn't explain the presence of both EGFP expression patterns. Another, more likely explanation would be the polymerization of EGFP resulting from overly high expression levels, which has been reported to cause aberrations in localization [116], [117].

The results of the transformation efficiency experiments are somewhat difficult to make certain conclusions out of, because WI transformation of PEGFP resulted in a very low variation in results while WI transformation of P196 resulted in a very high variation in results. Despite this, the general results seem to indicate that PEGFP plasmids are transformed with a slightly higher efficiency than P196, P220 and P229. This may have resulted from the lower amount of PEGFP plasmid used for transformation, 10 µg instead of the 20 µg used for the gRNA vectors. A higher amount of DNA present in the transformation solution may have aggregated with PEG and therefore few DNA molecules could have entered the cells. Transformation with WI seems to be a little bit more efficient than transformation with MMg as higher results were recorded for P196, P220 and P229. This could have resulted from passing the protoplasts from WI to MMg, as protoplasts are fragile and every passage or treatment, they are subjected results in lower counts of viable protoplasts. Moreover, it seems that of the plasmids transformed P220 had the lowest efficiency, with the only difference of statistical significance being recorded between PEGFP and P220 of MMg

transformation series. Suffice to say that further research with more repeated experiments would be necessary to come to certain conclusions.

5.3. gRNA efficiency

Results from gRNA efficiency experiments indicate that transformation with MMg can result in generally higher gRNA efficiencies than transformation with WI. Transforming with WI, the highest gRNA efficiency was recorded from P229, while transforming with MMg the highest gRNA efficiency was tied between P220 and P229. The most consistent results were recorded in P220 for transformation with WI and P196 for transformation with MMg. Results were discarded from gRNA efficiency experiments with P values under 0.8, due to which only 2 repeated experiments were displayed for all gRNA efficiency experiments except P220 transformed with WI. On one hand, the low numbers of repeated experiments make it difficult to draw significant conclusions from the data presented, due to which further research would be necessary. On the other hand, the fact that editions (indels) fitted the statistical analyses performed by TIDE (R^2 values ≥ 0.8) show that the method developed can be suitable for the assessment of gRNAs efficiency.

The previously presented results could be used as the basis for the future generation of perennial ryegrass plants with a CRISPR knocked out *CBP20* gene. Plants with such modifications would be expected to have more cuticular waxes, since *CBP20* acts as a repressor of waxes synthesis and transport. This increased presence of waxes could allow the plants to become more tolerant to drought stress, as has been shown in other *Poaceae* plants such as barley [106]. To achieve this predicted drought tolerance phenotype, plant tissue such as calli could be transformed with the gRNAs tested during this thesis. Then, explants would be regenerated from transformed calli and their genotypes would be evaluated to detect the presence of indels that could lead to knocked out *CBP20*. If said indels were confirmed, the plants would be tested to evaluate their phenotype regarding drought response. The tests would be done first in the lab and finally in the field if a permit is obtained.

Kokkuvõte

Globaalse kliimamuutusega kaasneb suurenev tähtsus karjamaa-raiheina, enim kasutatud mäletsejate söödamoona kohandada muutustega ilmastikutingimustes. Karjamaa raihein ei ole võimeline liigisiseselt suguliselt paljunema, mistõttu ei saa selle peal rakendada klassikalisi aretustehnikaid. Genoomi täppismuundamise tehnoloogiate nagu CRISPR/Cas9 abil on aga võimalik lahendada liigisisesest sugulisest paljunemisvõimetusest tulenev probleem ning luua külma- ja põuatingimustes paremini toimetulevad taimed. Genoomi täppismuundamise tehnoloogiate tulemuste kontrollimiseks on vaja taimi eelnevalt regenereerida, mis on töömahukas ja ajakulukas. Selle asemel on võimalik polüetüleenglükooliga transformeerides katsetada CRISPR/Cas9 komponentide tõhusust genoomi täppismuundamisel taime protoplastides.

Selle töö eesmärgid olid töötada välja kõrge saagikusega protokoll karjamaa-raiheina mesofüllist protoplastide eralduseks, tõhus protokoll raiheina polüetüleenglükooliga transformeerimiseks ning nende protokollide rakendamine, et katsetada CRISPR/Cas9 gRNAde tõhusust genoomi täppismuundamisel. Selle töö raames tehtud uuring on osa rahvusvahelisest EEA-projektist „Raiheina kohanemisvõime ja vastupidavuse parandamine ohutute ja säästvate toidusüsteemide jaoks CRISPR/Cas9 tehnoloogia abil“, mille eesmärgiks oli tõsta karjamaa-raiheina põua ja külmataluvust paremaks kohanemisvõimeks Põhja-Euroopa kliimatingimustes.

Karjamaa-raiheina protoplastide eralduseks töötati välja kõrge saagikusega protokoll, mille optimeerimiseks kasutati mitmeid meetodeid. Eraldatud protoplastidesse transformeeriti edukalt polüetüleenglükooliga CRISPR/Cas9 kompleksi osi kodeerivad järjestused. Lisaks kinnitati meetodi rakendatavus, et kontrollida gRNAde tõhusust CRISPR/Cas9 kompleksiga genoomi täppismuundamisel. Selles uuringus välja töötatud metoodikat saab rakendada tulevastes CRISPR/Cas9 uuringutes karjamaa-raiheinas.

Acknowledgements

My sincerest gratitude goes out to my supervisors Ferenz and Cecilia for their ironclad willpower and fortitude to suffer through all my mishaps and failures throughout my research for this thesis. Their work ethic is truly something to marvel at. Specifically, I would like to thank Ferenz for always being ready to help, give guidance or offer moral support when needed and for being the preeminent contributor to fun times had during my master's studies, and Cecilia for providing seasoned guidance for any problem arisen during research and always being available for any help required.

Moreover, I would like to thank Lenne and Kairi for procedural guidance on lab work performed for this thesis, Signe for always keeping a lookout for any solutions I may have needed to autoclave, Merike for her limitless capacity for conversation, which often included valuable advice on various topics alongside much needed moral support and last but not least everyone else affiliated with the plant lab at TalTech for making my master's studies so memorable.

References

[1] J. E. F. af Klercker, *Eine Methode zur Isolierung lebender Protoplasten*, vol. 3. in Pflanzenphysiologische Mitteilungen, vol. 3. Stockholm: Ofvers Vetensk Akad. FOTh., 1892. Accessed: Nov. 14, 2023. [Online]. Available: http://sammlungen.ub.uni-frankfurt.de/botanik/4449859

[2] E. C. Cocking, "A Method for the Isolation of Plant Protoplasts and Vacuoles," *Nature*, vol. 187, no. 4741, Art. no. 4741, Sep. 1960, doi: 10.1038/187962a0.

[3] D. B. Hannaway *et al.*, "Perennial ryegrass (Lolium perenne L.)," *Corvallis Or. State Univ. Ext. Serv. Pullman Wash Wash. State Univ. Coop. Ext. Mosc. Ida. Univ. Ida. Coop. Ext. Serv.*, 1999

[4] M. Gagic, M. Faville, I. Kardailsky, and J. Putterill, "Comparative Genomics and Functional Characterisation of the GIGANTEA Gene from the Temperate Forage Perennial Ryegrass Lolium perenne," *Plant Mol. Biol. Report.*, vol. 33, no. 4, pp. 1098–1106, Aug. 2015, doi: 10.1007/s11105-014-0820-3.

[5] A. R. Beddows, "Lolium Perenne L.," *J. Ecol.*, vol. 55, no. 2, pp. 567–587, 1967, doi: 10.2307/2257894.

[6] S. Esmaeili, H. Salehi, and M. Khosh-Khui, "Direct and indirect in vitro plant regeneration of two commercial cultivars of perennial ryegrass," *Adv. Hortic. Sci.*, vol. 32, no. 2, Art. no. 2, Mar. 2018, doi: 10.13128/ahs-20654.

[7] L. M. Slatter, S. Barth, C. Manzanares, J. Velmurugan, I. Place, and D. Thorogood, "A new genetic locus for self-compatibility in the outcrossing grass species perennial ryegrass (Lolium perenne)," *Ann. Bot.*, vol. 127, no. 6, pp. 715–722, May 2021, doi: 10.1093/aob/mcaa140.

[8] H. Zhao *et al.*, "Origin, loss, and regain of self-incompatibility in angiosperms," *Plant Cell*, vol. 34, no. 1, pp. 579–596, Jan. 2022, doi: 10.1093/plcell/koab266.

[9] P. W. Wilkins, "Breeding perennial ryegrass for agriculture," *Euphytica*, vol. 52, no. 3, pp. 201–214, Feb. 1991, doi: 10.1007/BF00029397.

[10] S. L. Byrne *et al.*, "A synteny-based draft genome sequence of the forage grass Lolium perenne," *Plant J.*, vol. 84, no. 4, pp. 816–826, 2015, doi: 10.1111/tpj.13037.

[11] N. McNicholl, "Diploid vs Tetraploid Ryegrass: Which is Best for Farmers?," Germinal GB. Accessed: Nov. 10, 2023. [Online]. Available: https://germinal.com/diploid-vs-tetraploid-ryegrass/

[12] I. Nagy *et al.*, "Chromosome-scale assembly and annotation of the perennial ryegrass genome," *BMC Genomics*, vol. 23, no. 1, p. 505, Jul. 2022, doi: 10.1186/s12864-022-08697-0.

[13] C. J. Andrews, "Low-temperature stress in field and forage crop production – an overview," *Can. J. Plant Sci.*, vol. 67, no. 4, pp. 1121–1133, Oct. 1987, doi: 10.4141/cjps87-152.

[14] S. C. Goslee, J. M. Gonet, and R. H. Skinner, "Freeze Tolerance of Perennial Ryegrass and Implications for Future Species Distribution," *Crop Sci.*, vol. 57, no. 5, pp. 2875–2880, 2017, doi: 10.2135/cropsci2017.02.0135.

[15] C. Loix, M. Huybrechts, J. Vangronsveld, M. Gielen, E. Keunen, and A. Cuypers, "Reciprocal Interactions between Cadmium-Induced Cell Wall Responses and Oxidative Stress in Plants," *Front. Plant Sci.*, vol. 8, 2017, Accessed: Nov. 10, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fpls.2017.01867

[16] M. R. Davey, P. Anthony, J. B. Power, and K. C. Lowe, "Plant protoplasts: status and biotechnological perspectives," *Biotechnol. Adv.*, vol. 23, no. 2, pp. 131–171, Mar. 2005, doi: 10.1016/j.biotechadv.2004.09.008.

[17] T. Eeckhaut, P. S. Lakshmanan, D. Deryckere, E. Van Bockstaele, and J. Van Huylenbroeck, "Progress in plant protoplast research," *Planta*, vol. 238, no. 6, pp. 991–1003, Dec. 2013, doi: 10.1007/s00425-013-1936-7.

[18] F. Wu and Y. Hanzawa, “A Simple Method for Isolation of Soybean Protoplasts and Application to Transient Gene Expression Analyses,” *J. Vis. Exp. JoVE*, no. 131, p. 57258, Jan. 2018, doi: 10.3791/57258.
[19] E. J. Stadelmann, “Chapter 7 Evaluation of Turgidity, Plasmolysis, and Deplasmolysis of Plant Cells,” in *Methods in Cell Biology*, vol. 2, D. M. Prescott, Ed., Academic Press, 1966, pp. 143–216. doi: 10.1016/S0091-679X(08)62139-1.
[20] E. C. Cocking and D. W. Gregory, “Organized Protoplasmic Units of the Plant Cell: I. THEIR OCCURRENCE, ORIGIN, AND STRUCTURE,” *J. Exp. Bot.*, vol. 14, no. 3, pp. 504–511, Oct. 1963, doi: 10.1093/jxb/14.3.504.
[21] E. C. Cocking, “Plant Cell Protoplasts-Isolation and Development,” *Annu. Rev. Plant Physiol.*, vol. 23, no. 1, pp. 29–50, 1972, doi: 10.1146/annurev.pp.23.060172.000333.
[22] R. Chambers and K. Höfler, “Micrurgical studies on the tonoplast of Allium cepa,” *Protoplasma*, vol. 12, no. 1, pp. 338–355, Dec. 1931, doi: 10.1007/BF01618743.
[23] H. S. Chawla, *Introduction to plant biotechnology*, Third edition. Enfield, NH Jersey Plymouth: Science Publishers, 2009.
[24] S.-D. Yoo, Y.-H. Cho, and J. Sheen, “Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis,” *Nat. Protoc.*, vol. 2, no. 7, Art. no. 7, Jul. 2007, doi: 10.1038/nprot.2007.199.
[25] K. Wang and F. Zhang, Eds., *Protoplast Technology: Methods and Protocols*, vol. 2464. in Methods in Molecular Biology, vol. 2464. New York, NY: Springer US, 2022. doi: 10.1007/978-1-0716-2164-6.
[26] J. B. Power, S. E. Cummins, and E. C. Cocking, “Fusion of isolated plant protoplasts,” *Nature*, vol. 225, no. 5237, pp. 1016–1018, Mar. 1970, doi: 10.1038/2251016a0.
[27] L. A. Withers and E. C. Cocking, “Fine-structural studies on spontaneous and induced fusion of higher plant protoplasts,” *J. Cell Sci.*, vol. 11, no. 1, pp. 59–75, Jul. 1972, doi: 10.1242/jcs.11.1.59.
[28] M. R. Davey, P. Anthony, D. Patel, and J. B. Power, “Plant Protoplasts: Isolation, Culture and Plant Regeneration,” in *Plant Cell Culture*, John Wiley & Sons, Ltd, 2010, pp. 153–173. doi: 10.1002/9780470686522.ch9.
[29] V. K. A. Silva *et al.*, “Replicative Aging Remodels the Cell Wall and Is Associated with Increased Intracellular Trafficking in Human Pathogenic Yeasts,” *mBio*, vol. 13, no. 1, pp. e00190-22, 2022, doi: 10.1128/mbio.00190-22.
[30] F.-H. Wu, S.-C. Shen, L.-Y. Lee, S.-H. Lee, M.-T. Chan, and C.-S. Lin, “Tape-Arabidopsis Sandwich - a simpler Arabidopsis protoplast isolation method,” *Plant Methods*, vol. 5, no. 1, p. 16, Nov. 2009, doi: 10.1186/1746-4811-5-16.
[31] H. S. Gupta and A. Pattanayak, “Plant Regeneration from Mesophyll Protoplasts of Rice (Oryza sativa L.),” *Bio/Technology*, vol. 11, no. 1, Art. no. 1, Jan. 1993, doi: 10.1038/nbt0193-90.
[32] F. Sarhan and D. Cesar, “High yield isolation of mesophyll protoplasts from wheat, barley and rye,” *Physiol. Plant.*, vol. 72, no. 2, pp. 337–342, 1988, doi: 10.1111/j.1399-3054.1988.tb05842.x.
[33] I. Potrykus and R. D. Shillito, “Protoplasts: Isolation, culture, plant regeneration,” in *Methods in Enzymology*, vol. 118, in Plant Molecular Biology, vol. 118. , Academic Press, 1986, pp. 549–578. doi: 10.1016/0076-6879(86)18101-8.
[34] W. A. Keller and G. Melchers, “The effect of high pH and calcium on tobacco leaf protoplast fusion,” *Z. Naturforschung Teil C Biochem. Biophys. Biol. Virol.*, vol. 28, no. 11, pp. 737–741, 1973, doi: 10.1515/znc-1973-11-1215.
[35] J. Sardans and J. Peñuelas, “Potassium Control of Plant Functions: Ecological and Agricultural Implications,” *Plants*, vol. 10, no. 2, p. 419, Feb. 2021, doi: 10.3390/plants10020419.
[36] K. M. Reed and B. O. R. Bargmann, “Protoplast Regeneration and Its Use in New Plant Breeding Technologies,” *Front. Genome Ed.*, vol. 3, 2021, Accessed: Nov. 23, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fgeed.2021.734951

[37] M. Ge *et al.*, "Effect of aeration rates on enzymatic activity and bacterial community succession during cattle manure composting," *Bioresour. Technol.*, vol. 304, p. 122928, May 2020, doi: 10.1016/j.biortech.2020.122928.
[38] H. Wang, K. Mochidzuki, S. Kobayashi, H. Hiraide, X. Wang, and Z. Cui, "Effect of Bovine Serum Albumin (BSA) on Enzymatic Cellulose Hydrolysis," *Appl. Biochem. Biotechnol.*, vol. 170, no. 3, pp. 541–551, Jun. 2013, doi: 10.1007/s12010-013-0208-0.
[39] H. Y. Kim, G. G. Coté, and R. C. Crain, "Effects of Light on the Membrane Potential of Protoplasts from Samanea saman Pulvini," *Plant Physiol.*, vol. 99, no. 4, pp. 1532–1539, Aug. 1992.
[40] S. S. Bhojwani and M. K. Razdan, Eds., "Chapter 12 Protoplast isolation and culture," in *Studies in Plant Science*, vol. 5, in Plant Tissue Culture, vol. 5. , Elsevier, 1996, pp. 337–372. doi: 10.1016/S0928-3420(96)80014-0.
[41] N. L. Paiva, "Plant Cell Culture," in *Manual of Industrial Microbiology and Biotechnology*, John Wiley & Sons, Ltd, 2010, pp. 196–211. doi: 10.1128/9781555816827.ch14.
[42] A. Vembadi, A. Menachery, and M. A. Qasaimeh, "Cell Cytometry: Review and Perspective on Biotechnological Advances," *Front. Bioeng. Biotechnol.*, vol. 7, 2019, Accessed: Dec. 27, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fbioe.2019.00147
[43] M. Watanabe, Y. Watanabe, and N. Shimada, "Colorimetric estimation of leaf-protoplast potential-to-divide by use of 2,3,5-triphenyl tetrazolium chloride," *J. Plant Physiol.*, vol. 141, no. 1, pp. 111–114, Jan. 1993, doi: 10.1016/S0176-1617(11)80860-6.
[44] M. Balakshin, E. Capanema, and A. Berlin, "Chapter 4 - Isolation and Analysis of Lignin–Carbohydrate Complexes Preparations with Traditional and Advanced Methods: A Review," in *Studies in Natural Products Chemistry*, vol. 42, Atta-ur-Rahman, Ed., Elsevier, 2014, pp. 83–115. doi: 10.1016/B978-0-444-63281-4.00004-5.
[45] T. Nagata and I. Takebe, "Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts," *Planta*, vol. 92, no. 4, pp. 301–308, Dec. 1970, doi: 10.1007/BF00385097.
[46] A. Al-Sabah, Z. M. Jessop, I. S. Whitaker, and C. Thornton, "4 - Cell preparation for 3D bioprinting," in *3D Bioprinting for Reconstructive Surgery*, D. J. Thomas, Z. M. Jessop, and I. S. Whitaker, Eds., Woodhead Publishing, 2018, pp. 75–88. doi: 10.1016/B978-0-08-101103-4.00006-5.
[47] R. Fernandez-Da Silva and A. Menendez-Yuffa, "Viability in protoplasts and cell suspensions of Coffea arabica cv. Catimor," *Electron. J. Biotechnol.*, vol. 9, no. 5, pp. 0–0, Oct. 2006, doi: 10.2225/vol9-issue5-fulltext-4.
[48] C.-N. Huang, M. J. Cornejo, D. S. Bush, and R. L. Jones, "Estimating viability of plant protoplasts using double and single staining," *Protoplasma*, vol. 135, no. 2, pp. 80–87, Jun. 1986, doi: 10.1007/BF01277001.
[49] K. Lindsey and M. G. Jones, "The permeability of electroporated cells and protoplasts of sugar beet," *Planta*, vol. 172, no. 3, pp. 346–355, Nov. 1987, doi: 10.1007/BF00398663.
[50] J. R. Tennant, "EVALUATION OF THE TRYPAN BLUE TECHNIQUE FOR DETERMINATION OF CELL VIABILITY," *Transplantation*, vol. 2, pp. 685–694, Nov. 1964, doi: 10.1097/00007890-196411000-00001.
[51] O. Erikson, M. Hertzberg, and T. Näsholm, "A conditional marker gene allowing both positive and negative selection in plants," *Nat. Biotechnol.*, vol. 22, no. 4, Art. no. 4, Apr. 2004, doi: 10.1038/nbt946.
[52] P. L. Bhalla and M. J. Dalling, "Characteristics of a β-Galactosidase Associated with the Stroma of Chloroplasts Prepared from Mesophyll Protoplasts of the Primary Leaf of Wheat," *Plant Physiol.*, vol. 76, no. 1, pp. 92–95, Sep. 1984.
[53] L. Herrera-Estrella, A. Depicker, M. Van Montagu, and J. Schell, "Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector," *Nature*, vol. 303, no. 5914, Art. no. 5914, May 1983, doi: 10.1038/303209a0.

[54] N. K. Alton and D. Vapnek, “Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9,” *Nature*, vol. 282, no. 5741, pp. 864–869, Dec. 1979, doi: 10.1038/282864a0.
[55] A. Ziemienowicz, “Plant selectable markers and reporter genes,” *Acta Physiol. Plant.*, vol. 23, no. 3, pp. 363–374, Sep. 2001, doi: 10.1007/s11738-001-0045-6.
[56] M. A. Savka and S. K. Farrand, “Mannityl opine accumulation and exudation by transgenic tobacco,” *Plant Physiol.*, vol. 98, no. 2, pp. 784–789, Feb. 1992, doi: 10.1104/pp.98.2.784.
[57] A. Escher, D. J. O’Kane, J. Lee, and A. A. Szalay, “Bacterial luciferase alpha beta fusion protein is fully active as a monomer and highly sensitive in vivo to elevated temperature,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 86, no. 17, pp. 6528–6532, Sep. 1989, doi: 10.1073/pnas.86.17.6528.
[58] O. Olsson, O. Nilsson, and C. Koncz, “Novel monomeric luciferase enzymes as tools to study plant gene regulation in vivo,” *J. Biolumin. Chemilumin.*, vol. 5, no. 2, pp. 79–87, 1990, doi: 10.1002/bio.1170050203.
[59] J. W. Hastings, T. O. Baldwin, and M. Z. Nicoli, “[14] Bacterial luciferase: Assay, purification, and properties,” in *Methods in Enzymology*, vol. 57, in Bioluminescence and Chemiluminescence, vol. 57. , Academic Press, 1978, pp. 135–152. doi: 10.1016/0076-6879(78)57016-X.
[60] D. C. Prasher, V. K. Eckenrode, W. W. Ward, F. G. Prendergast, and M. J. Cormier, “Primary structure of the Aequorea victoria green-fluorescent protein,” *Gene*, vol. 111, no. 2, pp. 229–233, Feb. 1992, doi: 10.1016/0378-1119(92)90691-h.
[61] C. W. Cody, D. C. Prasher, W. M. Westler, F. G. Prendergast, and W. W. Ward, “Chemical structure of the hexapeptide chromophore of the Aequorea green-fluorescent protein,” *Biochemistry*, vol. 32, no. 5, pp. 1212–1218, Feb. 1993, doi: 10.1021/bi00056a003.
[62] J. Sun, G. H. Kelemen, J. M. Fernández-Abalos, and M. J. Bibb, “Green fluorescent protein as a reporter for spatial and temporal gene expression in Streptomyces coelicolor A3(2),” *Microbiol. Read. Engl.*, vol. 145 (Pt 9), pp. 2221–2227, Sep. 1999, doi: 10.1099/00221287-145-9-2221.
[63] G. Keshavareddy, A. Kumar, and R. S V, “Methods of Plant Transformation- A Review,” *Int. J. Curr. Microbiol. Appl. Sci.*, vol. 7, pp. 2656–2668, Jul. 2018, doi: 10.20546/ijcmas.2018.707.312.
[64] S. Dai *et al.*, “Comparative analysis of transgenic rice plants obtained by Agrobacterium-mediated transformation and particle bombardment,” *Mol. Breed.*, vol. 7, no. 1, pp. 25–33, Jan. 2001, doi: 10.1023/A:1009687511633.
[65] B. Darbani, “DNA-Delivery Methods to Produce Transgenic Plants,” *Biotechnology(Faisalabad)*, vol. 7, pp. 385–402, Jan. 2008.
[66] H. Dudek, A. Ghosh, and M. E. Greenberg, “Calcium phosphate transfection of DNA into neurons in primary culture,” *Curr. Protoc. Neurosci.*, vol. Chapter 3, p. Unit 3.11, May 2001, doi: 10.1002/0471142301.ns0311s03.
[67] R. A. Aubin, M. Weinfeld, R. Mirzayans, and M. C. Paterson, “Polybrene/DMSO-assisted gene transfer. Generating stable transfectants with nanogram amounts of DNA,” *Mol. Biotechnol.*, vol. 1, no. 1, pp. 29–48, Feb. 1994, doi: 10.1007/BF02821509.
[68] M. R. Lieber, J. E. Hesse, K. Mizuuchi, and M. Gellert, “Developmental stage specificity of the lymphoid V(D)J recombination activity,” *Genes Dev.*, vol. 1, no. 8, pp. 751–761, Oct. 1987, doi: 10.1101/gad.1.8.751.
[69] W. Holter, C. M. Fordis, and B. H. Howard, “Efficient gene transfer by sequential treatment of mammalian cells with DEAE-dextran and deoxyribonucleic acid,” *Exp. Cell Res.*, vol. 184, no. 2, pp. 546–551, Oct. 1989, doi: 10.1016/0014-4827(89)90353-4.
[70] H. J. Ryser, “A membrane effect of basic polymers dependent on molecular size,” *Nature*, vol. 215, no. 5104, pp. 934–936, Aug. 1967, doi: 10.1038/215934a0.
[71] Y. W. Yang and J. C. Yang, “Studies of DEAE-dextran-mediated gene transfer,” *Biotechnol. Appl. Biochem.*, vol. 25, no. 1, pp. 47–51, Feb. 1997, doi: 10.1111/j.1470-8744.1997.tb00413.x.

[72] P. Kumar, A. Nagarajan, and P. D. Uchil, “DEAE-Dextran Transfection,” *Cold Spring Harb. Protoc.*, vol. 2018, no. 7, Jul. 2018, doi: 10.1101/pdb.top096263.
[73] T. Wang *et al.*, “Impact of molecular weight on the mechanism of cellular uptake of polyethylene glycols (PEGs) with particular reference to P-glycoprotein,” *Acta Pharm. Sin. B*, vol. 10, no. 10, p. 2002, Oct. 2020, doi: 10.1016/j.apsb.2020.02.001.
[74] V. S. Kamburova *et al.*, “Genome Editing in Plants: An Overview of Tools and Applications,” *Int. J. Agron.*, vol. 2017, p. e7315351, Jul. 2017, doi: 10.1155/2017/7315351.
[75] D. G. Knorre and V. V. Vlasov, “Reactive Derivatives of Nucleic Acids and Their Components as Affinity Reagents,” *Russ. Chem. Rev.*, vol. 54, no. 9, p. 836, Sep. 1985, doi: 10.1070/RC1985v054n09ABEH003115.
[76] N. J. Palpant and D. Dudzinski, “Zinc finger nucleases: looking toward translation,” *Gene Ther.*, vol. 20, no. 2, Art. no. 2, Feb. 2013, doi: 10.1038/gt.2012.2.
[77] R. Jankele and P. Svoboda, “TAL effectors: tools for DNA targeting,” *Brief. Funct. Genomics*, vol. 13, no. 5, pp. 409–419, Sep. 2014, doi: 10.1093/bfgp/elu013.
[78] T. Gaj, C. A. Gersbach, and C. F. Barbas, “ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering,” *Trends Biotechnol.*, vol. 31, no. 7, pp. 397–405, Jul. 2013, doi: 10.1016/j.tibtech.2013.04.004.
[79] L. Lowder, A. Malzahn, and Y. Qi, “Rapid Evolution of Manifold CRISPR Systems for Plant Genome Editing,” *Front. Plant Sci.*, vol. 7, p. 1683, 2016, doi: 10.3389/fpls.2016.01683.
[80] V. Kumar and M. Jain, “The CRISPR-Cas system for plant genome editing: advances and opportunities,” *J. Exp. Bot.*, vol. 66, no. 1, pp. 47–57, Jan. 2015, doi: 10.1093/jxb/eru429.
[81] B. Chen *et al.*, “Expanding the CRISPR imaging toolset with Staphylococcus aureus Cas9 for simultaneous imaging of multiple genomic loci,” *Nucleic Acids Res.*, vol. 44, no. 8, p. e75, May 2016, doi: 10.1093/nar/gkv1533.
[82] H. Zhang *et al.*, “TALEN-mediated targeted mutagenesis produces a large variety of heritable mutations in rice,” *Plant Biotechnol. J.*, vol. 14, no. 1, pp. 186–194, Jan. 2016, doi: 10.1111/pbi.12372.
[83] J. Gao *et al.*, “CRISPR/Cas9-mediated targeted mutagenesis in Nicotiana tabacum,” *Plant Mol. Biol.*, vol. 87, no. 1–2, pp. 99–110, Jan. 2015, doi: 10.1007/s11103-014-0263-0.
[84] P. Perez-Pinera, D. G. Ousterout, and C. A. Gersbach, “Advances in Targeted Genome Editing,” *Curr. Opin. Chem. Biol.*, vol. 16, no. 3–4, pp. 268–277, Aug. 2012, doi: 10.1016/j.cbpa.2012.06.007.
[85] Y. G. Kim, J. Cha, and S. Chandrasegaran, “Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 93, no. 3, pp. 1156–1160, Feb. 1996.
[86] C. O. Pabo, E. Peisach, and R. A. Grant, “Design and Selection of Novel Cys2His2 Zinc Finger Proteins,” *Annu. Rev. Biochem.*, vol. 70, no. 1, pp. 313–340, 2001, doi: 10.1146/annurev.biochem.70.1.313.
[87] T. Cathomen and J. K. Joung, “Zinc-finger nucleases: the next generation emerges,” *Mol. Ther. J. Am. Soc. Gene Ther.*, vol. 16, no. 7, pp. 1200–1207, Jul. 2008, doi: 10.1038/mt.2008.114.
[88] J. F. Petolino, “Genome editing in plants via designed zinc finger nucleases,” *Vitro Cell. Dev. Biol. - Plant*, vol. 51, no. 1, pp. 1–8, Feb. 2015, doi: 10.1007/s11627-015-9663-3.
[89] N. P. Pavletich and C. O. Pabo, “Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A,” *Science*, vol. 252, no. 5007, pp. 809–817, May 1991, doi: 10.1126/science.2028256.
[90] P. Römer, S. Hahn, T. Jordan, T. Strauss, U. Bonas, and T. Lahaye, “Plant pathogen recognition mediated by promoter activation of the pepper Bs3 resistance gene,” *Science*, vol. 318, no. 5850, pp. 645–648, Oct. 2007, doi: 10.1126/science.1144958.
[91] J. Boch *et al.*, “Breaking the code of DNA binding specificity of TAL-type III effectors,” *Science*, vol. 326, no. 5959, pp. 1509–1512, Dec. 2009, doi: 10.1126/science.1178811.

[92] S. Schornack, A. Meyer, P. Römer, T. Jordan, and T. Lahaye, “Gene-for-gene-mediated recognition of nuclear-targeted AvrBs3-like bacterial effector proteins,” *J. Plant Physiol.*, vol. 163, no. 3, pp. 256–272, Feb. 2006, doi: 10.1016/j.jplph.2005.12.001.
[93] B. M. Lamb, A. C. Mercer, and C. F. Barbas, “Directed evolution of the TALE N-terminal domain for recognition of all 5′ bases,” *Nucleic Acids Res.*, vol. 41, no. 21, pp. 9779–9785, Nov. 2013, doi: 10.1093/nar/gkt754.
[94] D. P. Weeks, M. H. Spalding, and B. Yang, “Use of designer nucleases for targeted gene and genome editing in plants,” *Plant Biotechnol. J.*, vol. 14, no. 2, pp. 483–495, Feb. 2016, doi: 10.1111/pbi.12448.
[95] M. Christian *et al.*, “Targeting DNA double-strand breaks with TAL effector nucleases,” *Genetics*, vol. 186, no. 2, pp. 757–761, Oct. 2010, doi: 10.1534/genetics.110.120717.
[96] A. A. Nemudryi, K. R. Valetdinova, S. P. Medvedev, and S. M. Zakian, “TALEN and CRISPR/Cas Genome Editing Systems: Tools of Discovery,” *Acta Naturae*, vol. 6, no. 3, pp. 19–40, 2014.
[97] F. Zhang, Y. Wen, and X. Guo, “CRISPR/Cas9 for genome editing: progress, implications and challenges,” *Hum. Mol. Genet.*, vol. 23, no. R1, pp. R40-46, Sep. 2014, doi: 10.1093/hmg/ddu125.
[98] R. Barrangou *et al.*, “CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes,” *Science*, vol. 315, no. 5819, pp. 1709–1712, Mar. 2007, doi: 10.1126/science.1138140.
[99] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier, “A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity,” *Science*, vol. 337, no. 6096, pp. 816–821, Aug. 2012, doi: 10.1126/science.1225829.
[100] L. Cong *et al.*, “Multiplex genome engineering using CRISPR/Cas systems,” *Science*, vol. 339, no. 6121, pp. 819–823, Feb. 2013, doi: 10.1126/science.1231143.
[101] E. Deltcheva *et al.*, “CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III,” *Nature*, vol. 471, no. 7340, pp. 602–607, Mar. 2011, doi: 10.1038/nature09886.
[102] A. Eid and M. Mahfouz, “Genome editing: The road of CRISPR/Cas9 from bench to clinic,” *Exp. Mol. Med.*, vol. 48, Oct. 2016, doi: 10.1038/emm.2016.111.
[103] S. W. Cho, S. Kim, J. M. Kim, and J.-S. Kim, “Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease,” *Nat. Biotechnol.*, vol. 31, no. 3, pp. 230–232, Mar. 2013, doi: 10.1038/nbt.2507.
[104] Q. Shan *et al.*, “Targeted genome modification of crop plants using a CRISPR-Cas system,” *Nat. Biotechnol.*, vol. 31, no. 8, pp. 686–688, Aug. 2013, doi: 10.1038/nbt.2650.
[105] I. Uuselu, L. Nigul, and C. Sarmiento, “GFD-d kodeeriva reporterjärjestuse lisamine CRISPR/Cas9 taimevektorisse,” Tallinna Tehnikaülikool, 2017.
[106] A. Daszkowska-Golec, J. Karcz, T. Plociniczak, K. Sitko, and I. Szarejko, “Cuticular waxes-A shield of barley mutant in CBP20 (Cap-Binding Protein 20) gene when struggling with drought stress,” *Plant Sci. Int. J. Exp. Plant Biol.*, vol. 300, p. 110593, Nov. 2020, doi: 10.1016/j.plantsci.2020.110593.
[107] J.-P. Concordet and M. Haeussler, “CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens,” *Nucleic Acids Res.*, vol. 46, no. W1, pp. W242–W245, Jul. 2018, doi: 10.1093/nar/gky354.
[108] M. R. Green and J. Sambrook, “The Inoue Method for Preparation and Transformation of Competent Escherichia coli: ‘Ultracompetent’ Cells,” *Cold Spring Harb. Protoc.*, vol. 2020, no. 6, p. 101196, Jun. 2020, doi: 10.1101/pdb.prot101196.
[109] M. R. Green and J. Sambrook, “Chapter 1 Isolation and Quantification of DNA,” in *Molecular Cloning: A Laboratory Manual (Fourth Edition)*, 4th edition., vol. 1, Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press, 2012, pp. 11–14.
[110] H. R. Davis, A. L. Maddison, D. W. Phillips, and H. D. Jones, “Genetic Transformation of Protoplasts Isolated from Leaves of Lolium temulentum and Lolium perenne,” in *Cereal Genomics: Methods and Protocols*, L. M. Vaschetto, Ed., in Methods in Molecular Biology. , New York, NY: Springer US, 2020, pp. 199–205. doi: 10.1007/978-1-4939-9865-4_16.

[111] K. M. Brandt, H. Gunn, N. Moretti, and R. S. Zemetra, “A Streamlined Protocol for Wheat (Triticum aestivum) Protoplast Isolation and Transformation With CRISPR-Cas Ribonucleoprotein Complexes,” *Front. Plant Sci.*, vol. 11, 2020, Accessed: Jan. 03, 2024. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fpls.2020.00769

[112] D. Weigel and J. Glazebrook, “Quick miniprep for plant DNA isolation,” *Cold Spring Harb. Protoc.*, vol. 2009, no. 3, p. pdb.prot5179, Mar. 2009, doi: 10.1101/pdb.prot5179.

[113] E. K. Brinkman *et al.*, “Easy quantification of template-directed CRISPR/Cas9 editing.” bioRxiv, p. 218156, Nov. 13, 2017. doi: 10.1101/218156.

[114] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, “NIH Image to ImageJ: 25 years of image analysis,” *Nat. Methods*, vol. 9, no. 7, Art. no. 7, Jul. 2012, doi: 10.1038/nmeth.2089.

[115] N. M. Seibel, J. Eljouni, M. M. Nalaskowski, and W. Hampe, “Nuclear localization of enhanced green fluorescent protein homomultimers,” *Anal. Biochem.*, vol. 368, no. 1, pp. 95–99, Sep. 2007, doi: 10.1016/j.ab.2007.05.025.

[116] M. A. Rizzo, M. W. Davidson, and D. W. Piston, “Fluorescent protein tracking and detection: applications using fluorescent proteins in living cells,” *Cold Spring Harb. Protoc.*, vol. 2009, no. 12, p. pdb.top64, Dec. 2009, doi: 10.1101/pdb.top64.

[117] M. A. Rizzo, M. W. Davidson, and D. W. Piston, “Fluorescent protein tracking and detection: fluorescent protein structure and color variants,” *Cold Spring Harb. Protoc.*, vol. 2009, no. 12, p. pdb.top63, Dec. 2009, doi: 10.1101/pdb.top63.

Appendices

Appendix 1 – Map of the pHSE401/EGFP transformation vector

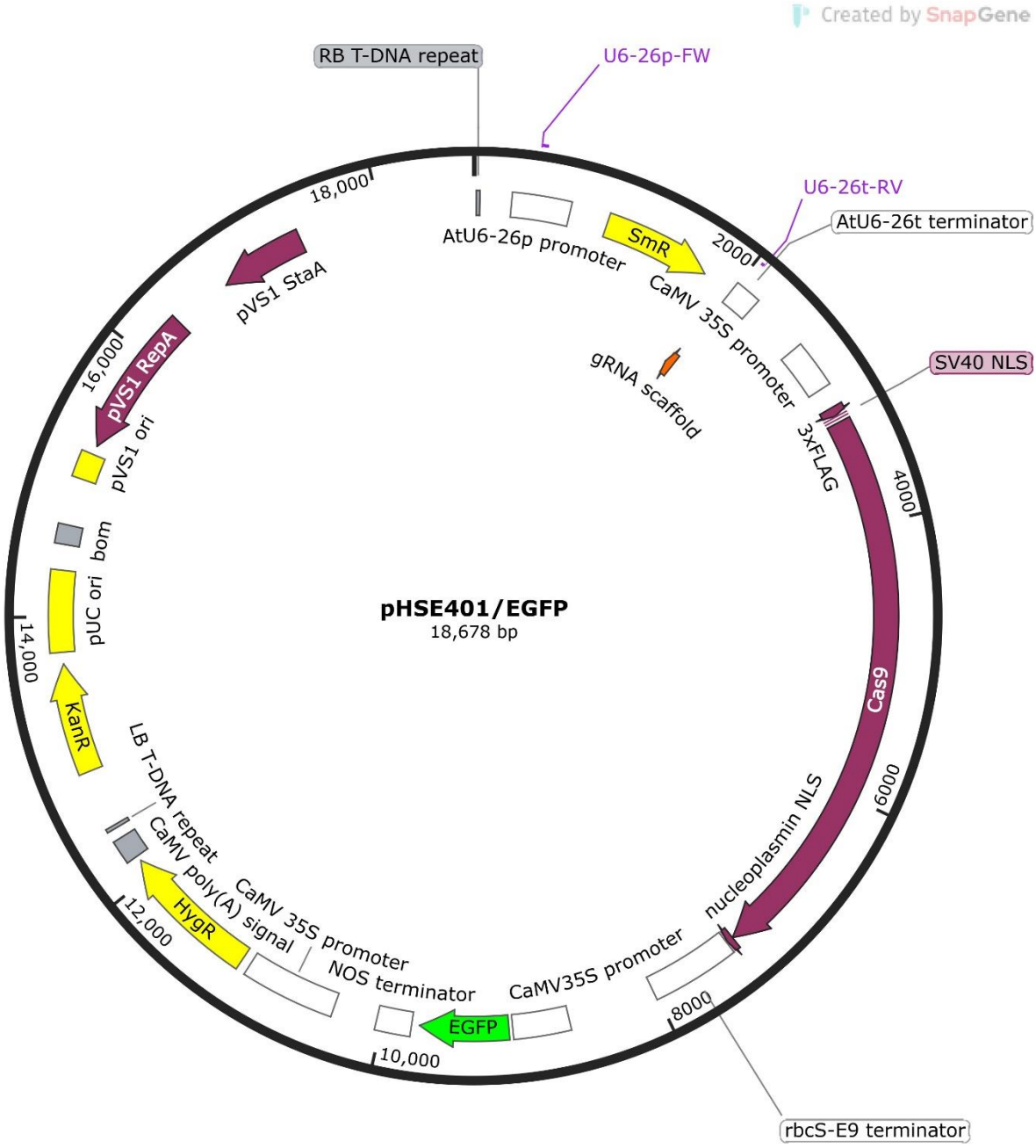


Image generated with SnapGene

Appendix 2 – Oligonucleotides and primers sequences

Oligo/Primer name	Sequence (5'-3')
CBP 196-FW	ATTGGCGTCGCTGACGGTGTATG
CBP 196-RV	AAACCATACACCGTCAGCGACGC
CBP 220-FW	ATTGAACATGTCCTTCTACAGCA
CBP 220-RV	AAACTGCTGTAGAAGGACATGTT
CBP 229-FW	ATTGTTCTACAGCACGGAGGAGC
CBP 229-RV	AAACGCTCCTCCGTGCTGTAGAA
U6-26p-FW	TGTCCCAGGATTAGAATGATTAGGC
U6-26t-RV	CCCCAGAAATTGAACGCCGAAGAAC
gRNA_Sang_FW	AATGGCGTCCCTCTTCAAGG
gRNA_Sang_RV	ACGCACAGTACGAAGCAGAA

Sequences of oligonucleotides and primers used for vector generation, PCR and Sanger sequencing

Appendix 3 – Golden Gate reaction composition

Component	Volume
Annealed oligonucleotides	1 µL
pHSE401/EGFP (100 ng)	1 µL
EcoR31I (BsaI)	0.5 µL
T4 DNA ligase	1 µL
T4 DNA ligase buffer (10x)	2 µL
Nuclease free water	Up to 20 µL

Reaction mix used for the Golden Gate assembly of transformation vectors

Appendix 4 – Golden Gate program

Temperature	Time	Cycles
37°C	10 min	15
16°C	10 min	
50°C	10 min	1
65°C	10 min	1
10°C	Hold	-

Golden Gate program used for the assembly of transformation vectors

Appendix 5 – Colony PCR reaction compositions

	gRNA 196	PCR 196	gRNA 220	PCR 220	gRNA 229	PCR 229	gRNA C	PCR C
DreamTaq MM	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
U6-26p-FW (10 µM)	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
U6-26t-RV (10 µM)	-	1 µL	-	1 µL	-	1 µL	-	1 µL
CBP 196-RV (10 µM)	1 µL	-	-	-	-	-	1 µL	-
CBP 220-RV (10 µM)	-	-	1 µL	-	-	-		-
CBP 229-RV (10 µM)	-	-	-	-	1 µL	-		-
Colony	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	-	-
Plasmid (1 ng)	-	-	-	-	-	-	2 µL	2 µL
H2O	3 µL	3 µL	3 µL	3 µL	3 µL	3 µL	6 µL	6 µL

Reactions mix used to evaluate the presence of gRNAs in the transformation vectors

Appendix 6 – Colony PCR program

Temperature	Time	Cycles
95°C	3 min	1
95°C	30 s	35
52°C	30 s	
72°C	2 min	
72°C	5 min	1
10°C	Hold	-

Colony PCR program used to evaluate the presence of gRNAs in the transformation vectors

Appendix 7 – Composition of the solutions used for protoplasts purification and transformation

	W5	W5A	WI	MMg
MES, pH 5.7	2 mM	5 mM	4 mM	4 mM
Mannitol	-	-	500 mM	400 mM
Glucose	-	5 mM	-	-
NaCl	154 mM	154 mM	-	-
CaCl2	125 mM	125 mM	-	-
KCl	5 mM	5 mM	5 mM	-
MgCl2	-	-	-	15 mM

Appendix 8 – Phusion polymerase PCR reaction composition

Component	Volume
Protoplasts DNA (10 ng)	1 µL
gRNA_Sang_FW (10 µM)	1 µL
gRNA_Sang_RV (10 µM)	0.5 µL
Phusion Master Mix (2x)	1 µL
Nuclease free water	Up to 20 µL

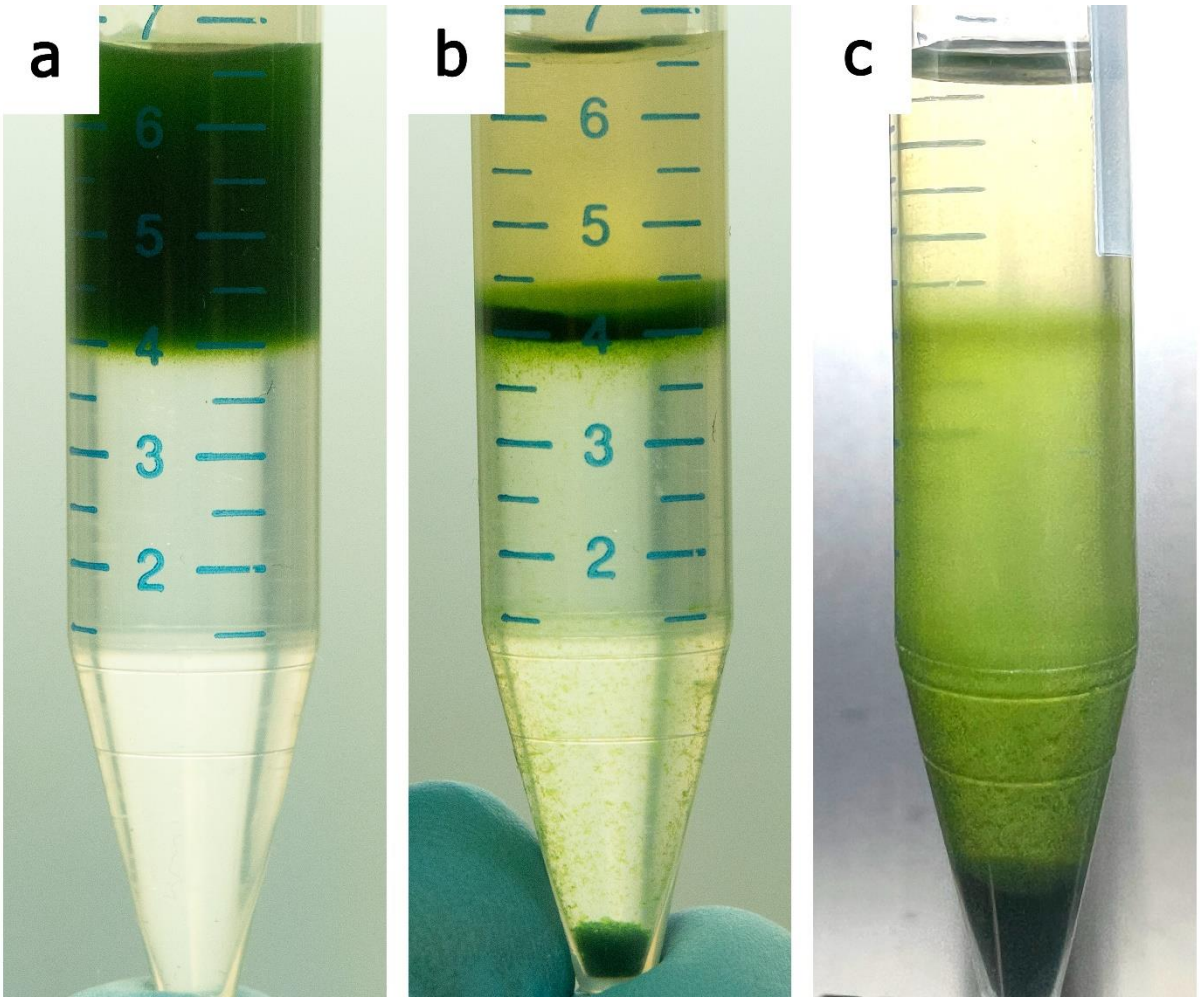
Reaction mix of the PCR used to amplify the region of *CBP20* targeted by the gRNAS

Appendix 9 – Phusion polymerase PCR program

Temperature	Time	Cycles
98°C	30 s	1
98°C	10 s	35
58°C	30 s	
72°C	30 s	
72°C	5 min	1
10°C	Hold	-

PCR program used to amplify the region of *CBP20* targeted by the gRNAS

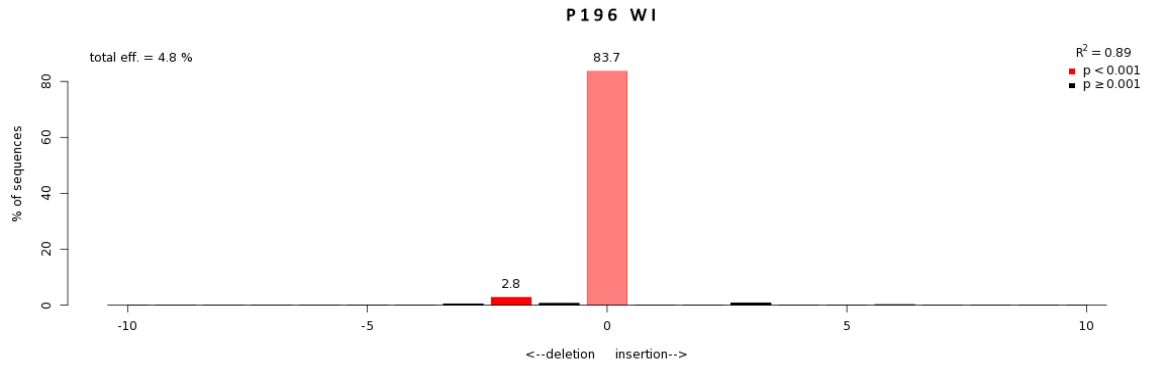
Appendix 10 – Sucrose cushion



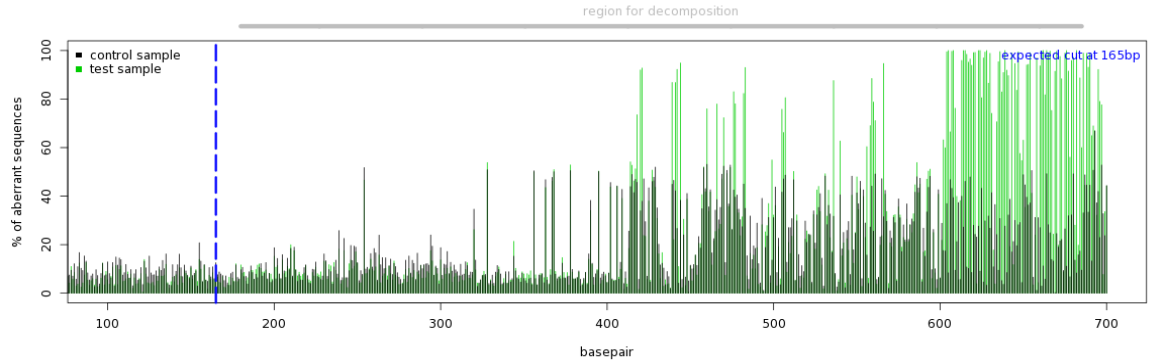
Depiction of the sucrose cushion used for protoplasts purification. a) Protoplasts suspension layered on top of 21% sucrose solution. b) Successful separation of viable protoplasts with a sucrose cushion. c) Failed separation of viable protoplasts with a sucrose cushion.

Appendix 11 – Output graphics from TIDE analysis for WI P196

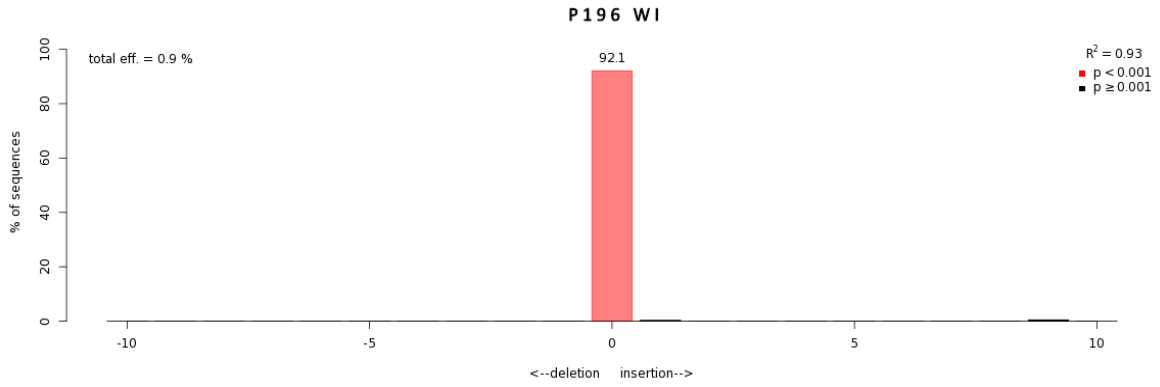
Indel Spectrum



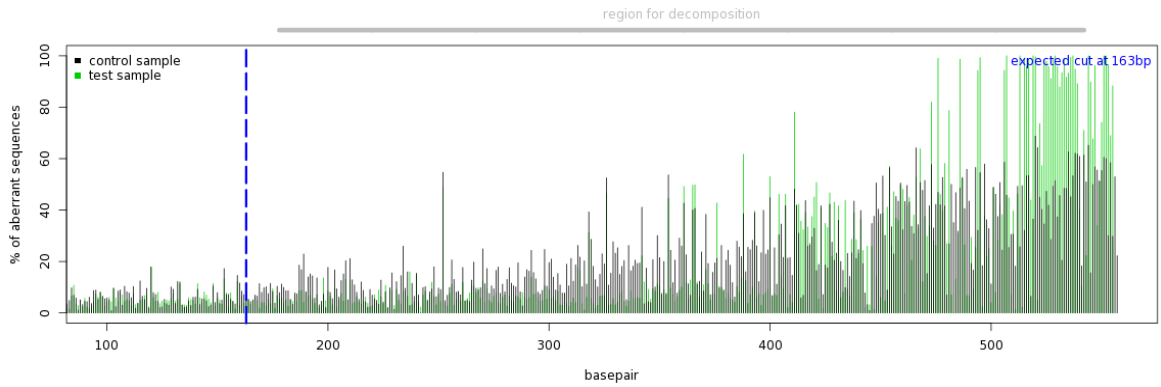
Quality control - Aberrant sequence signal



Indel Spectrum

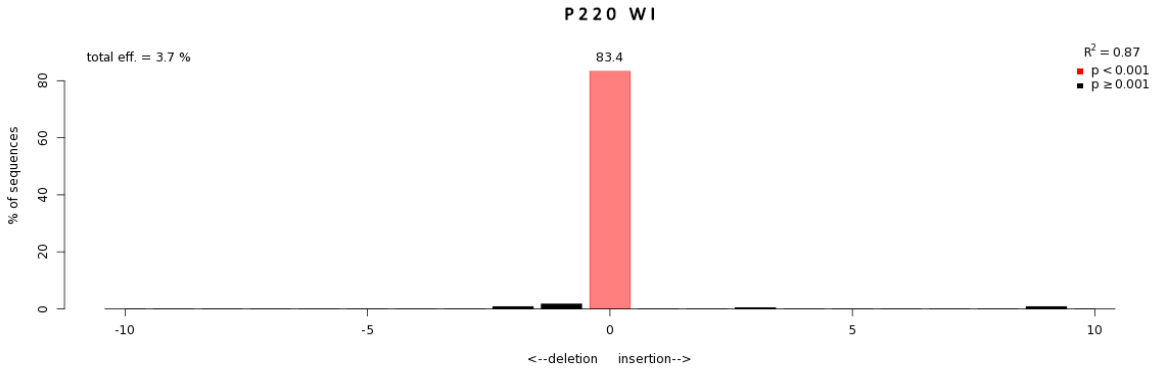


Quality control - Aberrant sequence signal

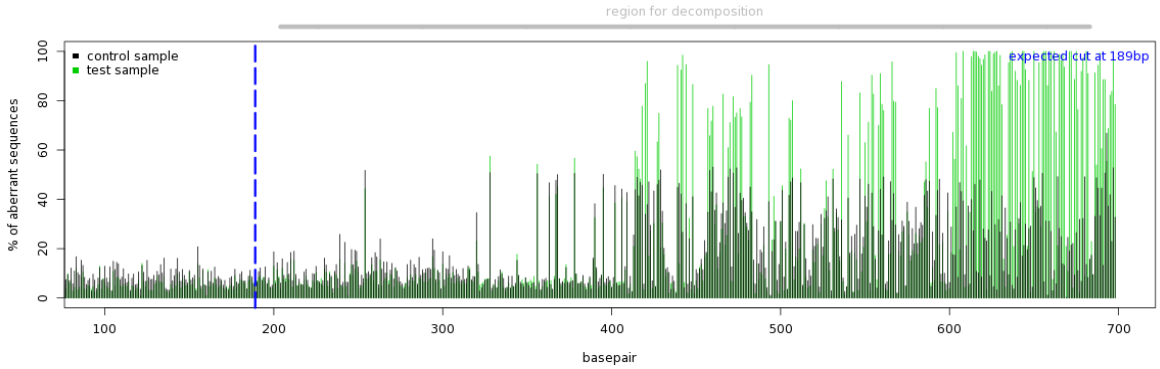


Appendix 12 – Output graphics from TIDE analysis for WI P220 1

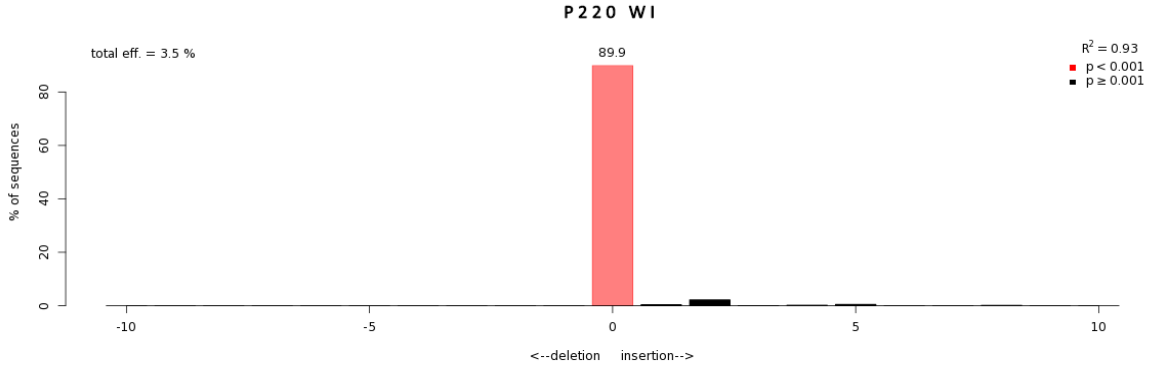
Indel Spectrum



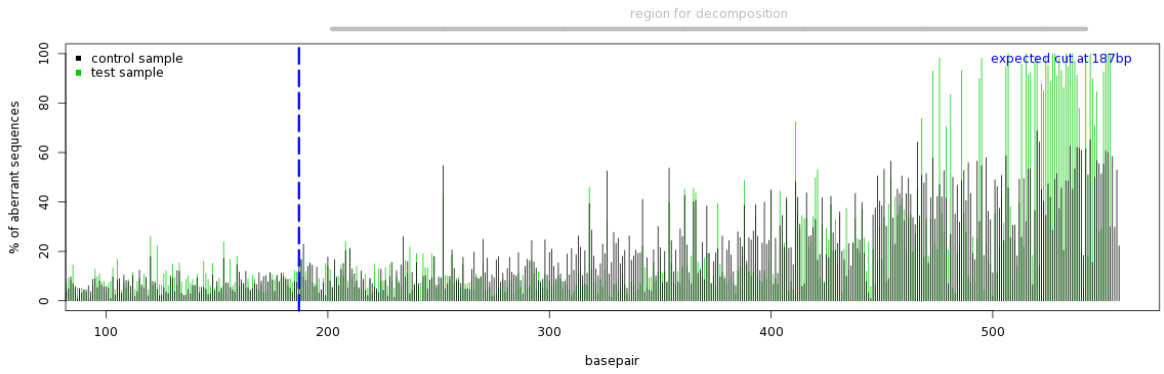
Quality control - Aberrant sequence signal



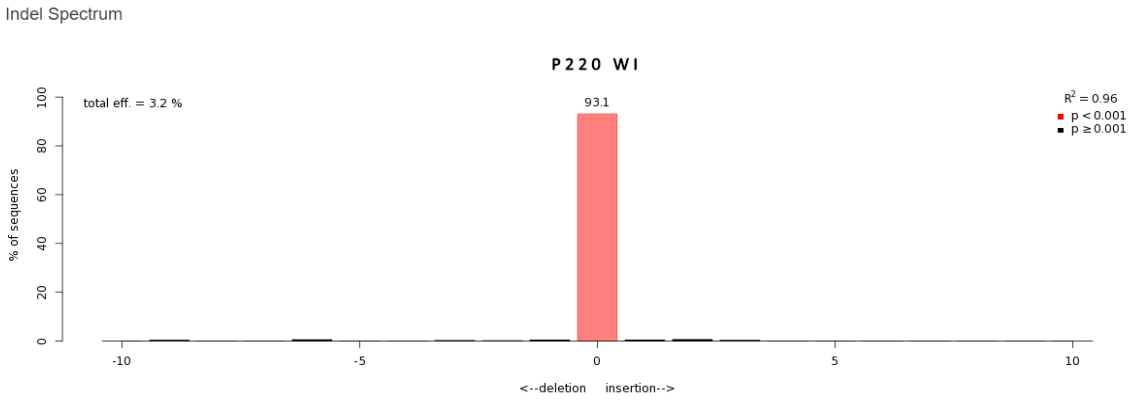
Indel Spectrum

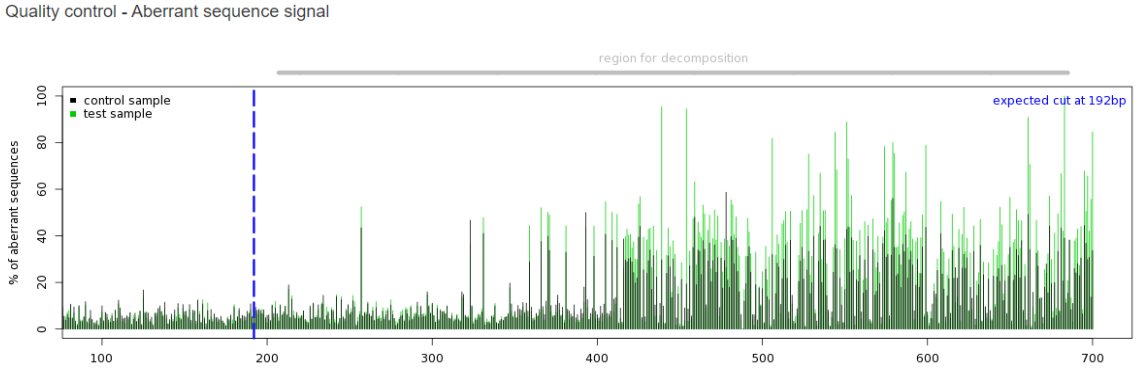


Quality control - Aberrant sequence signal



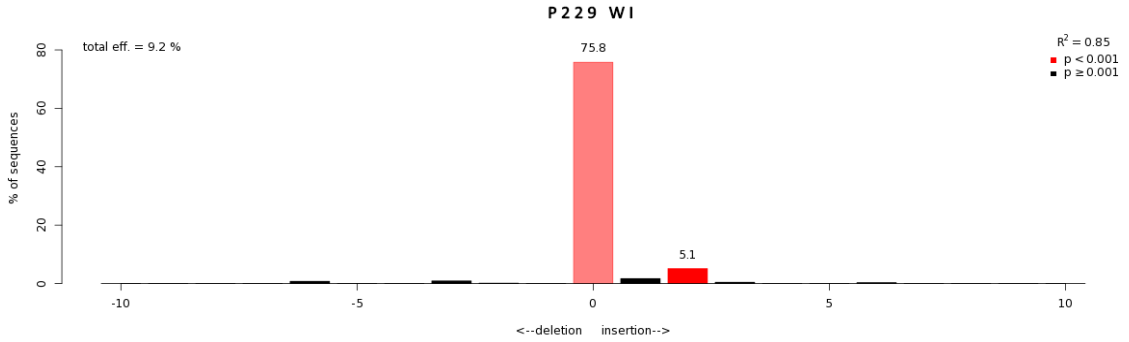
Appendix 13 – Output graphics from TIDE analysis for WI P220 2



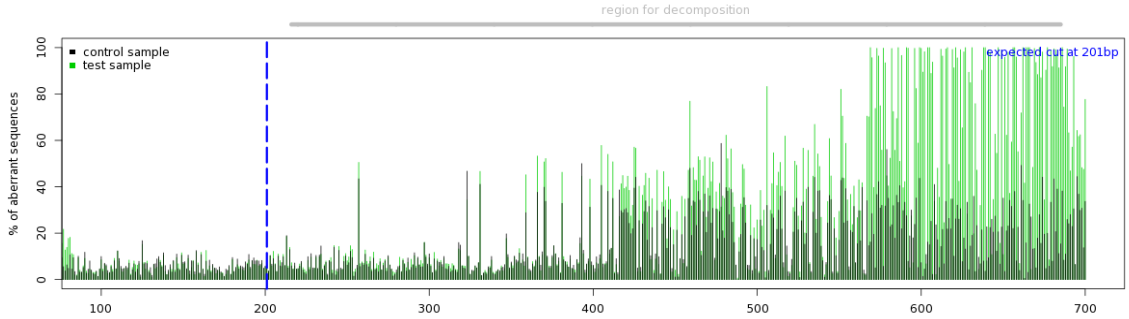


Appendix 13 – Output graphics from TIDE analysis for WI P229

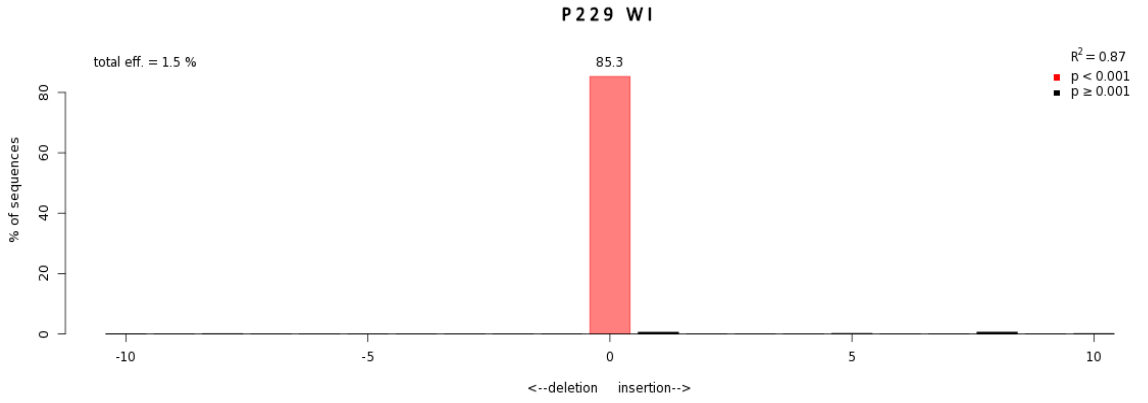
Indel Spectrum



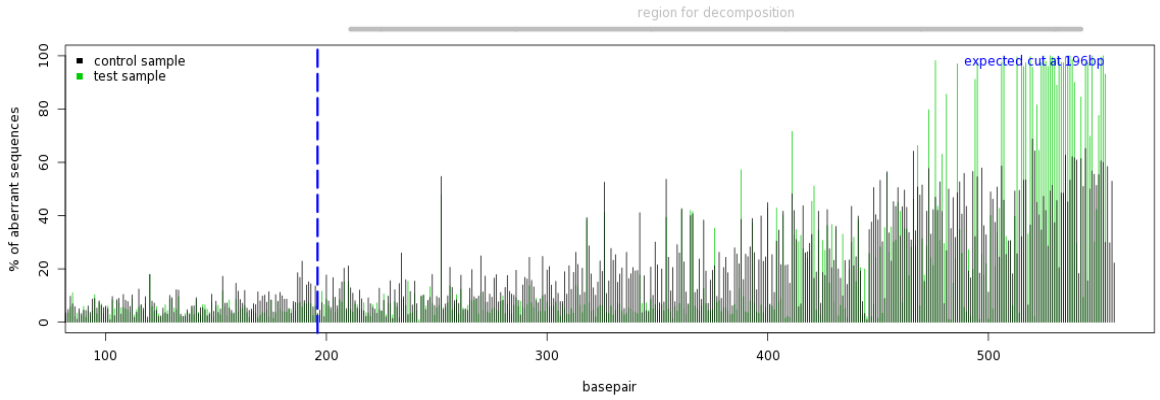
Quality control - Aberrant sequence signal



Indel Spectrum

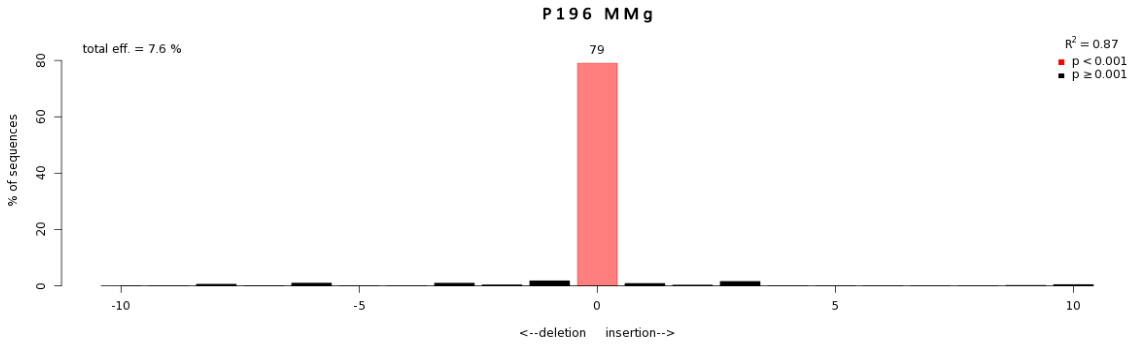


Quality control - Aberrant sequence signal

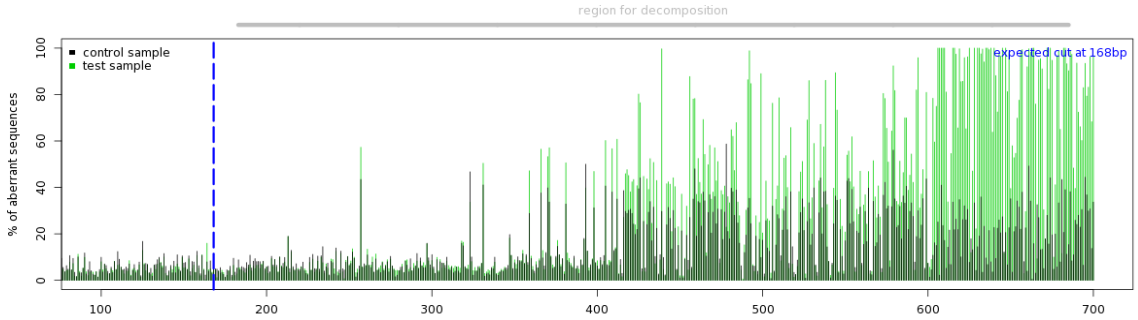


Appendix 14 – Output graphics from TIDE analysis for MMg P196

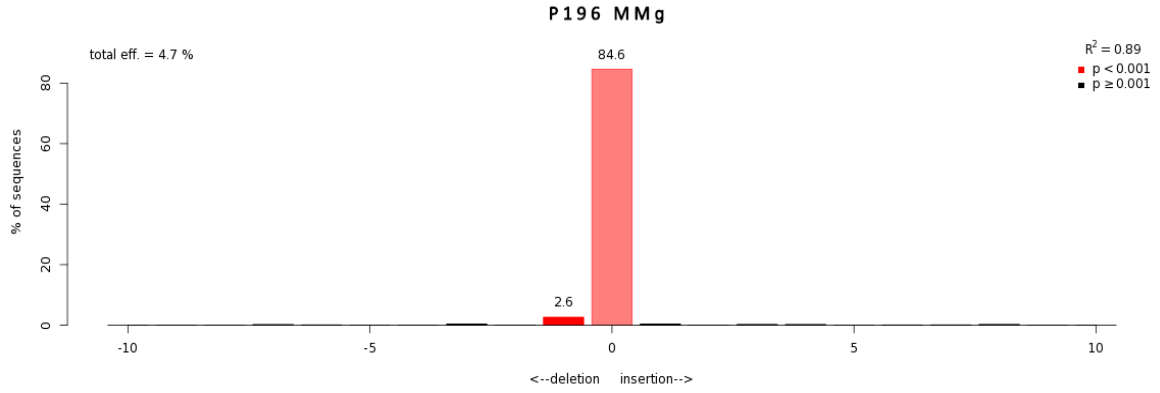
Indel Spectrum



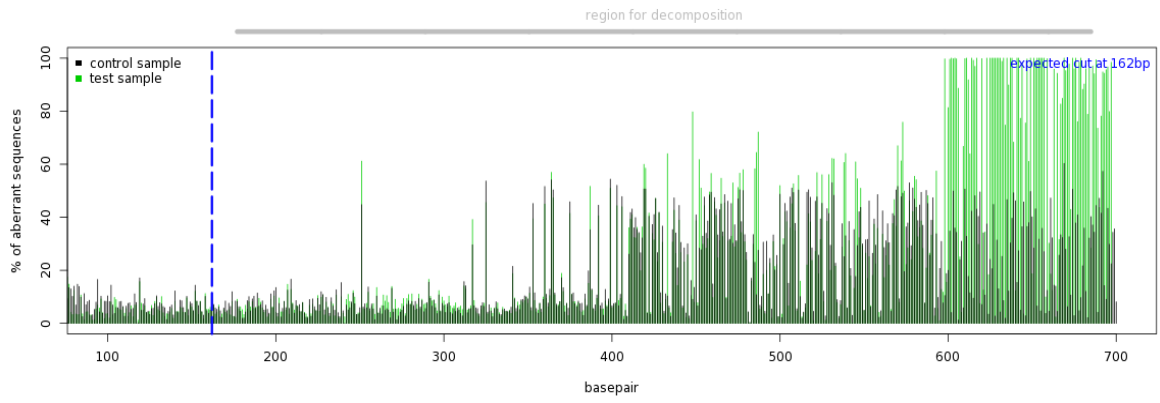
Quality control - Aberrant sequence signal



Indel Spectrum



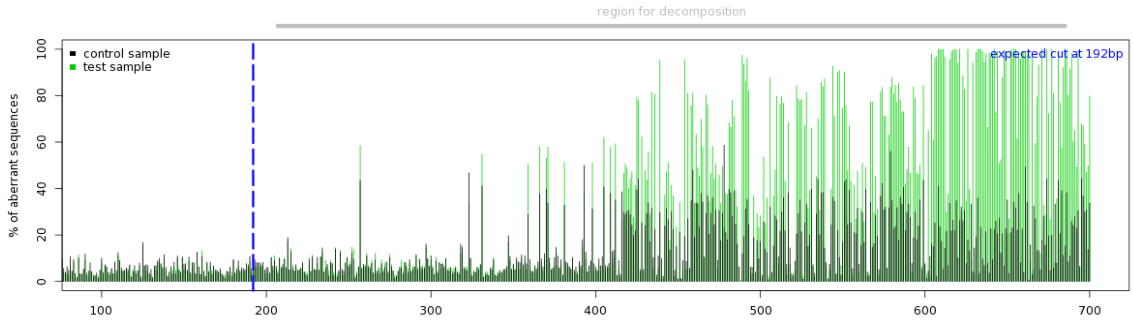
Quality control - Aberrant sequence signal



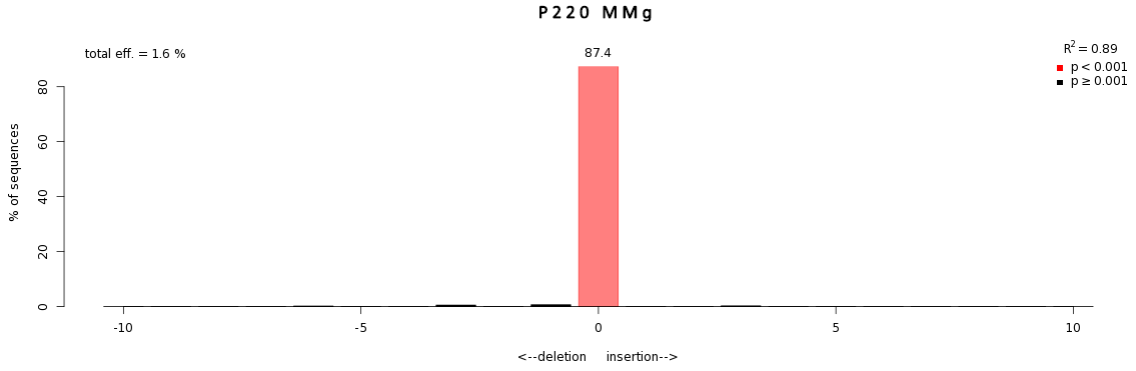
Appendix 15 – Output graphics from TIDE analysis for MMg P220

Indel Spectrum

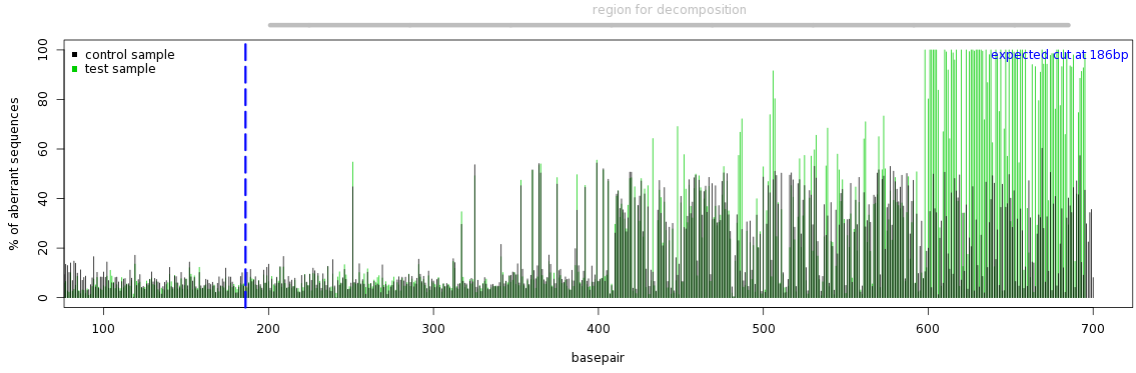
Quality control - Aberrant sequence signal



Indel Spectrum



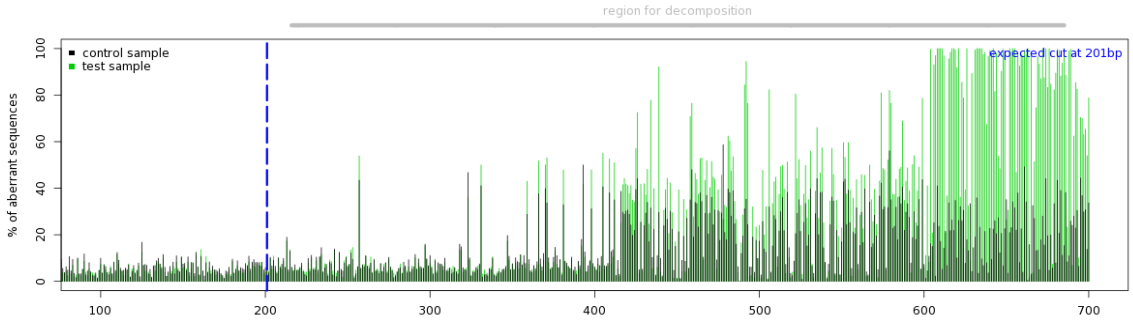
Quality control - Aberrant sequence signal



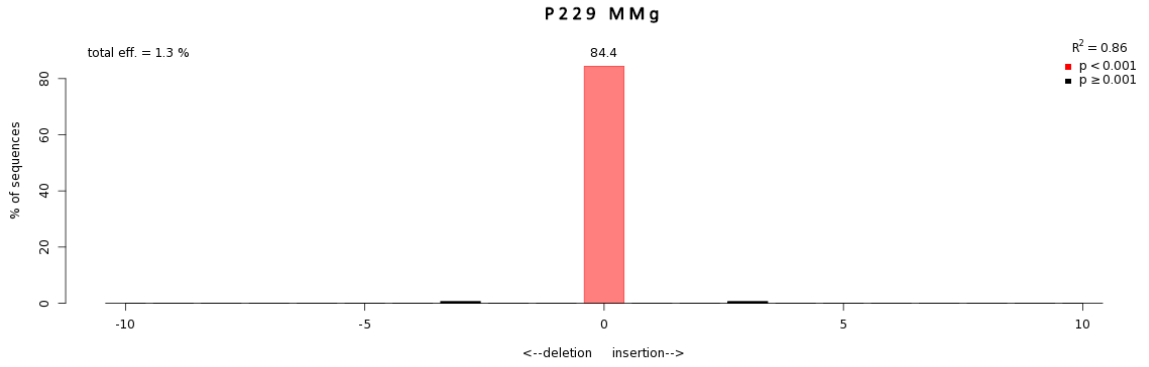
Appendix 16 – Output graphics from TIDE analysis for MMg P229

Indel Spectrum

Quality control - Aberrant sequence signal



Indel Spectrum



Quality control - Aberrant sequence signal