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DNA-free Genome Editing of *Solanum tuberosum*. A CRISPR/Cas9-mediated Proteolistic Approach for Novel Crop Production

Thesis presented by

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for the degree of

Master of Research

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Abstract:

Climate change is an ever-growing concern for global food security and crop production. Further adding to these issues is population growth and legislative changes governing the availability of agrichemicals for pest control. Novel crop production methods, which include genetic modification, must be considered for addressing these global food security issues. This project focuses on using one such method, CRISPR/Cas9, to alter the genome of the potato crop, *Solanum tuberosum.* The importance of potato cannot be overstated, being the third-largest food crop globally, and historically tied to Ireland. The rapidly changing climate and demand for increased yields calls for improvement to the potato, which historically has proven difficult to enhance, due to its tetraploid nature.

The project specifically targeted the *SGT3* gene, (*Rhamnose:beta-solanine/beta-chaconine rhamnosyltransferase*), a key gene involved in the production of solanine. The solanine production pathway is crucial to glycoalkaloid synthesis. While low levels of glycoalkaloids contribute to the flavour of potato, higher levels are toxic to humans and it is estimated that 14-27% of the U.S. potato crop is rejected due to dangerous glycoalkaloid levels. By inducing a small, 20 base-pair, deletion in the *SGT3* gene, it is anticipated that its function would be disrupted, thus causing glycoalkaloid production to be supressed at an early stage of the pathway. In order to conduct this transformation in a "DNA-free" manner, a modified version of the CRISPR-Cas9 system was used, in which synthetic Cas9 endonuclease and synthetic sgRNA molecules were combined to form a ribonucleoprotien (RNP) complex. This RNP was then delivered to the plant tissue via particle bombardment. Following this, some plants were used for DNA analysis while others were subjected to regeneration protocols. A deletion in the *SGT3* region was not detected *in vivo*, but was successfully digested *in vitro,* and important steps have been taken to ensuring this method will be more successful in the future. In addition, studies were conducted to examine how two cultivars of potato, Golden Wonder and Maris Piper, grow when exposed to different wavelengths of light and longer photoperiods. The strongest growth response was observed when plants were grown under various combinations of blue and red wavelengths.

Introduction:

Overview on Food Security:

The global population is facing into significant environmental change. Global food security and sustainable crop production are ever-present challenges posed to the worldwide community. Conventional crop breeding must be changed in order to meet the needs of a rapidly rising population and changeable climate. In order to do this, plant genetic engineering must be utilised and combined with existing techniques to advance our ability to create new crops which will be tolerant to the pressure of new environments. Climate change heralds many different effects, some of which include increased rainfall and sea-levels, warmer temperatures and increased likelihood of severe weather. All of these factors influence our ability to grow crops to feed the world.

Furthermore, the global population is rising dramatically, and the ability for the human race to feed itself is declining. It is predicted that between 2010-2025 the urban population will increase by more than 1 billion people, but the rural population, those which work primarily in agriculture, will hardly increase. This will put a much greater demand on the agriculture industry to feed the ever-rising population (Buettner, 2015).

Although our ability to produce food has increased, millions are still going hungry. Hunger ranges in cause, from macro- to micro-nutrient deficiency, as well as sudden food shortages caused by changeable weather patterns. It is estimated that one in seven people do not have sufficient access to protein and other forms of energy in their diet, while micronutrient deficiencies, such as zinc and iron, are even more prevalent, estimated at 2 billion people worldwide (Wheeler, 2013) (FAO, 2009).

It is predicted that over the next 50 years the demand for food is set to double, while the land available to grow this food decreases due to a myriad of different factors which include soil erosion, desertification, flooding, unsustainable agriculture practices and climate change (World Bank, 2007). With the predicted rise in urbanisation, new crop variants must emerge which are more tolerant to adverse weather, resistant to pests, have increased nutritional content and longer shelf life.

Current Perspectives on Genetically Modified Crops:

The promise of sustainable food production offered by the use of genetically modified (GM) crops cannot be ignored. However, significant barriers prevent its widespread use. Primarily, there is the noteworthy cost and difficulty in creating these crops, requiring time and refined expertise. Furthermore, government legislation, especially in the European Union, prevents many of these crops from getting to market due to the nature of their creation. There is a spectrum of different traits that GM could confer onto crops, and while it is believed that the genetic ability for most crops to naturally increase their yield has already been reached, there are many other ways that these crops can be improved (Keshavareddy, et al., 2018).

Plants which generate their own resistance to pests like insects are one such example. This allows for a reduction in chemical sprays, which can damage the surrounding environment, while remaining harmless to those who consume these crops. Maize is one crop which has been genetically engineered to produce Cry proteins, which are naturally expressed by the bacterium *Bacillus thuringiensis,* and are capable of killing specific insects which damage the crop, thus allowing these pest populations to be controlled. By using different variations of Cry proteins, it is possible to slow down or even halt the ability for these pests to develop resistance to these proteins (Carzoli, et al., 2018).

Improving the shelf-life of crops for consumption is another way by which GM can impact food security. Either by downregulating genes which cause crops to go off, or introducing new genes, it is possible to extend the overall shelf-life. One example is the “purple-tomato”, which involves the expression of two genes from snapdragons, *Del* and *Ros1*, which encode transcription factors that increase the production of anthocyanin. By expressing these genes it was observed that these purple tomatoes stayed ripe for more than twice the time of a normal tomato. In addition to having a longer shelf-life, the consumption of anthocyanin offers protection against several different human diseases, including cancer, implying these purple tomatoes may also offer a dietary benefit to the consumer (Zhang, et al., 2013, Butelli, et al., 2008).

The benefit of GM crops is not just limited to the production of edible crops. It is possible to produce crops which can be used in the biofuel industry, an important alternative to the use of fossil fuels (Howard, et al., 2013).

While these examples highlight how GM can be beneficial, the legislation surrounding such organisms is controversial. The laws which govern the use of GM vary widely across the globe, with significant differences in the European Union, United States of America, China, Asia, and more. Over 25 years have passed since the Flavr Savr™ tomato, the first GM crop, came to market and since 1994, the total land area given to growing GM plants has risen to 185 million hectares. This stands at approx. 12% of the total worldwide cropland (Baranski, et al., 2019).

Many countries currently growing GM crops are less developed, and the prospects of growing GM gives these farmers from a poorer economic background a foothold in the agriculture industry. Even within these countries there are inconsistencies with how GM is controlled. In summary, GMOs across the globe are in a constant state of flux, influenced by politics and public perception. This perception varies across the world, countries like the USA readily accept new GM foods, whereas Europe tightly controls their use and marketing.

In order for GM to advance to a meaningful level of production, the public trust must be won and GM must be perceived widely as an acceptable complementary method of crop production.

Solanum tuberosum, a Vital Crop for the Global Population:

Potato (*Solanum tuberosum*) is the fourth most produced non-cereal crop worldwide. Being rich in a variety of macro- and micronutrients, it is a vitally important staple in the global food production chain. It is a seasonal crop, mainly produced in the northern hemisphere, with China being the biggest producer (20% of the total world potato stock). Due to its capacity to produce high amounts of starch, it is also a candidate for use in the biofuel industry (Zabed, et al., 2017).

There are a variety of issues that affect potato, which vary from hindering the growth to devastating the harvest, amongst them are late-blight. In addition, food crops like potato can occasionally produce and accumulate undesirable compounds, some of

which are toxic to humans. The production of glycoalkaloids by the potato is one such example. Glycoalkaloids are a group of alkaloids with sugars attached, usually found within potato in the form of alpha-solanine and alpha-chaconine. High levels of these glycoalkaloids can result in a bitter taste and are also toxic to humans. Symptoms of high level exposure include intestinal discomfort, fever, nausea, vomiting and diarrhoea, and in cases of acute toxicity, death (Omayio, et al., 2016). Currently, the recommended level of glycoalkaloids in potatoes is 200 mg/kg fresh-weight. High levels of these compounds can result in large yield loss (Schrenk, 2012). Many factors influence the levels of glycoalkaloids within a potato, some include genetic and geographical, and post-harvest can include transport, wounding, light exposure, storage and temperature. Functionally, they are important to the potato for pest and pathogen defence, involved in its protection against worms, fungi, insects and bacteria (Chen & Miller, 2001).

Theoretically, reducing the amount of glycoalkaloids produced by the potato could lead to increased yields and safer consumption of the crop. However the detrimental effect it may have on the crop should be considered.

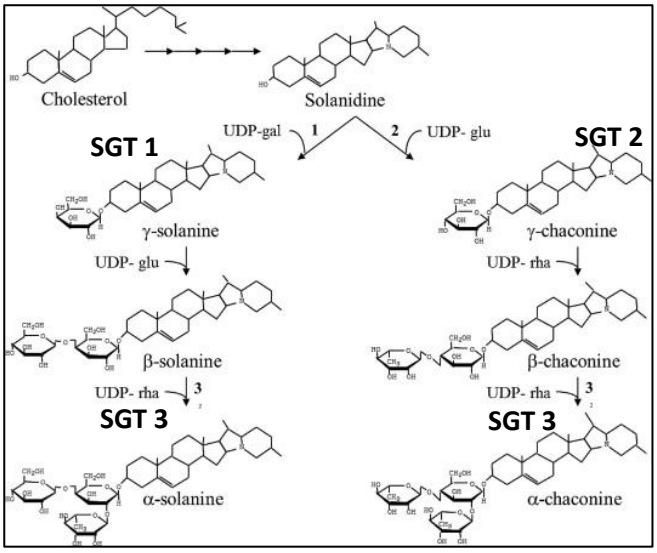


Figure 1: Diagram illustrating the solanine production pathway in potato. The three key genes, SGT1 SGT2 and SGT3 are shown at their respective positions in the pathway. (Shepherd, et al., 2015)

There have been extensive efforts made to downregulate the production of these glycoalkaloids, and with the use of GM, this process can become easier and more efficient. The main glycoalkaloids produced in potato are α-solanine and α-chaconine

(see Figure 1). There are three key genes involved in this biosynthetic pathway, as highlighted above in Fig. 1 (Shepherd, et al., 2015), *SGT1*, *SGT2* and *SGT3*. *SGT1* and *SGT2* are involved in the early stages of this pathway, and studies involved in the downregulation of either of these genes has resulted in a compensatory increase in the production of the product of the other (α-solanine and α-chaconine respectively). However, targeted downregulation of *SGT3* (*Rhamnose:beta-solanine/beta-chaconine rhamnosyltransferase*), the last gene in this pathway has shown downregulation in both alpha-solanine and alpha-chaconine (McCue, et al., 2005). For these reasons, *SGT3* could be a viable target for genetic modification, its downregulation allowing for overall lower levels of harmful glycoalkaloid production.

As potato is a tetraploid organism, which would normally make genetic engineering quite challenging, the low copy number makes *SGT3* relatively accessible for genetic modification. *SGT3* has a 1515 bp open reading frame which encodes for a predicted SGT3 amino acid sequence. There are similarities between *SGT3* and its predecessor, *SGT1*, its encoded amino acid sequence is 18 residues longer than *SGT1*, and is 45% identical to it (McCue, et al., 2007). The expression of *SGT3,* and indeed its precursors *SGT1* and *SGT2* is upregulated when exposed to constant fluorescent light, as demonstrated by (Zhang, et al., 2019). Many of the promoters which drive the expression of these genes, as well as genes responsible for disease resistance, contain light response elements as well as response elements to disease and stress conditions, indicating that this overall pathway can be driven by these key factors. It would seem from investigations such as these that the glycoalkaloid pathway is innately tied to biotic and abiotic stress response, and while detrimental to the consumer, these glycoalkaloids may play a very important defensive role for the potato.

Approaches for the Genetic Engineering of *Solanum tuberosum*:

In the last two decades there have been many crops which have been successfully genetically modified to produce beneficial traits. Broadly speaking, these compromise modifications to food crops, biofuel crops or crops which can be used in the pharmaceutical industry. Potato as a candidate for GM poses some challenges. As a tetraploid organism, inheritance of novel traits can be quite difficult due to the

various possible chromosomal combinations. Recently, efforts have been made to produce diploid potatoes, which have been shown to have some benefits to efforts regarding genetic modification, making inheritance and propagation of GM traits easier, but these crops have issues such as inbreeding depression and genetic load. Accumulation of deleterious mutations causes weaker crops to be produced, so, in order for diploid potatoes to be seriously considered, these mutations must first be eradicated (Zhang, C., et al., 2019).

Potato has already been the subject of successful genetic modification by many different groups. Some of these successes include the addition of the *AmA1* gene (Amaranth Albumin 1) into potato via *Agrobacterium* transformation for increased production of key amino acids for growth and development, (Chakraborty, 2010) and the addition of three separate transgenes to upregulate oil production in potato in order to produce an alternative to rapeseed and palm oil (Liu, et al., 2016).

In this project the gene of interest (GOI) was the aforementioned *SGT3*, due to its low-copy number and high conservation across the four chromosomes. By inducing a targeted knockout of this gene it is anticipated that the glycoalkaloid pathway will be disrupted, supressing the overall production of glycoalkaloids. Creating a genetic modification in a plant of any kind generally involves the use of site-specific nucleases (SSNs), proteins which are capable of digesting a fragment of DNA, which is then repaired by the cellular mechanisms of the plant, usually non-homologous end joining (NHEJ). Before the discovery of CRISPR/Cas9, nuclease-based systems such as zinc-finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs) were systematically used (Puchta, 2017). However with CRISPR/Cas9 becoming a revolutionary method for genome editing, it is possible to create genetic change within a plant in an entirely DNA-free way. This method is also much faster than its predecessors, as the engineering of complex TALENs and ZFNs is a time consuming process and highly specific. Typically, the CRISPR system involves synthetic guide RNA molecules (gRNA) combined with the Cas9 endonuclease in a ribonucleoprotein (RNP) complex. This RNP complex is delivered to the cell where it can cause targeted mutagenesis at the GOI and is then degraded. Provided a suitable delivery system is employed, this method does not involve the integration of any

foreign DNA in the process (Metje-Sprink, 2019). Outlined in Fig 2 is a comparison of previous *Agrobacterium* based delivery techniques vs. the DNA-free RNP method (Metje-Sprink, et al, 2019). DNA-free systems allow for efficient genome engineering without the integration of foreign DNA, meaning the only genetic change involves the host genome and does not include the introduction of novel DNA into the host at any point, one of the main advantages the approach has over *Agrobacterium* based methods.

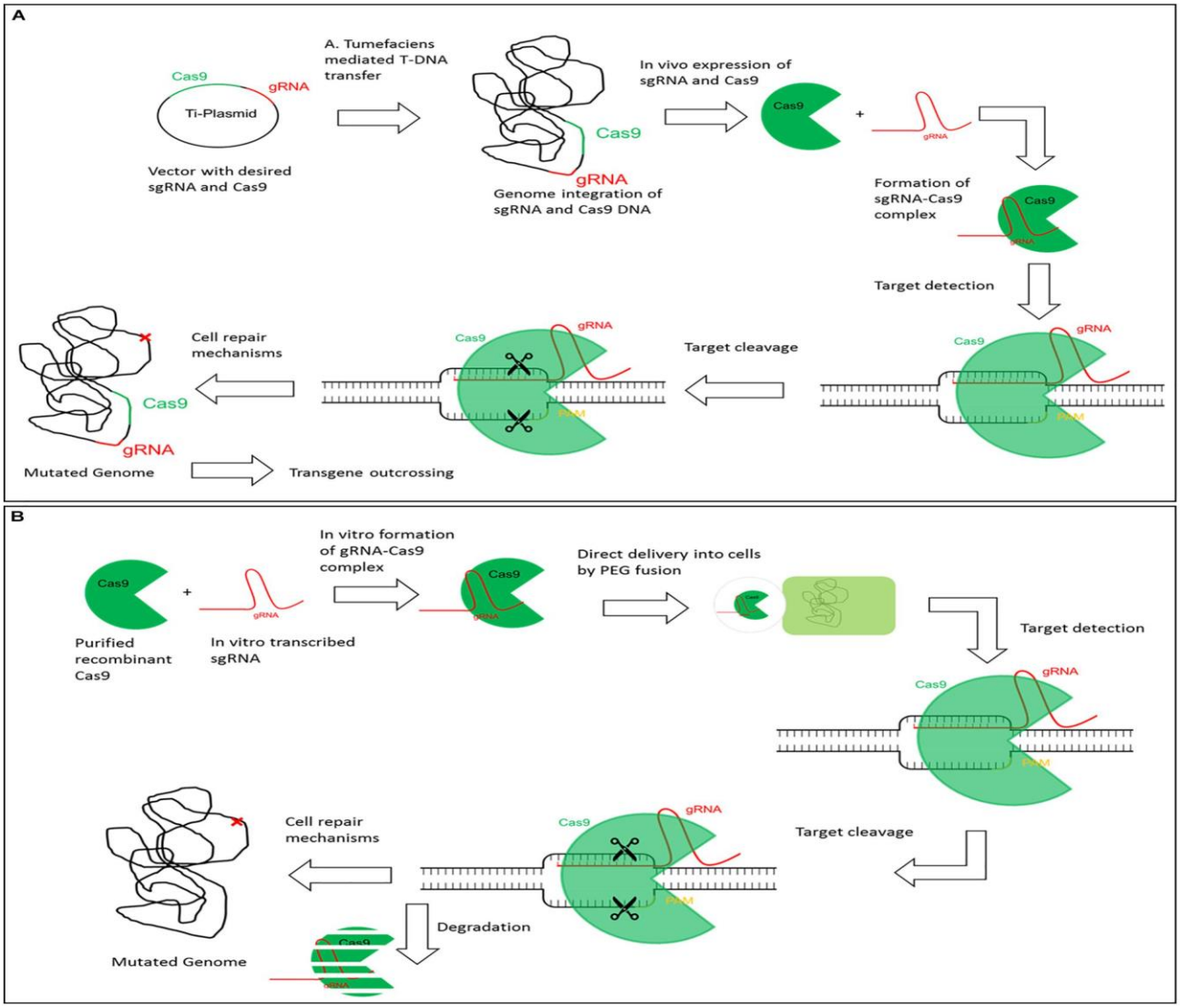


Figure 2: Diagram of Agrobacterium mediated CRISPR Cas9 transformation vs. DNA-free RNP method. (Metje-Sprink, et al, 2019)

The CRISPR system exists naturally in many bacteria as a defence mechanism against phage invasion (Marraffini & Sontheimer, 2010), and though it had been known for decades, only recently has it been exploited as a mechanism for genetic modification (Jinek M, 2012). Typically, in these DNA-free systems, delivery relies on a handful of

mechanisms. These include PEG-fusion, electroporation and biolistics. To date, these transformation techniques have been used successfully across a range of plant species, including potato. *Agrobacterium* based transformations of the potato genome have also been reported (Wang, et al., 2015) as well as transiently expressed CRISPR vectors which generated a knockout of the granule bound starch synthase gene (GBSS) in potato protoplasts (Andersson, et al., 2017).

PEG-mediated genome editing of potato protoplasts with RNPs has also been successfully reported (Andersson, et al., 2018). While the efficiency of these techniques is generally lower than *Agrobacterium* mediated transformation, there is a much lower occurrence of non-targeted modification, as well as being a method which does not utilise foreign DNA at any stage of the process (Kim, 2017).

This project focuses on delivering this RNP complex via particle bombardment. This technique is largely based off the methods established by Martin-Ortigosa & Wang, (2014) and Liang, et al., (2017). Termed “proteolistics”, the technique revolves around the deposition of the RNP complex on a micro-carrier (typically either gold or tungsten micro-particles, 1-3 µM in size), where it is dried at room temperature. It is then bombarded into the plant tissue, allowing the RNP complex to enact its DNA-editing ability.

Martin-Ortigosa and colleagues shows that a variety of different proteins, ranging from eGFP (enhanced green fluorescent protein) to cell-death inducing trypsin can be successfully dried down onto these micro-carriers and bombarded into leaf tissue while retaining their functionality (Martin-Ortigosa & Wang, 2014). As a DNA-free integration system, the gRNA molecules are either created synthetically in a suitable quantity or produced *in vitro.* The Cas9 used is also synthetic, and thus there is no foreign DNA present in a vector or otherwise which could integrate into the plant genome.

The proteolistic method has already been successful in a number of experimental attempts to generate herbicide resistance in rice crops (Sun, et al., 2016) and has also

successfully been used in the genetic engineering of wheat and maize (Svitashev, et al., 2016).

Approaches for "Speed-breeding" *Solanum tuberosum*:

One issue often encountered in plant-based scientific research is the time taken to cultivate plant stock. This is especially relevant in plant genetics, as often the different generations of plants need to be studied to examine the heritability of genetic traits. One way of overcoming this bottleneck is altering the growth conditions for plants. Factors such as volatile organic compounds, either synthetic or produced by bacteria, could be introduced to the plant. Alternatively, the amount and type of light a plant receives can be changed. The effects of different wavelengths of light and different photoperiods on a variety of plants have been extensively studied (Sysoeva, et al., 2010) and there are detailed protocols on using LED units for "speed-breeding", a term used to describe the general acceleration of a plant's growth, generally achieved by increasing photoperiods or by exposure to growth stimulants (Ghosh, et al., 2018). These studies have shown that continuous light is capable of speeding up the growth cycle for plants. Furthermore, by implementing different wavelengths of light such as Red, Far Red and Blue, the growth response of plants can differ. In this project the effects of longer photoperiods and different wavelengths of light on *Solanum tuberosum* were examined.

Project Summary:

The principle goal of this project was to test the capabilities of proteolistics and investigate if it is a suitable alternative to *Agrobacterium* based transformation methods. The project attempted to target and transform the gene *SGT3*, utilising three distinct sgRNA molecules combined with synthetic Cas9 endonuclease, which were bombarded into plant material via proteolistics. The growth response of *Solanum tuberosum* was also tested under different photoperiods and wavelengths of light using LED units. Previous papers have suggested that certain wavelengths upregulate genes, specifically red light inducing the expression of *SGT3.* The overall growth response was measured to see if there is a way to quickly produce samples which can be bombarded. The recovery and regeneration capacity of these plants was testsed on plants post-bombardment, and samples were genetically analysed to

test for *SGT3* knockouts. The overall aim of this project was to test the effectiveness of proteolistics in *Solanum tuberosum*, analyse the regenerative capacity of *Solanum tuberosum* post-bombardment and observe changes in growth patterns of *Solanum tuberosum* when exposed to different photoperiods and wavelengths of light.

Materials and Methods:

Section 1: Growth and Cultivation of Plant Stock:

Establishment of Plant Stock:

Solanum tuberosum cultivars, Maris Piper and Golden Wonder, were clonally propagated via nodal culture and grown in Murashige & Skoog media.

Table 1.1: M&S Media Ingredients for Plant Tissue Culture

Ingredient	Quantity (g/L)
Murashige & Skoog salts and vitamins	4.4
Sucrose	30
Agar	12

In all cases when propagating plant stock, media (media composition detailed in Table 1.1) was brought to pH 5.8 and autoclaved before being poured into micro-pots (approx. 20 pots per litre) and allowed to solidify. Internodal tissue culture was carried out as per (AGENCY, 2004). Following tissue culture, plants were transferred to the growth room (16hrs light, 8hrs dark, 22°C) and typically allowed to grow for 4 weeks before being re-established. These micro-plants were first grown in smaller micro-pots to avoid contamination and once a large stock was established it was maintained in larger micro-boxes.

LED Growth Experiments:

To test the growth responses of the two cultivars to different wavelengths of light, a separate growth chamber was set up where the explants were grown under specific wavelengths for a period of time. Following this, fresh weight, stem length, leaf number and dry weight were recorded.

Plants were cultivated following the same method described in Section 1 (Establishment of Plant Stock). Groups of four micro-pots (two micropots of each cultivar and four plants per pot) were prepared for each experiment. Plants were exposed to different wavelengths of light (see Table 1.2) from a Heliospectra LX601C 630W LED grow light unit for four-week periods. During this time they were exposed to light for 18 hrs per day and darkness for 4 hrs per day. Following this four-week period the plants were removed and a new cycle was begun which introduced new plants and a new wavelength. The plants were then taken for analysis. The leaf

number, stem length and fresh weight of each plant were recorded. Following a two-day drying period at 40°C the dry weight was then measured.

Table 1.2: LED Wavelengths Tested:

LED Colour	Wavelength (nm)
Blue	450
Red	680
Far Red	700-780
White	400-700
Far Red : Red (25:75)	680 & 700-780
Red : Blue (75:25)	680 & 450
Red : Blue (25:75)	680 & 450
Red : Blue (50:50)	680 & 450
All Colours (100%)	400-800

Data analysis of LED Results:

Measurements taken from the plants grown under LED conditions were subject to data analysis. The analysis focused on four variables, stem length, leaf number, fresh weight and dry weight. Firstly, an ANOVA test was carried out on each variable data set (eg. Leaf Number) to test for the significance of the data using software Microsoft Excel (Excel version 1910). Following this, independent sample T-tests were carried out to test for the significance of these results ($P < 0.05$) using software IBM SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp).

Section 2: Protein Extraction and Western Blots:

Initial Western Blots were carried out to detect the presence of Actin in leaf and stem tissue, as well as tissue from shop-bought tubers.

Establishment of BSA Standard Curve:

A 10% weight/vol. stock solution of BSA (bovine serum albumin) was prepared and used to set up a dilution series (10 µg/µl, 5 µg/µl, 2 µg/µl, 1 µg/µl). The absorbance of each BSA dilution was determined in a 1 ml plastic cuvette by mixing 200 µl of Bradford reagent with 800 µl of Mili-Q water, adding 1 µl of each dilution series. Samples were covered with Parafilm and mixed well. Absorbance at 595nm was measured. A graph of BSA concentration (x-axis) against Absorbance value (y axis) was plotted. The linear regression was then calculated [Absorbance (y)= Slope x Protein conc. (x) + constant value (when x = 0)] and the protein concentration was

determined using the following equation: Protein conc. (x) = [Absorbance (y) – Constant value]/slope]

Protein Extraction Method 1 from Tuber Samples:

The protein extraction method was based on (Delaplace, et al., 2006) and modified slightly (see Table 2.1). However, problems ensued with the following Bradford test due to the presence of PVP (polyvinylpyrrolidone), so a new protocol was later devised.

Table 2.1: Protein Extraction Buffer 1

Buffer	Stock	50 ml	2 ml
4% SDS	8% (2x)	25 ml	1 ml
5% Sucrose	50% (10x)	5 ml	200 µl
10% PVP	50% (5x)	10 ml	400 µl
20mM Sodium Phosphate	200mM (10x)	5 ml	200 µl
0.3% DTT	3% (10x)	5 ml	200 µl

For each extraction, liquid nitrogen was poured into a small mortar to cool it. The level of liquid nitrogen was maintained as the potato tuber (shop-bought) was chopped into small (approx. 1 cm square) pieces. The tuber was then ground using a pestle as liquid nitrogen was constantly added. When the potato was crushed to a fine powder, Eppendorfs were pre-cooled by submerging in liquid nitrogen. The tuber powder was added to these Eppendorfs which were then stored at -80°C. The protein extraction buffer was then made according to Table 2.1 and preheated in a water bath at 65°C. After 10 mins the tuber samples were removed from -80°C storage and the buffer was added at a 1:1 ratio of powder and buffer. These samples were then incubated at 65°C for 10 mins. Following this incubation, the samples were cooled on ice for 15 mins. 2 µl of sample was then added to a cuvette with 1 µl Bradford reagent and their absorbance was measured at 595 nm.

Protein Extraction Method 2 from Tuber Samples:

Due to the problems encountered with the first method of protein extraction, a new method was devised based largely on (Monte, et al., 1999). The extraction method was then modified to combine both steps into one so a greater amount of protein was obtained (see Table 2.2 for Extraction Buffer modifications).

Table 2.2: Protein Extraction Buffer 2

Buffer	Stock	10 ml	1 ml
100 mM Sodium Phosphate	200 mM	5 ml	500 µl
5% Mercaptoethanol	Added Fresh	500 µl	50 µl
50 mM Tris-HCl	1 M	250 µl	25 µl
100 mM NaCl	1 M	500 µl	50 µl
1% SDS	8%	1.25 ml	125 µl
dH_20	-	2.5 ml	250 µl

Mechanical extraction with liquid nitrogen was carried out as described above. Protein extracts were then homogenised in 100 mM sodium phosphate (pH 7.2), containing 5% 2-mercaptoethanol followed by centrifuging for 10 mins at 13,000 g at 4°C. The pellet was then resuspended in 50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1% SDS and 5% 2-mercaptoethanol. This was then centrifuged for 10 mins at 13,000 g at 4°C. Following this, 5 µl of sample was added to a cuvette with 1 ml of Bradford and their absorbance was measured at 595 nm.

Western Blot Analysis to Detect Actin:

Following successful protein extraction from leaf and tuber tissue, Western Blot analysis was carried out to detect the presence of actin in the sample. Of the extracted protein samples, two were selected for Western Blot analysis, one from tuber tissue and one from leaf tissue. Using the BSA standard curve previously established, protein concentration of these samples was determined using the aforementioned formula. From this, the amount to be loaded for the Western Blot was determined. Western Blot procedure was based largely on (Davis, et al., 1986). Anti-actin (1:5000) and Anti-rabbit (1:10,000) antibodies were used for the Western Blot. Following this, the gel was developed in the dark room for visualisation.

Section 3: sgRNA Design and Testing:

Designing sgRNA and SGT3 Analysis:

SGT3 sequence was obtained from GenBank (Accession number: MF134428). This sequence was compared with previous lab sequences of *SGT3* regions from both Golden Wonder and Maris Piper and it was found that the region was almost entirely identical, indicating high conservation across the gene. The first exon and coding region within were highlighted and based on this, gRNA molecules were designed. The gRNA molecules are highlighted green in Figure 3.1 below. The gRNA molecules were designed using the CRISPRdirect tool (Naito, et al., 2015). The three most efficient guides were chosen with low chance of off-target editing. All three guides were located close to the start of the first exon of *SGT3*, as can be seen from Figure 3.1. 1.5 nmol of each gRNA molecule was supplied synthetically by SYNTHEGO (Synthego USA).

Figure 3.1: SGT3 Sequence and gRNA Design

>NW_006239183.1:c281586-279904 Solanum tuberosum cultivar DM 1-3 516 R44 unplaced genomic scaffold, SolTub_3.0 scf00250, whole genome shotgun sequence

TTGTTAATGGCGATGGAACAGAATGAAGAAACTGCAATGCCGCATGTTGTGTTCATACCATACGCCATGA
CGAGTCATATAACTCCATTGGTACATATTGCTAGACTCTTCGCCCTCCATGGCCTCAAAGTTACTATCAT
TGCCCCTCAGCATAATGCTCTTCTTTTTCAGTCCTCTGTCGATAGAGACCGTCTCTTTTCGGGCAGCAAT
ATTACTGTCCGGACAATTCAATTTCCGTCTGAGGAAGTTGGATTACCTGTAGGAATTGAAAACTTCATCG
CAAGCCCTTCTATGGAAATAGTTGGCAAAGTTCACTATGGGTTTATTCTGCTCCAAAAGATTATGGAGCA
ACTAATTCGGGAGATCAATCCAAACTGCATTGTTTCCGATATGTTCTTCCCTTGGACTGTTGATTTAGCT
GAGGAGATGCAAATTCCGAGATTTTCTTTTCAACCAGCCACTTCCATACATCAATGTGCTTGGGTTTTAA
TTAGGGAATTTAAACCTTACAAGAATGTGGCGTCGGATTCTGAAAGGTTTTTGATTCCTGGTTTGCCTCT
CGACATCAAAATGAAAGTCTCAGAGATTGAAGATTTTCTTAAAGAGGAAACTGAGTACACAAAGACAGTA
GATGACGTTTTACAAGCTGAGGTTCGTAGCCATGGTATTATTCATAACACTTGCTCTGAGCTGGAACCTG
GCGTTGCCCAACTCTACGAAAAAGCTAGAGGAGTAAAAGGGTGGCATATAGGTCCACTTGCTCTGTTTAT
CAACAAATATGAAGCGGAAATTAGTTCTAAACAAATTTCCAATTCGAATATTAATTCATGTTCTGACCCT
TGGAAAGGGTACGGTGATTGTTTCAATTGGCTTGAAAATCAACAACCTAACTCCGTTCTCTTTGTTTGCT
TTGGAAGCATGATAAGATTTTCCGATGATCAGCTTAAGGAAATGGCTGTTGGATTGAAGGCTGCCAACTG
TCCAACTATTTGGGTTTTTAGGGAGCAGGACAAAAATGAAGTAGACGAGAAAGATGAGCATTCTGACTGG
AGCCGTAATGGTTTCAAAGAAATGATTGGGGAAAAGATGTTTATCATCCAAGGCTGGGCACCACAACAAT
TAATCCTGAAACATCGAGCAATTGGTGGATTCTTAACTCATTGTGGTTGGAACTCTATACTTGAGTCTCT
AGCCATAGGTGTTCCATTGATCACATGGCCACTTTTCTCAGACAACTTCTATACCGACAAGCTTTTGGAG
ACACTTGGCCTTGCTATTGGAATTGGAGCAGATGTGTGGAATCCGGGGTTTATATTATCGTGTCCACCCC
TTTCAGGAGAGAAGATAGAGTTGGCCGTCAAGCGTTTAATGAATAATTCAGAGGAAAGTAGAAAAATTAG
AGAAAATGCAAAGTTGATGGCAAAGAAGCTCAAAAGTGCCACTGAAGAAGGTGGTTCCTCTCATTCACAG
CTCATCGGGTTAATTGAGGAGATCAAGCGTTGTGCTTTCAAGAAATCCTCTTGAAATTTTATGTTTTACT
TATCACTTTGAAATAAATTTGGCAAATGGAGTTTGGTCAACATGACTCTATGTATTATGTTAACAATGTA
TCTGGTCTTAATTTTTGTTTAAATGCATGCTTTGCAGTGTCATTTGTCATTAGTGAAACATTAAATCTTA
ATA

T-A Exon
A-A CDS

Preparation of Cas9 Nuclease and sgRNA Molecules:

In order to verify the efficiency of the sgRNA molecules an *in vitro* digestion with Cas9 and the target region was carried out. The method was largely based on the protocol found at Origene.com: "In vitro digestion with Cas9 nuclease and sgRNA."

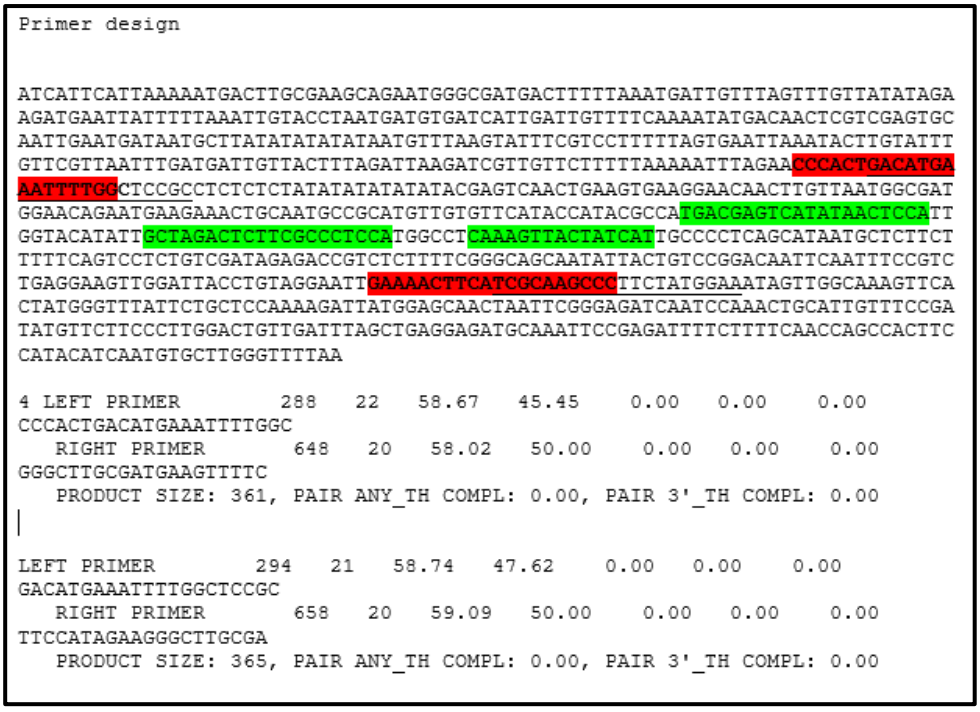
Before the digestion was carried out, the sgRNA molecules were first diluted to a storage stock. The tubes were centrifuged for 5 mins to spin down any sgRNA, during this time Eppendorfs were cooled on ice. 48 µl of nuclease-free water was added to each sgRNA and they were left on ice for 20 mins. Following this, the sgRNA tubes were briefly centrifuged and subdivided into Eppendorfs (6 µl into each). Before the *in vitro* digestion, these sgRNAs were further diluted from their 31 µM stock down to a 1 µM stock. Synthetic Cas9 nuclease was supplied by IDT (Integrated DNA Technologies). Synthetic Cas9 was supplied in a 62 µM stock which was diluted in nuclease-free water to a 1 µM stock prior to the digestion.

DNA Extraction and Purification from Leaf Tissue:

DNA Extraction was carried out as follows. A leaf roughly the size of an Eppendorf lid was collected and grinded with a rod. 400 µl of Edward's Buffer was added and the leaf was ground up further (it is important to avoid air bubbles at this stage). Samples were then vortexed well for several minutes and centrifuged at 13,000 RPM for 5 mins at room temperature. The supernatant was removed to new tubes where 300 µl of isopropanol had already been added. The samples were mixed well with isopropanol by inverting several times and left on the bench at room-temperature for 5 mins. The samples were centrifuged for 10 mins at 13,000 RPM at room-temperature and the supernatant was discarded. 500 µl of 70% ethanol was added and samples were vortexed well for several minutes. Samples were centrifuged at 13,000 RPM for 5 mins at room temperature and the ethanol was then removed without disturbing the pellet. Samples were left to air-dry in the flow hood for approx. 20 mins and resuspended in 50 µl of nuclease-free water. Samples were left at 4°C for 20 mins and the tubes were then flicked lightly to resuspend the pellets. Samples were centrifuged at 13,000 RPM for 3 mins at room-temperature. The supernatant was the extracted DNA.

Following DNA extraction, the region of interest within *SGT3* was amplified using PCR. Two sets of primers were designed on Primer3 (Untergasser, et al., 2012) and supplied by IDT. Figure 3.2 highlights the Primers within the region of interest. The first pair are highlighted red and the second pair are underlined.

Figure 3.2 Primer Design



The PCR reaction components were assembled in PCR wells as follows;

Table 3.1: PCR Ingredients for in vitro Digestion of SGT3 DNA with sgRNA and Cas9

Ingredients	1x	24x
2x Master Mix	10 µl	240 µl
Forward Primer	0.2 µl	4.8 µl
Reverse Primer	0.2 µl	4.8 µl
dH_20	8.6 µl	206.4 µl
DNA	1.0 µl	

A MiniAmp Plus Thermal Cycler from Thermofisher was used for the PCR;

- Stage 1: 95°C for 3 mins, 1x Cycle

- Stage 2: 95°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds, 32x Cycle

- Stage 3: 72°C for 5 mins, hold at 4°C, 1x Cycle.

After the PCR was completed, 1 µl of purple dye was added to each well and they were centrifuged for 10 seconds. 19 µl from each well was loaded into a 1% Agarose gel and allowed to run for 30 mins.

After gel electrophoresis the bands were excised under UV light and weighed in an Eppendorf tube. DNA was then purified using the QIAquick® Gel Extraction Kit (Cat no. 28704) following the manual supplied. After purification the DNA was quantified using a nanodropper and visualised by electrophoresis on a 1.2% Agarose gel. From the amount of DNA purified, the amount of DNA required for the *in vitro* digestion was calculated (approx. 19 ng of DNA in 4 µl).

In vitro Digestion of DNA with sgRNA and Cas9:

The protocol for the *in vitro* digestion is based on methods found at www.origene.com; "In vitro digestion of DNA with Cas9 nuclease and sgRNA".

Table 3.2: Components of the in vitro Digestion Reaction for testing sgRNA and Cas9

Reaction Components	**Quantity**
10x Cas9 Reaction Buffer	3 µl
1 µM sgRNA	1 µl
1 µM Cas9	1 µl
20 nM DNA	4 µl
Nuclease-free Water	21 µl

The Cas9 reaction buffer was prepared (20mM HEPES, 100mM NaCl, 5mM MCl2, 0.1mM EDTA, pH 6.5 at 25°C). The sgRNA and Cas9 were diluted to working stocks prior to their addition to the reaction. The reaction components (see Table 3.2) were then prepared in a nuclease-free PCR tube which had been exposed to UV light for 20 mins. The reaction was mixed thoroughly and briefly centrifuged. The reaction was then incubated at 37°C for 1 hour, then heated at 65°C for 10 mins (deactivates Cas9) and held at 4°C using a MiniAmp Plus Thermal Cycler from Thermofisher. Fragment analysis was then carried out by gel electrophoresis on a 1.2% agarose gel which was run for 30 mins.

This experiment was repeated to test how differing quantities and ratios affected the digestion. In this case, different ratios of Cas9:sgRNA was tested, as well as combinations of sgRNA molecules in a single reaction.

Section 4: Proteolistic Bombardment:

Preparation of Tungsten Micro-carriers:

Before being used as micro-carriers for the CRISPR/Cas9 system the tungsten micro-particles must be sterilised and suspended in solution. The tungsten preparation method was carried out as per the user manual for the Biolistic PDS-1000/He Particle Delivery System by Biorad™.

LED and Pulse Culture Pre-treatment of Solanum tuberosum:

Prior to bombardment the plants were exposed to factors which would improve their regeneration capacity (post-bombardment) and upregulate the expression of *SGT3* to aid with genomic analysis. Leaf Explants (4 weeks old) were taken from *in vitro* plantlets 4 weeks after subculture and cut across the base, discarding the petiole and lower 1-2 mm of the leaf base. The remaining sample was used for bombardment and regeneration. These leaf explants were floated overnight in magenta vessels on a liquid plant growth regulator pulse medium containing MS salts and vitamins, supplemented with 10 g/l of sucrose, 147 mg/l $CaCl_2$, 54 µM NAA and 44 µM BA. During this time the plants were also exposed to red light to induce the expression of *SGT3*. Semi-solid callus induction medium was prepared in micro-pots during this time (MS salts and vitamins, supplemented with 1 g/l sucrose, 4 g/l mannitol, 0.1 µM IAA, 10 µM BA and solidified with 0.8% agar). The following day the plants were removed from the liquid pulse medium and dried on filter paper in the flow-hood for approx. 1-2hrs prior to bombardment.

Bombardment Protocol:

Due to the length of time required to carry out each bombardment group, the bombardments were carried out over two days (see Table 4.1 and 4.2 for details). After day 1 bombardments were completed the plant pre-treatment step was carried out again on the leaf explants to be bombarded on day 2. The bombardment protocol was largely based on two papers (Liang, et al., 2017) and (Martin-Ortigosa & Wang, 2014). Due to the drying-time required for each macro-carrier, each bombardment day was divided into three groups, highlighted below.

Table 4.1: Day 1 Bombardments

Group 1	Group 2	Group 3
x2 Control (Buffer & Tungsten)	x1 sgRNA 1 (2 µg per shot)	x5 sgRNA 3 (2 µg per shot)
x4 sgRNA 1 (2 µg per shot)	x5 sgRNA 2 (2 µg per shot)	x1 sgRNA 1&2 (1 µg each per shot)

Table 4.2: Day 2 Bombardments

Group 1	Group 2	Group 3
x2 Control (Buffer & Tungsten)	x5 sgRNA 1&3 (1 µg each per shot)	x5 sgRNA 1,2&3 (1 µg each per shot
x5 sgRNA 1&2 (1 µg each per shot)	x5 sgRNA 2&3 (1 µg each per shot)	-

The synthetic Cas9 and sgRNA molecules were first prepared to working stocks and kept on ice. For each shot using both Cas9 and sgRNA the Cas9 protein (2 µg) and sgRNA (2 µg) were premixed in a Cas9 reaction buffer (20 mM HEPES (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT) to a total volume of 10 µl and incubated at room-temperature for 10 mins. After 5 mins, the tungsten micro-carriers were vortexed vigorously for 5 mins to resuspend agglomerated particles. To the solution containing the Cas9 and sgRNA, 5 µl of tungsten micro-particles were added. This solution was briefly vortexed and then spread onto wire mesh macro-carriers (pre-autoclaved) and allowed to air-dry in the flow hood at room temperature for approx. 2 hours. The leaf discs which had been exposed to overnight pulse-media were also dried on filter paper during this time.

During this drying period the gene gun was prepared. All components which could not be autoclaved were thoroughly cleaned with 70% ethanol inside the flow-hood and the gun was assembled.

After the drying time the bombardments were carried out. Using a forceps, the macro-carrier for each shot was placed into the firing chamber and six of the dried leaf discs were placed on the bombardment stage. The distance from the nozzle to stage was set to 6 cm and the pressure was set to 100 psi. The distance and pressure were based on similar distances observed in other papers and previous experiments involving the gene gun carried out in the lab.

After each bombardment, two leaf discs were immediately wrapped in tin foil and frozen in liquid nitrogen. They were then stored at -80°C. This represents T_0 post-bombardment. The remaining four were placed in a micro-pot containing semi-solid callus induction medium. 24 hours after bombardment, two more leaves were removed from the callus induction medium and frozen in liquid nitrogen. They were then stored at -80°C. This represents T_{24} post-bombardment. This process was carried out until each Group was finished. Following this, the next Group was prepared and allowed drying time once more, and the bombardment was repeated.

Section 5: Regeneration and Genetic Analysis:

Regeneration Protocols:

Post-bombardment, a selection of plants were cultured in regeneration medium. This process was begun pre-bombardment when the plants were grown overnight in the liquid-pulse medium, previously described, and then placed in callus induction medium. The protocol is largely based on methods found in (Hulme, et al., 1992) with some modifications to suit proteolistics.

After one week of growth in the callus induction media observations were recorded and the plants were transferred to regeneration medium. Regeneration medium was composed of MS salts and vitamins, supplemented with 15 g/l sucrose, 10 µM BA, 14 µM GA_3 and solidified with 0.8% agar. Leaf tissue was cultured upside-down on this regeneration medium for 2 weeks before being transferred to fresh medium. Observations were recorded at two-week intervals and contaminated tissue was removed.

DNA Extraction from Bombarded Material:

Following bombardment, some leaf tissue was stored at -80°C. For each shot two leaf discs at T_0 and T_{24} respectively were stored. In order to extract enough DNA for quantification and analysis, samples were grouped together (eg. leaves from shot 1 & 2 of sgRNA 1 only). The protocol follows the same technique as described in section 3.3. An example of how the leaves were grouped is shown in the table below.

Table 5.1: Grouping of Bombarded Samples

Day 1, sgRNA 1:	Day 1, sgRNA 1:
Shot1, T_0 + Shot2, T_0 = N.1	Shot1, T_{24} + Shot2, T_{24} = N.4
Shot3, T_0 + Shot4, T_0 = N.2	Shot3, T_{24} + Shot4, T_{24} = N.5
Shot 5, T_0 = N.3	Shot 5, T_{24} = N.6

Based on the groups described in Table 5.1, samples were ground with liquid nitrogen as previously described. Immediately after grinding, samples were placed in labelled Eppendorfs corresponding to their respective group and placed in liquid nitrogen. This process was repeated until all samples were ground, and were then stored at -80°C until use. New Eppendorfs were labelled with corresponding leaf & sgRNA names and 400 µl of Edward's Buffer was added to each one. The ground leaf tissue was removed from storage and placed in a cylinder containing liquid nitrogen. Using a chilled spatula, a small amount of leaf tissue was transferred into their respective Eppendorf containing Edward's Buffer. This step was repeated for all samples which were then vortexed and centrifuged for 5 mins at 13,000 RPM, room-temperature. Steps 4-11 from section 3.3 were then carried out to extract DNA from leaf samples.

PCR and Sequencing of Samples:

Following DNA extraction, a PCR reaction was set up to analyse the targeted region within *SGT3*. The same primers previously used were used for this reaction also. It is possible that a 20 bp deletion would be visible on a gel after electrophoresis. However, to be sure of the result, DNA sequencing of the samples would also have to be carried out. DNA samples were taken from 4°C storage and vortexed briefly. In total, 26 samples were to be analysed, with two sets of primers.

Table 5.2: PCR ingredients for Analysis of Bombarded Samples

Ingredients	1x	60x
2x Master Mix	10 µl	600.0 µl
Forward Primer	0.2 µl	12.0 µl
Reverse Primer	0.2 µl	12.0 µl
dH_20	8.6 µl	516.0 µl
DNA	1.0 µl	-

A MiniAmp Plus Thermal Cycler from Thermofisher was used for the PCR;

- Stage 1: 95°C for 3 mins, 1x Cycle
- Stage 2: 95°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds, 32x Cycle
- Stage 3: 72°C for 5 mins, hold at 4°C, 1x Cycle

After completion of the PCR, 1 µl of purple loading dye was added to each well and the products were run on a 3% agarose gel for 2 hrs to allow for full separation. A sequencing gel was then run at 1% for approx. 40 mins using Ethidium Bromide instead of SYBER safe. The bands were excised from the gel and separated using spin columns. The sequencing reaction was set up as outlined in the table below.

Ingredients	1x	25x
Big Dye	0.5 µl	12.5 µl
Buffer	1.75 µl	43.75 µl
Forward Primer (288F)	0.32 µl	8 µl
dH_20	6.43 µl	160.75 µl
DNA	1.0 µl	25 µl

Table 6.3: Sequencing Reaction Components

Sequencing was performed in 10µl reaction volumes using the Big Dye™ terminator sequencing kit (Applied Biosystems). Reactions were purified using the Ethanol-EDTA purification method outlined in the kit handbook and run on an ABI3500xl DNA analyser. Sequencing results were interpreted, and base-calling was carried out using Chromas™. Following this, multiple sequence alignments were carried out to compare the sequencing results with both negative controls and the online reference sequence.

Section 6: Enhanced GFP-Cas9 Fusion Protein Bombardments:

eGFP-Cas9 Fusion Protein Bombardment:

As part of the troubleshooting, a protocol was set up to replicate the bombardment which replaced the synthetic Cas9 with an eGFP-Cas9 fusion (10 µg supplied by

GenScript). No sgRNA was used in this experiment as the only variable to be examined was the successful delivery of the Cas9 into the cell.

Tungsten microparticles were prepared in the same way as previously described. For each shot, the eGFP-Cas9 fusion was premixed with 10x reaction buffer (2 µl and 8 µl respectively) and incubated at room-temperature for 10 mins. Pre-bombardment steps were carried out as previously described. Bombardment was carried out as described in section 4.3. Following bombardment, leaf tissue was placed in petri-dishes containing M&S media, parafilmed and covered with tin foil for approx. 1 hour. Blank bombardments were carried out as a control which contained only 10x reaction buffer and tungsten.

Microscopy analysis was then carried out using an Olympus IX51 inverted microscope under a 10x air objective. For each sample, Brightfield images were first captured using a Hamamatsu ORCA-ER camera with an exposure time of 100 ms. Following this, GFP fluorescence was imaged by exciting the bombarded tissue at a wavelength of 480 nm (100 W xenon arc lamp and Carin monochromator). Images were again captured using a Hamamatsu camera, this time with a longer exposure time of 10 seconds. Images were processed and analysed using Andor IQ V2.0 software. Brightfield and GFP images were merged over particular areas of interest and comparisons were drawn between control samples (blank shots) and eGFP samples.

Results:

Section 1: LED Growth Response Results:

Presented below are graphical interpretations of the plant responses to the different wavelengths of that they were exposed to. The analysis focused on four variables, stem length, leaf number, fresh weight and dry weight.

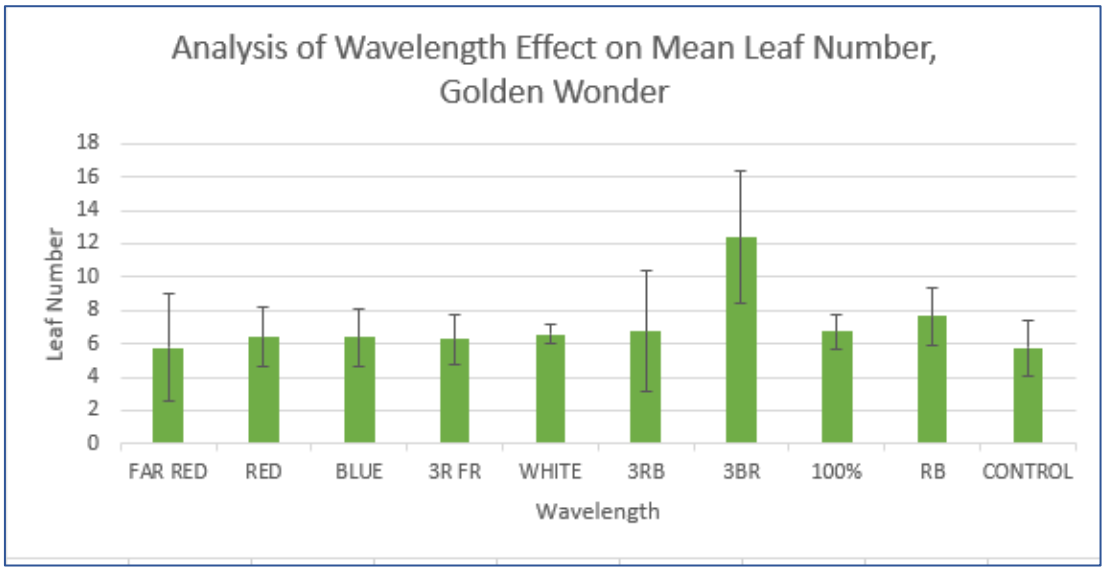


Figure 4.1: Golden Wonder *Leaf Number LED Growth Response measured after 4 weeks growth under 22 hr light, 2hr dark photoperiod. (Far Red, Red, Blue, 3R FR- 3:1 Red:Far Red, White, 3RB- 3:1 Red:Blue, 3BR- 3:1, 100%, Blue:Red, RB- 1:1 Red:Blue, Control).*

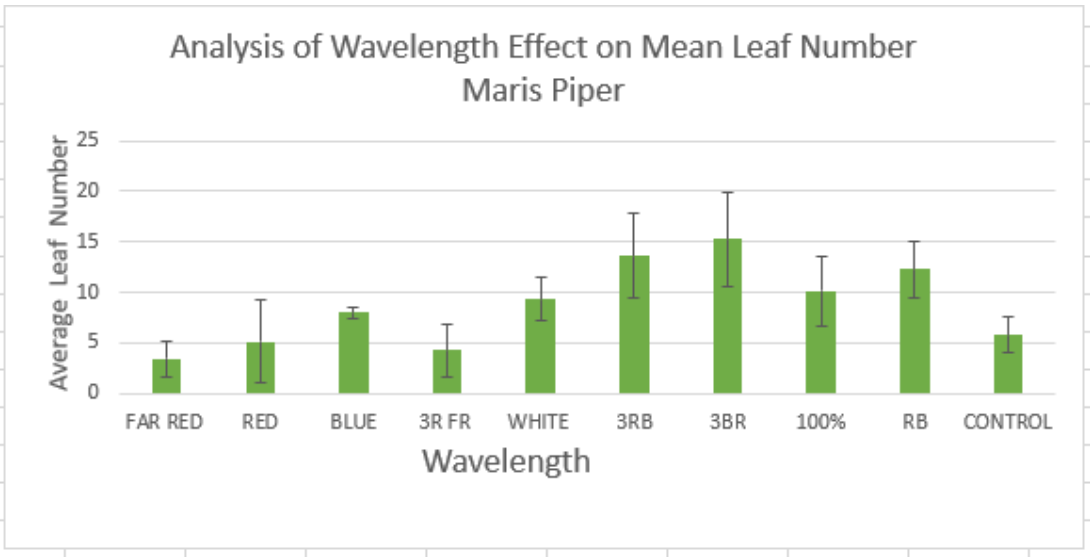


Figure 4.2 Maris Piper Leaf Number LED Growth Response, measured after 4 weeks growth under 22 hr light 2 hr dark photoperiod. (Far Red, Red, Blue, 3R FR- 3:1 Red:Far Red, White, 3RB- 3:1 Red:Blue, 3BR- 3:1, 100%, Blue:Red, RB- 1:1 Red:Blue, Control).

In the case of leaf number, Golden Wonder responded best to a ratio of 3:1 Blue:Red (75% of light was Blue and 25% was red) and was significantly different from the control (P= .002) as can be seen in Figure 4.1. Maris Piper showed a more variable response overall, illustrated in Figure 4.2. Far Red appeared to have a negative impact on leaf number, being negatively significantly different to the control (P= .019). However, White and all ratios of Blue:Red wavelengths had a positive impact on leaf development when compared with the controls.

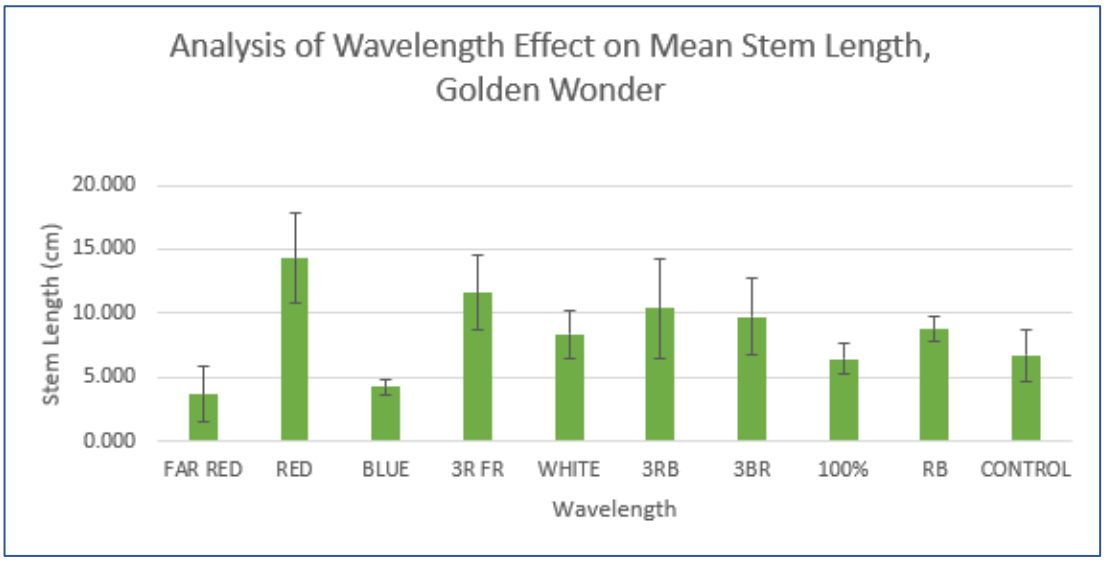


Figure 4.3 Golden Wonder *Stem Length LED Growth Response, measured after 4 weeks growth under 22 hr light, 2 hr dark photoperiod. (Far Red, Red, Blue, 3R FR- 3:1 Red:Far Red, White, 3RB- 3:1 Red:Blue, 3BR- 3:1, 100%, Blue:Red, RB- 1:1 Red:Blue, Control).*

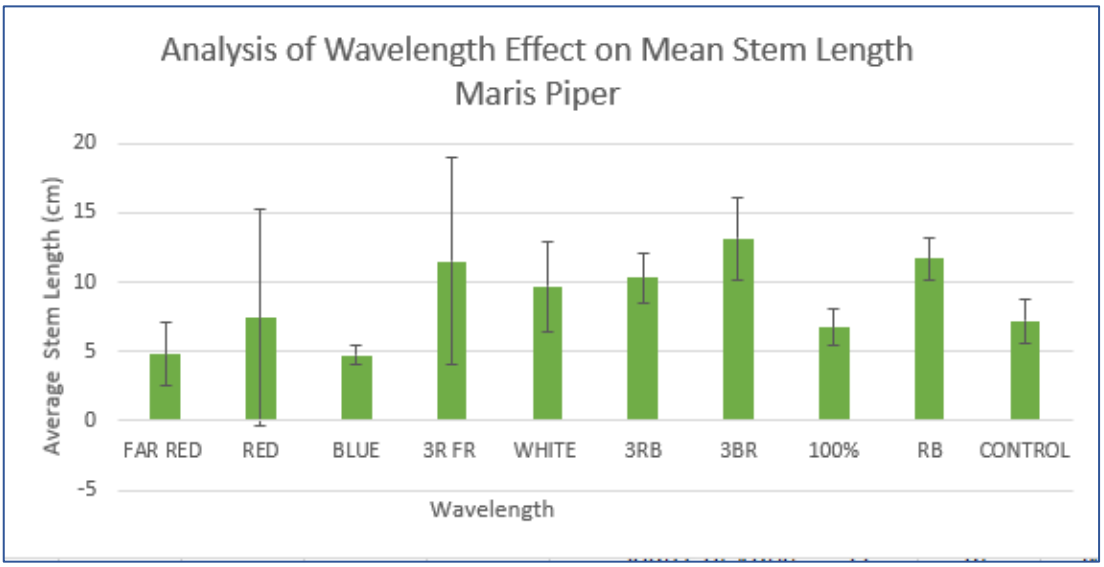


Figure 4.4 Maris Piper Stem Length LED Growth Response, measured after 4 weeks growth under 22 hr light, 2 hr dark photoperiod. (Far Red, Red, Blue, 3R FR- 3:1 Red:Far Red, White, 3RB- 3:1 Red:Blue, 3BR- 3:1, 100%, Blue:Red, RB- 1:1 Red:Blue, Control).

Stem length showed variable responses to different wavelengths in Golden Wonder. This variable response is clearly illustrated in Figure 4.3. Interestingly, Far Red alone

had a negative impact on stem length, showing negative significant difference when compared with the control (P= 0.012), however Red light promoted stem growth and when both were combined, the stem length still showed positive significant difference from the control (P=0.002). Similarly, in Maris Piper, (Figure 4.4), Far Red showed negative significant difference when compared with the mean (P=0.029) however when combined with Red light, it outperformed the control. Similar to the leaf number results, combinations of Red and Blue wavelengths all showed positive significant difference when compared with the control plants.

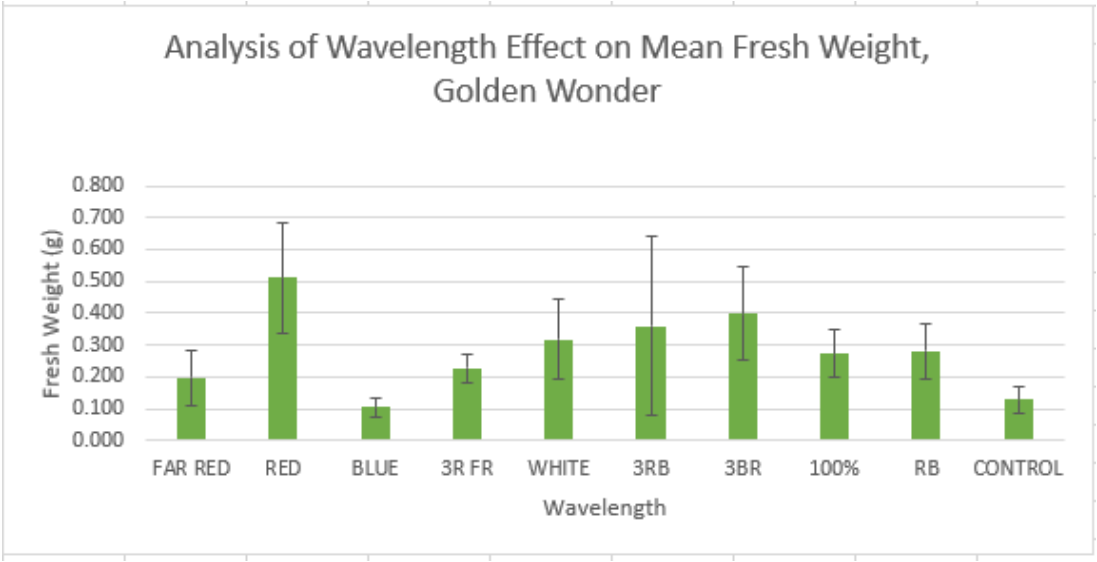


Figure 4.5: Golden Wonder Fresh Weight LED Growth Response, measured after 4 weeks growth under 22 hr light, 2 hr dark photoperiod. (Far Red, Red, Blue, 3R FR- 3:1 Red:Far Red, White, 3RB- 3:1 Red:Blue, 3BR- 3:1, 100%, Blue:Red, RB- 1:1 Red:Blue, Control)

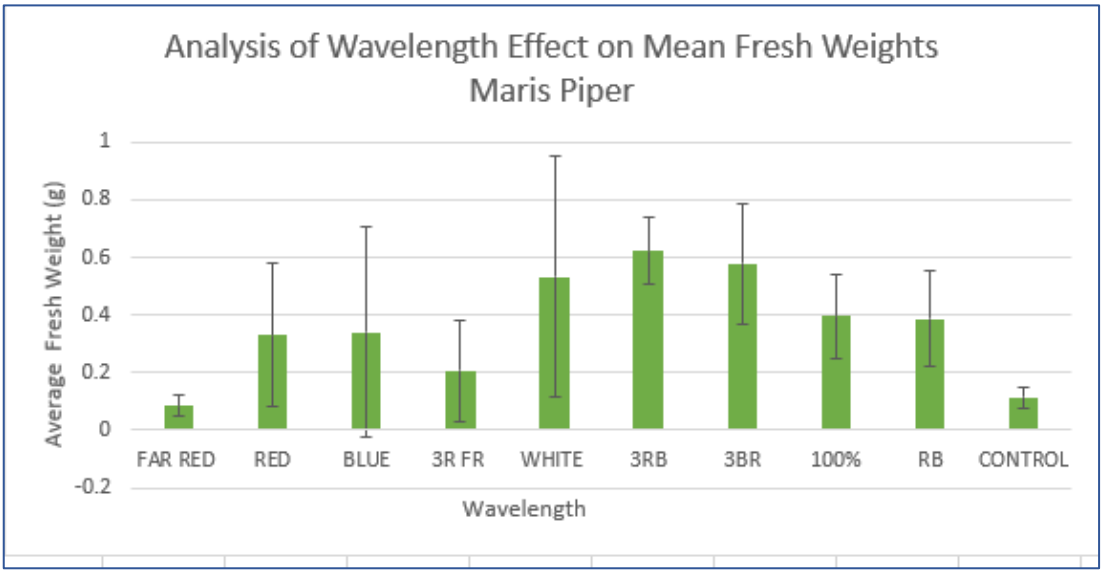


Figure 4.6: Maris Piper Fresh Weight LED Growth Response, measured after 4 weeks growth under 22 hr light, 2 hr dark photoperiod. (Far Red, Red, Blue, 3R FR- 3:1 Red:Far Red, White, 3RB- 3:1 Red:Blue, 3BR- 3:1, 100%, Blue:Red, RB- 1:1 Red:Blue, Control).

Fresh weight results, (Figure 4.5), for Golden Wonder showed more variance. Blue, Far Red and Far Red:Red all showed no significant difference from the control, however Red appeared to greatly outperform the control, showing strong positive significant difference (P= 0.000). Combinations of Blue and Red wavelengths appeared to perform well here also, with 3:1 Blue:Red performing best. Following the previously observed trends for Maris Piper, combinations of Red and Blue wavelengths had a positive outcome on plant growth when measuring fresh weights, which can be seen in Figure 4.6.

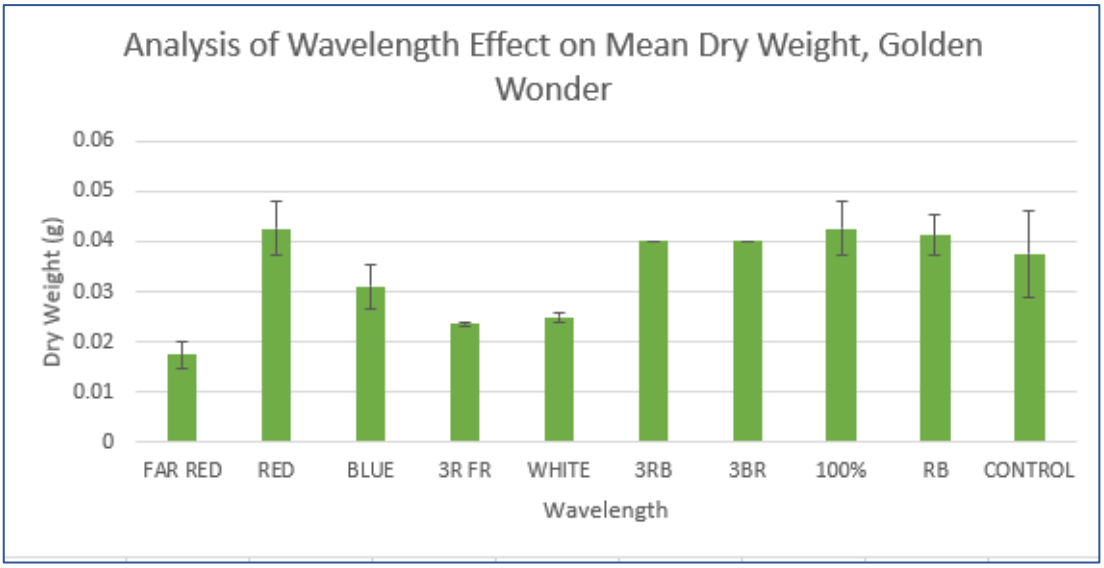


Figure 4.7: Golden Wonder *Dry Weight LED Growth Response, measured after 4 weeks growth under 22 hr photoperiod. (Far Red, Red, Blue, 3R FR- 3:1 Red:Far Red, White, 3RB- 3:1 Red:Blue, 3BR- 3:1, 100%, Blue:Red, RB- 1:1 Red:Blue, Control).*

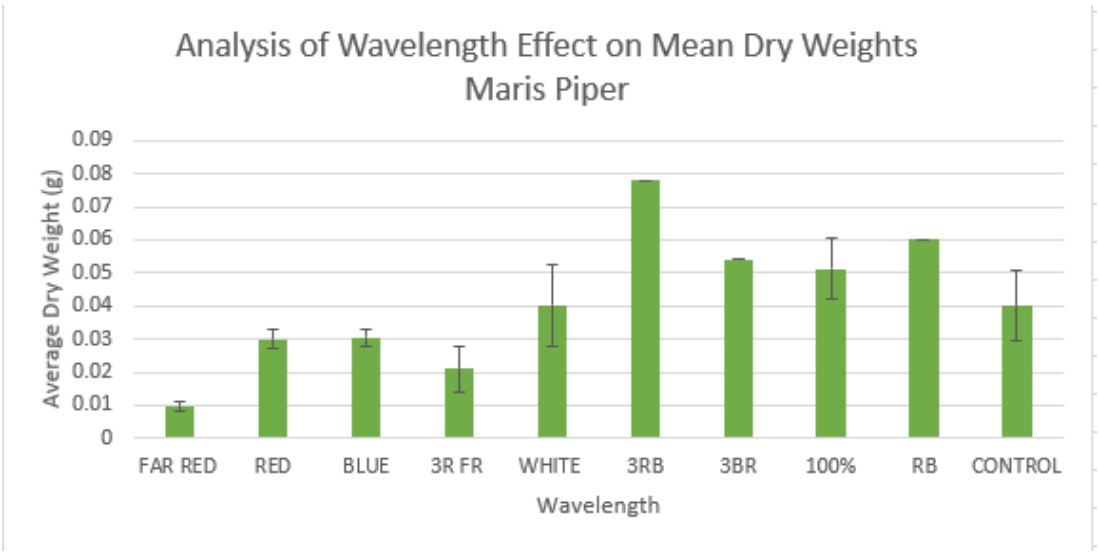


Figure 4.8: Maris Piper Dry Weight LED Growth Response, measured after 4 weeks growth under 22 hr photoperiod. (Far Red, Red, Blue, 3R FR- 3:1 Red:Far Red, White, 3RB- 3:1 Red:Blue, 3BR- 3:1, 100%, Blue:Red, RB- 1:1 Red:Blue, Control).

Dry weight results for Golden Wonder, (Figure 4.7), seemed relatively uniform across all wavelengths and controls. Worthy of note is Far Red, Far Red:Red and White wavelengths, all which showed negative significant difference when compared with the controls. As seen in previous Figures relating to Maris Piper, combinations of Blue and Red light performed very well, all showing positive significant difference from the control. Interestingly Far Red, Blue and Far Red:Red all showed negative significant difference when compared with the controls.

Section 2: Protein Extraction and Western Blot:

An anti-actin Western Blot was performed as a test to see if it would be possible to detect actin, abundant in plants, with the intention of using this method in the future to detect Cas9. However, as the project progressed it was decided to verify the Cas9 presence using a different method. Nevertheless, the Western Blot successfully detected actin in both tuber and leaf tissue, though the signal was stronger in tuber tissue. Two bands were detected in both samples, one of which corresponded to the expected size for actin. While it is possible that the other band is another form of actin, it is more likely that it is a degraded product.

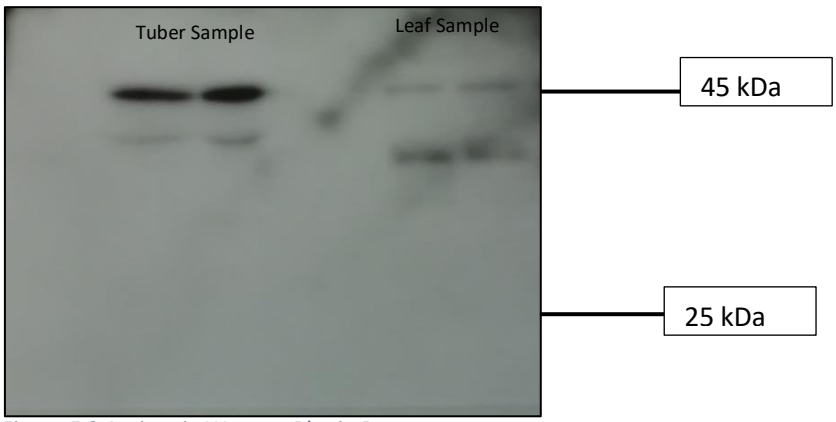


Figure 5.2 Anti-actin Western Blot in Potato

Section 3: *In vitro* Digestion with sgRNA and Cas9:

After PCR and gel electrophoresis was used to extract the genomic region of interest within the potato, gel purification was carried out and the results were quantified using nanodrop. Purified DNA values ranged from 2.62 ng/nl to 6.68 ng/nl when measured at an absorbance of A 260/280. These values are relatively low but were still enough for the *in vitro* digestion experiment. The low values were likely due to loss of DNA during the purification step.

The *in vitro* digestion results are highlighted below. The experiment was carried out twice. The first time, the sgRNA molecules were added individually to test their ability to guide the Cas9. As comparison, alongside each digestion reaction, a sample was loaded which contained all reaction components but without sgRNA and Cas9. A positive control (PC) containing sgRNA with no Cas9 was also used and a negative control (NC) which contained Cas9 with no sgRNA was used. In the second experiment, the ratio of Cas9 to sgRNA was altered and combinations of sgRNAs were also tested.

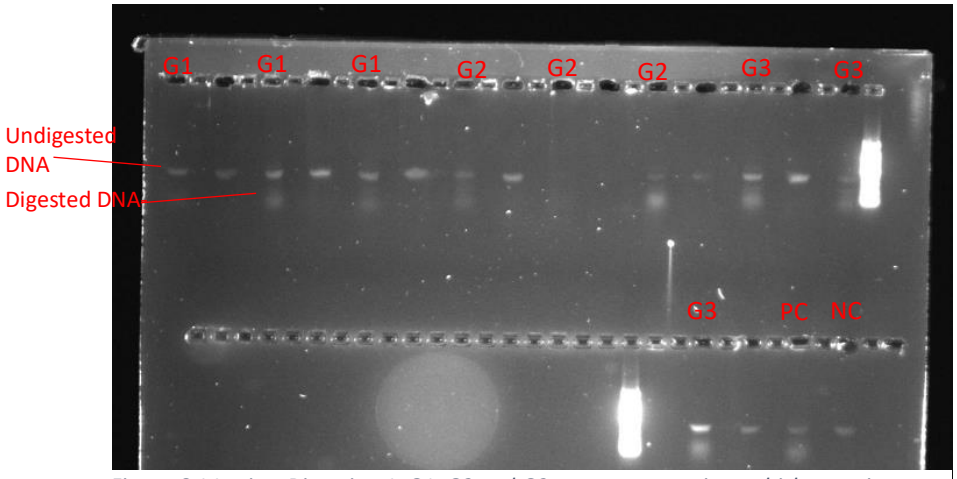


Figure 6.1 In vitro Digestion 1. G1, G2 and G3 represent reactions which contain sgRNAs 1, 2 and 3 respectively. PC represents the positive control (detailed above) and NC represents the negative control (detailed above).

For the second digestion, the gel was run for 1 hour instead of 30 mins in order to separate the ladder more and to allow for easier identification. For this experiment a blank control (BC) containing the reaction components without sgRNA and Cas9, a positive control (PC) containing sgRNA but no Cas9 and a negative control (NC) containing Cas9 but no sgRNA were all used. Cas9 concentration was altered (Cx1-

Cx4) and combinations of sgRNAs were also tested. The bands from the electrophoresis were faint, but fully digested DNA was detected in some samples, while partially digested DNA was detected in others.

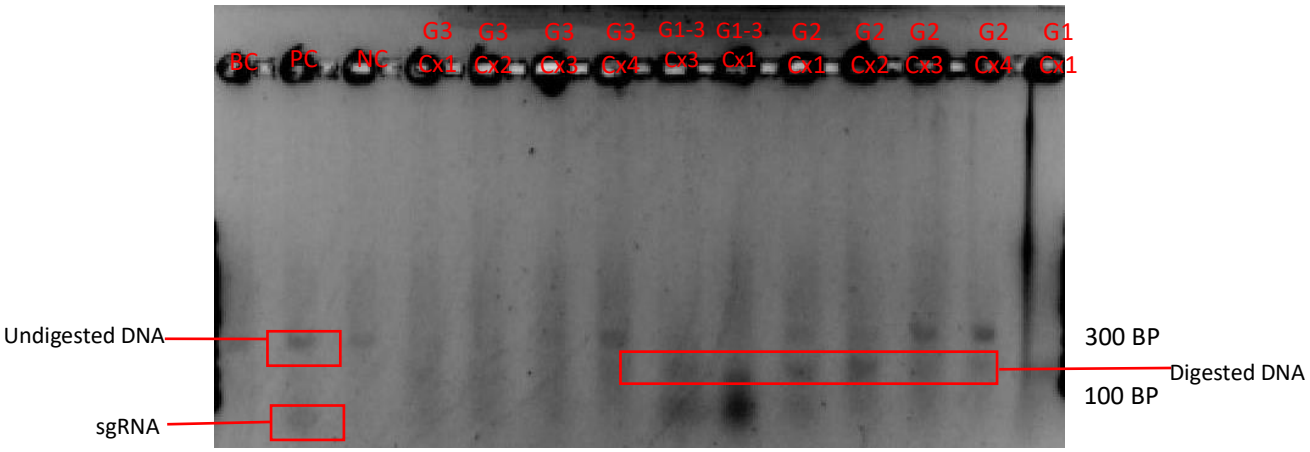


Figure 6.2 In vitro Digestion 2, Gel Electrophoresis. As in Fig 6.1, G2 and G3 represents reactions containing sgRNAs 2 and 3. G1-3 represents reactions containing all of the sgRNAs. Undigested DNA visible at approx. 300 bp. Digested DNA most visible between G1-3, Cx3 to G2, Cx4. sgRNA also visible at less than 100 bp mark.

To summarise the results from this Section, Figure 6.2 shows both full and partial digestion most clearly. Completely undigested DNA is visible in the positive control (PC), while a more complete digestion is visible in G1-3 Cx3 and G1-3 Cx1. Partial digestion is visible in the G2 samples, as there are still faint bands visible at the 300 bp mark in these wells. This would seem to indicate that combinations of sgRNA works better than single sgRNA molecules used by themselves.

Section 4: Proteolistics Analysis and Sequencing:

PCR and gel electrophoresis was carried out on extracted DNA from bombarded material prior to sequencing. After gel electrophoresis, no apparent 20 bp deletion was visible on the gel. Sequencing was carried out to see if there was a smaller deletion present in the region. In this experiment a positive control (PC) was used which contained extracted DNA from tissue bombarded with Cas9 reaction buffer and tungsten, but no sgRNA or Cas9. A negative control (NC) was used which contained DNA extracted from leaf tissue which had not been bombarded. Following sequencing, results were analysed using Clustal Omega Multiple Sequence Alignment. These results confirmed the observations from the initial gel electrophoresis, that there was no knockout present. One such multiple sequence alignment is shown below. Regions highlighted in green are sgRNA target sites.

```
CLUSTAL O(1.2.4) multiple sequence alignment
Ref      CCCACTGACATGAAATTTTGGCTCCGCCTCTCTCTATATATATATATACGAGTCAACTGA
         60
S1       -----------------------------------------CAATACMRASTCATTGA
         17
                                                      ****      * ***
Ref      AGTGAAGGAACAACTTGTTAATGGCGATGGAACAGAATGAAGAAACTGCAATGCCGCATG
         120
S1       AGTGARRGAACAWCTTGTTARTGGCKATGGAACASAATGAAAAAACTGCAATGCCGCATG
         77
         *****  ***** ******* **** ******** ****** ******************
Ref      TTGTGTTCATACCATACGCCATGACGAGTCATATAACTCCATTGGTACATATTGCTAGAC
         180
S1       TTGTGTTCATACCATACSCCATGACRASTCATATAACTCCATTGRTACATATTGMTASAC
         137
         ***************** ******* * *************** ********* ** **
Ref      TCTTCGCCCTCCATGGCCTCAAAGTTACTATCATTGCCCCTCAGCATAATGCTCTTCTTT
         240
S1       TCTTCSCCCYCCATGGCCYCAAAKTTACTATCATTGCCCCTCAGCATAAYGCTCTTCTTT
         197
         ***** *** ******** **** ************************ **********
Ref      TTCAGTCCTCTGTCGATAGAGACCGTCTCTTTTCGGGCAGCAATATTACTGTCCGGACAA
         300
S1       TTYWYTCYTCTGTCKATAKAKACCGTCTCTTTTCGGGCAKYWATATTACTGTCCGGACAW
         257
         **   ** ****** *** * ******************   *****************
Ref      TTCAATTTCCGTCTGAGGAAGTTGGATTACCTGTAGGAATTGAAAACTTCATCGCAAGCC
         360
S1       TTCWWTTTCYSTCTGARGAAGTTGGATTACCTGTAAGAATTGAAAACTTCWTCRCAMSCA
         317
         ***  ****  ***** ****************** ************** ** **  *
Ref       C----  361
S1        CCCAC  322
          *
```

Figure 7.1 Multiple Sequence Alignment of Sequenced Bombarded Material and Reference sequence of SGT3 region containing all three sgRNA (highlighted green). No clear deletions are present in alignment.

Section 5: Regeneration Protocol:

Following bombardments, any samples not being used for genetic analysis were subject to regeneration treatments.



Figure 8.1: Maris Piper *plant material growing in Regeneration media. Shoots are clearly visible after this period of growth (6 weeks).*

Observations of these samples were recorded every two weeks, prior to transfer to new media. After one week, approx. 50% of bombarded leaves were observed to be curling with small calli growing, mostly on the leaf undersides. 27% of leaves remained unchanged after bombardment. Some samples were observed to have turned purple around wounding sites, where calli was present. Some plants were also lost to contamination. After the third week the majority of plants which had survived were observed to have many calli. Some leaves were browning and dying. At this point, approx. 50% of the initial stock had been removed due to failure to produce any calli or contamination of plant material. Shoots were observed after two weeks growth in regeneration media. The majority of plants which had survived to this stage were capable of producing shoots. In total, seven plants were successfully regenerated from the initial stock of sixty six bombarded plants.

Section 6: eGFP-Cas9 Bombardments:

Cas9-eGFP was bombarded into leaf tissue using the proteolistics protocol. Following this bombardment, microscopy work was conducted to detect GFP. Presented below are Brightfield, GFP and Merged views of eGFP-Cas9 bombarded tissue, Control bombarded tissue and an area of tissue which was not bombarded.

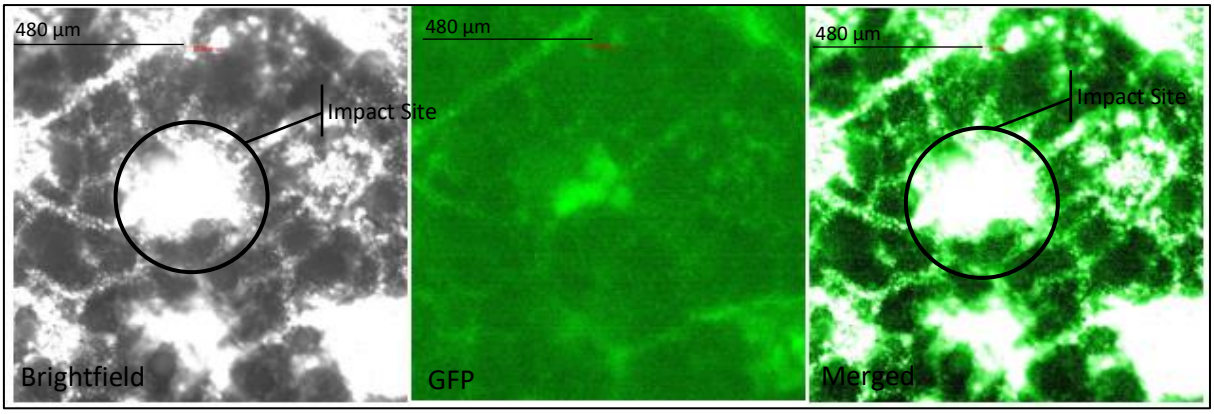


Figure 9.1: Microscope images of eGFP-Cas9 Bombarded Tissue. Tungsten impact sites are circled and 480 µm scale bar is shown.

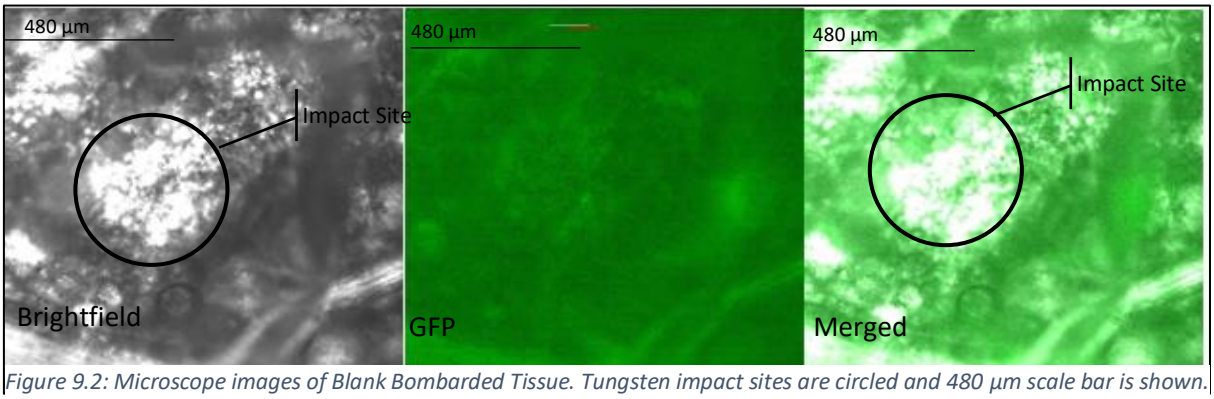


Figure 9.2: Microscope images of Blank Bombarded Tissue. Tungsten impact sites are circled and 480 µm scale bar is shown.

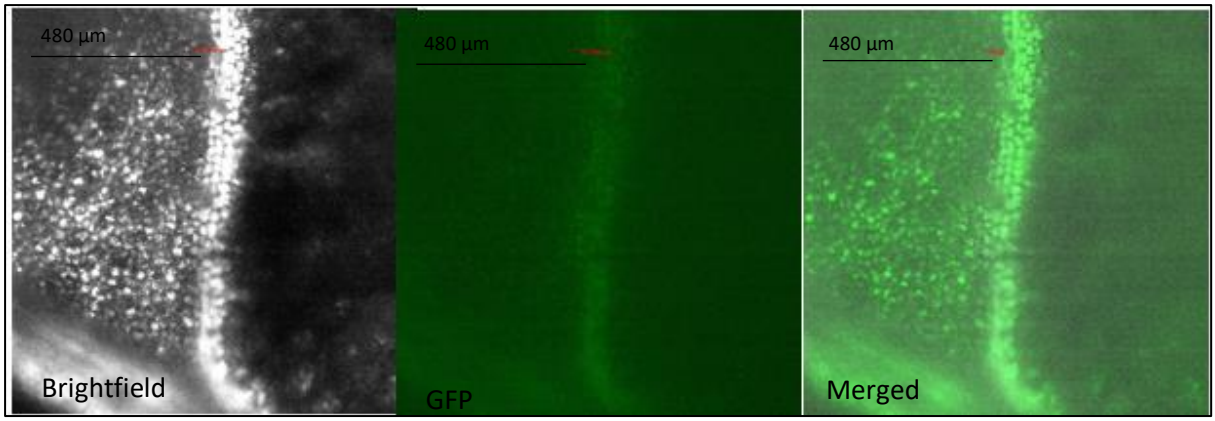


Figure 9.3: Microscope images of non-bombarded Tissue. 480 µm scale bar is shown.

It appears from these images that the Cas9-eGFP bombardment protocol was unsuccessful at inserting the fusion protein into the plant cells. While initially it seemed as though there was fluorescence around the “impact sites” from the tungsten, upon comparison with non-bombarded tissue and blank bombardments, this fluorescence was attributed to auto-fluorescence from the plant cell itself.

Discussion:

Section 1: LED Growth Response Treatments:

Different wavelengths of light, including red and blue light have been extensively shown to affect plant growth (Okamoto, et al., 1996). For example, it has been documented widely that red light is a key factor for driving photosynthesis and blue light contributes to stomatal opening and closing, driving factors like CO_2 exchange and stem length, however the effects of this can vary between or within species (Massa, Kim, Wheeler, & Mitchell , 2008). These effects were reflected in the results of this experiment, especially in the case of the different cultivars, which appeared to respond differently to certain wavelengths. In these experiments, it appeared Maris Piper responded better overall to the combinations of blue and red wavelengths. It is likely that the effects conferred by red and blue light combined to give a strong growth response, compared with when the wavelengths were used in isolation and the plants showed less of a growth response. In general, these combinations of blue and red also outperformed the control plants, so it is possible that these two wavelengths when combined confer an overall boost to fitness. Interestingly, Golden Wonder did not perform as well as Maris Piper when exposed to combinations of blue and red light. However, red light by itself seemed to improve overall stem elongation and fresh weight.

Aksenova and colleauges found that in their study, red light caused potato plants to be weaker, with long but spindly stems (Aksenova, et al., 1994). This is reflected in our results, especially in the case of Maris Piper, which had longer stems but overall had a much lower dry weight when grown under red light, compared to the control. Interestingly, though Golden Wonder had longer stems, its fresh and dry weights were relatively high when grown under red light. Rocha and colleagues further proved that in the case of potato, red light seems to promote stem elongation, with blue light inhibiting stem length (da Rocha, et al., 2015). Our results show that for both Maris Piper and Golden Wonder, blue light seemed to induce shorter stems when compared with other wavelengths and the control.

Overall, it appears that Golden Wonder is more receptive to LED-based growth treatments, and combinations of blue and red wavelengths promote general plant

growth which is greater than that of the control plants, grown under fluorescent light. Maris Piper appears more receptive to red light in general, but overall shows less of an improvement when exposed to these LED-based growth treatments.

Section 2: Protein Extraction and Western Blot:

Isolating protein from plant tissue often poses many problems due to the interference of many different compounds such as phenolics and carbohydrates. For this reason, 2-DE is still the standard for proteomic studies relating to plants, and potato is no exception (Delaplace, et al., 2006).

In this project, an SDS-based protein extraction method was devised based on previous published methods (Monte, et al., 1999) (Delaplace, et al., 2006). Modifications were made due to the difficulties of obtaining a quantifiable and usable level of total protein. Following these modifications which involved combining the method into a single-step extraction, satisfactory amounts of protein were obtained from both leaf and tuber samples.

The initial goal for the Western Blot was to use it as a method to detect Cas9 within bombarded leaf tissue, using Cas9 antibodies. We decided to initially optimise the Western conditions, using actin as the control. Actin was detected in both tuber and leaf tissue (see Fig. 5.2). In both cases, there were two bands present, indicating that the antibody could have detected some degraded product, which is likely, or a different form of actin had been detected. Previous findings suggest the evolution and development of at least nine distinct actin DNA sequences in potato, which differ in nucleotide composition and amino acid length (Drouin & Dover, 1990). Other studies suggests anywhere between 12-36 different actin genes exist within potato (de Sá & Drouin, 1996). It is quite likely that the different bands detected in this Western Blot are due to degraded product, however the stronger signal seen in the tuber sample compared to the leaf sample could be to do with different types or amounts of actin. This proved further that if actin, which is abundant in plant cells, is not easily detected in leaf tissue, then detecting much smaller amounts of Cas9 would have been even more difficult.

Section 3: *In vitro* Digestion:

Validation of sgRNAs in any CRISPR/Cas9 based transformation is an important step (Mehravar, et al., 2019). While an *in vitro* digestion gives a good indication of the efficiency of sgRNAs, it is not completely reliable, as *in vivo* there are many other factors, such as nucleosomes, which can inhibit the ability of CRISPR/Cas9 to operate (Yarrington, et al., 2018).

Following amplification, extraction and purification of the DNA target region, a small amount of DNA was obtained. It is likely that during the various purification steps some DNA was lost and though these values were low, it was still possible to carry out the *in vitro* digestion which required only 4 µl of 20 nM DNA. The results from the first digestion were not conclusive enough to be confident in the sgRNA molecules. It appeared that though some DNA was digested (as seen in Fig. 6.1), there was still undigested DNA which was giving a stronger signal. Furthermore, it was difficult to discern the difference between the positive control band and sample bands. Therefore it was decided to run this experiment again and alter the quantities of sgRNA and Cas9, as well as allowing the gel to run for twice as long for easier observations. These results (Figure 6.2) were more conclusive. Though the bands were fainter overall, digestion was clearly visible in certain reactions. There was still some undigested DNA when the Cas9 concentration was altered but digestion was observed as there was faint bands around the 200 bp mark for sgRNA 2. Cas9 concentration at 1x and 2x performed best, digesting almost all the DNA, but when the concentration was increased beyond this, the efficiency appears to decrease. It has been documented that for highly specific DNA cleavage, lower amounts of sgRNA and Cas9 are optimal, as higher amounts can improve the overall efficiency but have a greater chance of off-target mutations (Marx, 2014). In this case, off-target mutations was not a concern as the digestion was acting specifically on an isolated sequence from *SGT3.*

Combining the sgRNAs appears to be the most effective way to ensure complete digestion of DNA. This is unsurprising as there is extensive literature surrounding the use of multiple-gRNA systems (Cong, et al., 2013) (Mali, et al., 2013). By using multiple sgRNAs, it is possible to target multiallelic systems, such as those

found in the tetraploid potato (Bortesi & Fischer, 2015). By using combinations of the sgRNAs, almost complete digestion of target DNA was observed, compared to when only one sgRNA was used and only partial digestion was observed. It is likely, in this case, that *SGT3* was present in more than one allelic form, and so by using sgRNAs which targeted different areas of the gene, a more complete digestion was obtained. Thus, by using a combination of sgRNAs during transformation experiments, the likelihood of obtaining a deletion in the target genome appears to be much higher.

Section 4: Analysis of Bombarded Samples:

Following analysis of bombarded leaf samples of the *SGT3* target region it was concluded that there was no visible knockout present. Given the results of the *in vitro* digestion, it is possible to assume that the gene gun is the likely cause of this failure. There have been many studies comparing the efficiency of the gene gun with the likes of *Agrobacterium* as a transformation method (Gao & Nielsen, 2013) (Creissen, et al., 1990) and while the gene gun is generally considered less efficient, it is believed to be a suitable delivery mechanism when using RNPs. Furthermore, *Agrobacterium* cannot be considered a "DNA-free" method due to the presence of bacterial vectors, while the proteolistics method relies only on sgRNAs, and synthetic Cas9, and no expression vectors are utilised in the process. Proteolistics using RNPs has already been successfully implemented in plants (Martin-Ortigosa & Wang, 2014) (Liang, et al., 2017) (Liu, et al., 2019) but the efficiency remains relatively low at approx. 3-6%. Low efficiency is likely due to the damaging nature of the gene gun (Zhang, et al., 2014) combined with elements within the nucleosome which prevent the action of CRISPR/Cas9 system (Yarrington, et al., 2018). In the case of this project, the above factors, as well as the results from the *in vitro* digestion which showed that the target DNA is not always fully digested, are likely the reason why the transformation experiment did not work. By using a different delivery system, such as PEG-mediated delivery, the chance of successful transformation could be higher. PEG-mediated transformation using RNPs has already been used in potato (Andersson, et al., 2018) and a variety of other systems exist which are capable of delivering such RNPs (Altpeter, et al., 2016). In the future, it may be possible to achieve a knockout in *SGT3* using one of these delivery methods.

Section 5: Regeneration of Bombarded Material:

Regenerating plants after transformation is widely considered challenging. When dealing with plants which have been bombarded, it can be the case that the damage caused is too severe, and the plant is more likely to enter senescence rather than regenerate. However, regeneration after bombardment has been documented in a variety of plants (Chlan, et al., 1995) (Singh, et al., 2010) (Kikkert, et al., 2005) and extensive protocols for the regeneration of potato have been described (Ghosh, et al., 2015) (Kumlay & Ercisli , 2015). In the case of this project, a potato-specific regeneration protocol was devised, based on a post-*Agrobacterium* regeneration protocol. In many cases, it appeared that the plant cells sustained too much damage from the bombardment and were incapable of producing calli. However, in some cases, when the damage was not so severe, calli formed from the tungsten impact sites, which were visible as tiny perforations in the plant cell surface. It would appear that a degree of damage is beneficial for regeneration, but too much will inhibit or halt the process. In this experiment, 7/66 plants successfully regenerated, just under 10%. This low efficiency is likely due to the aggressive nature of the gene gun coupled with contamination issues which arose due to the constant need to transfer plants to fresh media every two weeks.

Section 6: Enhanced GFP-Cas9 Fusion Bombardments:

Following the failure to achieve a knockout in *SGT3* an experiment was devised which would test the ability of the gene gun to deliver a fusion protein composed of Cas9 and enhanced GFP into leaf tissue. Previous publications have successfully bombarded eGFP into plant tissue (Martin-Ortigosa & Wang, 2014) and as a troubleshooting experiment this provided valuable insight into our experimental conditions. Initial microscope images of bombarded material seemed to show fluorescence, however, when compared with the control (blank) samples and samples which were not bombarded, a similar fluorescence was observed. This suggests that this signal was due to autofluorescence from the plant cells rather than fluorescence caused by GFP. Autofluorescence is a well-documented subject (Roshchina, 2012) and it is likely that this caused a false-positive result for this experiment. The results here indicate that the gene gun was unsuccessful at delivering the fusion protein into the plant cell, and so, was most likely also incapable

of delivering the RNP complex into the plant cell. This demonstrates that while the gene gun is a novel idea for the delivery of RNPs as a form of "DNA-free" method, it is inefficient, and there are too many variables which can cause problems.

Conclusion:

The goal of this project was to test the biolistic approach for the integration of RNP systems into plant tissue and achieve a deletion in the first exon of the *SGT3* gene of *Solanum tuberosum*. While the deletion was not achieved, important steps have been taken to further this field of research. The *in vitro* digestion showed that the inherent principles of the CRISPR/Cas9 system are functional and capable of digesting the *SGT3* target site, however when this system was taken to *in vivo*, it failed. It appears that the most likely cause of this was the gene gun, as demonstrated by the enhanced GFP-Cas9 fusion protein bombardment, which failed to deliver the fusion protein into the plant cell. The LED plant growth experiments successfully demonstrated that when exposed to different wavelengths of light, *Solanum tuberosum's* growth cycle can be improved, a useful tool for future experiments that require the swift generation of plant material. As an approach for genetic engineering, RNPs are a promising tool, however it appears that the delivery mechanism, as well as the tissue to be transformed, play a vital role in its success. Future projects could refine this process, using other possible delivery methods such as PEG or lipofection mediated transformation.

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