THESIS

# Developing novel potato properties using gene editing

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**THESIS DECLARATION**

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I, Diem Ly, declare that:

This thesis is my account of my research and has been substantially accomplished during enrolment in this degree, except where other sources are fully acknowledged.

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- Murdoch University Institutional Biosafety Committee (IBC) approval number 171
- Risk Assessment Processes (RAMP) reference number RAMP0902_01_18
- Working with Biological Materials (WBM) number WBM/18/245 (approved date 27 February 2018)
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**ABSTRACT**

Potato (*Solanum tuberosum L.*) is the most important tuber food crop worldwide and a significant component of food security. A major use of potato is their processing into crisps and French fries. Potato tubers that accumulate hexose sugars and exhibit browning after frying are rejected, causing economic losses and waste. Acrylamide formation when potato tubers are deep-fried also presents a health risk for consumers because acrylamide is a potential neurotoxin/ carcinogen. The aim of this project was to upgrade existing potato cultivars (Atlantic and Desiree) using gene editing (CRISPR/Cas9) to reduce the expression of the *vacuolar invertase* and *asparagine synthetase 1* genes, to minimise cold-induced sweetening and acrylamide formation in fried potato products.

The Cas9 and gRNAs were delivered into plant cells either as expression vectors using *Agrobacterium tumefaciens* or directly by particle bombardment as a ribonucleoprotein complex. A total of 20 transgenic edited events and one transgene-free edited event were obtained. Ten transgenic events were further analysed, and small insertions and deletions that ranged from 1 bp to 35 bp accounted for 67.9% of mutation frequency. Biochemical analysis from cold-stored, edited tubers (four months at 4°C) revealed that hexose sugars in cold-stored tubers and acrylamide levels in crisps were significantly reduced, accompanied by a noticeably improved colour intensity of fried crisps. Hexose sugar in edited events was reduced up to 21 times and was extremely low in one event; only 0.02 mg/gfw glucose and 0.13 mg/gfw fructose were detected. The acrylamide content was decreased up to 3.7 times in Atlantic-derived events and 6.8 times in Desiree-derived events, and the lowest recorded acrylamide level was 332.9 ng/g. Overall, gene-edited events generated in this project exhibited better properties for human consumption/health. This research successfully demonstrated that CRISPR/Cas9 can be used to improve economically important potato cultivars.

**LIST OF CONFERENCES**

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**Diem N.P. Ly**, Michael G.K. Jones, Sadia Iqbal, Stephen P. Milroy. 2020. CRISPR for healthier CRISPS (Poster). Plant and Animal Genome XXVIII Conference, San Diego, California, USA.

**Diem N.P. Ly**, Michael G.K. Jones, Sadia Iqbal, Stephen P. Milroy. 2019. Developing novel potato properties using genome editing. Murdoch University Annual Research Symposium, Murdoch. Western Australia, Australia.

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**LIST OF ABBREVIATIONS**

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| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| AS1 | asparagine synthetase 1 |
| BA | benzyladenine |
| BAP | benzyladenine purine |
| bp | base pair |
| Cas9 | CRISPR associated protein 9 |
| cDNA | complementary DNA |
| cds | coding sequence |
| CIM | callus induction medium |
| CIS | cold-induced sweetening |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeat |
| CTAB | hexadecyltrimethylammonium bromide |
| DNA | deoxyribonucleic acid |
| DSB | double-stranded break |
| DSR | direct shoot regeneration |
| $GA_3$ | gibberellic acid |
| gRNA | guide RNA |
| HR | homologous recombination |
| mRNA | messenger RNA |
| MS | Murashige & Skoog basal medium |
| MSGV | Murashige & Skoog Modified Basal Medium with Gamborg Vitamins |
| NAA | naphthaleneacetic acid |
| NHEJ | non-homologous end-joining |
| nptII | neomycin phosphotransferase II |
| nt | nucleotide |
| PAM | protospacer adjacent motif |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RNP | ribonucleoprotein |
| SDN | site-directed nuclease |

| | |
|---|---|
| SIM | shoot induction medium |
| SNP | single nucleotide polymorphism |
| TALEN | transcription activator-like effector nuclease |
| VInv/ VINV | vacuolar invertase |
| ZFN | zinc finger nuclease |
| ZR | trans-zeatin riboside |

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Thank you all.

Dedicated to

**My parents and younger sister**

# Chapter 1

# Introduction and literature review

## 1.1 General introduction

Potato plays an essential role in the world's food security as one of the most significant food crops. It is the fourth most important crop (after wheat, rice and maize) and the most widely grown tuber crop. It is used as a freshly cooked vegetable, although almost half of the harvested potato tubers are processed into crisps and French fries or related frozen products (USDA, 2017). During processing, potato with undesirable characteristics, such as excessive browning after frying, are rejected by processing plants, causing economic loss and waste. The acrylamide level in fried potatoes is a health issue because acrylamide has been recognised as a neurotoxin and carcinogen, and has been classified as probably carcinogenic to humans ( Group 2A) by the International Agency for Research on Cancer (IARC) (Lyon, 1994, SCF, 2002). Improving potato through conventional breeding is a time-consuming challenge due to its heterozygosity and polyploid nature. A promising solution to improve its nutritional properties is that the potato genome can now be manipulated, for example, to down-regulate or silence the genes linked to browning and acrylamide production on processing. The genes linked to these characteristics are *vacuolar invertase* (*VInv*) and *asparagine synthetase 1* (*AS1*) (Bhaskar et al., 2010, Chawla et al., 2012). Conventional breeding to achieve the same ends would take up to 20 years or more to generate a new commercialized variety. With new breeding technologies such as gene editing, the time needed for generating an improved potato variety may be shortened: the process is more direct, precise and can be used to improve an existing and accepted variety.

The bacterial 'Clustered Regularly Interspaced Short Palindromic Repeat' (CRISPR) system and its associated protein Cas9 has been developed recently as a new genome-editing (or preferably 'gene editing') tool, with its first application in plants reported by Feng et al. (2013). It is essentially a system to generate targeted mutations and uses two components, a double-stranded DNA nuclease (e.g. Cas9) and an RNA-based guide system, abbreviated CRISPR/Cas9. This editing system can be delivered into plant cells using *Agrobacterium tumefaciens,* or it can be pre-assembled to make a

ribonucleoprotein (RNP) complex and then introduced into protoplasts or cells. The latter method can generate transgene-free gene-edited plants with desirable traits: such plants are now exempt from being regarded as genetically modified plants in Australia and increasingly in other countries including USA, Canada, Japan, South Africa and most countries in South America (Menz et al., 2020, Gupta et al., 2021).

This project applied the CRISPR/Cas9 system to potato to silence or down-regulate the *VInv* and *AS1* genes; the aim was to reduce excessive browning caused by cold-induced sweetening and acrylamide production on deep-fat frying of potato.

## 1.2 Introduction to the potato crop

### 1.2.1 Origin, distribution and production

Potato (*Solanum tuberosum* L.) (2*n* = 4*x* = 48) belongs to the genus Solanum which has more than a thousand recognized species (Machida-Hirano, 2015). The cultivated potato and its wild relatives originated in the Andes, western South America (de Haan and Rodriguez, 2016). The most extensive potato collection at the International Potato Center (CIP) holds more than 7,000 known varieties and accessions, including wild and cultivated potato (CIP, 2017).

From the Andes, potato was introduced to Spain in 1567, France in 1574 and England in 1580. It was documented that from England, potato was transported to Bermuda in 1610 and to Kenya in 1880. From early in the sixteenth to the middle of the twentieth centuries, this crop reached India, Sri Lanka, China, America, Indonesia, Kenya and Saudi Arabia (de Haan and Rodriguez, 2016). After its widespread distribution worldwide, potato soon became a staple crop for human consumption because farmers can produce more food with less time and cultivation area. Potato is cultivated on approximately 17.5 million hectares across most continents, with more than 368 million tonnes harvested in 2018 (FAOSTAT, 2020b). Notably, potato crops produce a higher yield with more dry matter and minerals per hectare than most other food crops. The global average yield of potato in

2018 was approximately 20.9 t/ha, while comparable values for maize, rice and wheat are 5.9 t/ha, 4.6 t/ha and 3.4 t/ha, respectively (FAOSTAT, 2020b). Asia now accounts for more than half the world's production, while Oceania (including Australia and New Zealand) contributes 0.5% of potato production (Figure 1-1). China is currently the top potato producer with 90 million tonnes harvested in 2018, followed by India with 48 million tonnes and Ukraine with 22 million tonnes (FAOSTAT, 2020b).

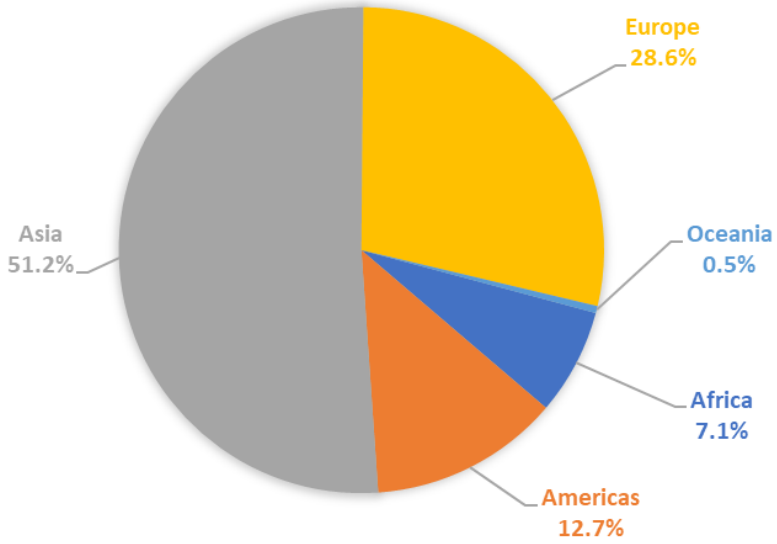



**Figure 1-1. Production share of potato by region in 2018 (FAOSTAT (2020b)).**

In Australia, the total potato production area is approximately 32.4 thousand hectares, producing 1.2 million tonnes in the 2018-2019 period (ABS, 2020). Interestingly, Australia's potato yield was 37.8 t/ha, which is substantially higher than the world average. Among States that produce potato in Australia, Western Australia (WA) contributes 6.5 % of the total (Figure 1-2). Although WA is not the top national potato producer, it is the only state free from many diseases affecting potato production present in other areas. The yield of potato in WA is the second-highest in the country (42.5 t/ha) after Tasmania (54.3 t/ha); (ABS, 2020). Also, it has a highly organized production system, and the proximity to the growing markets of Asia provides an opportunity to increase exports (Milroy and Jones, 2017).

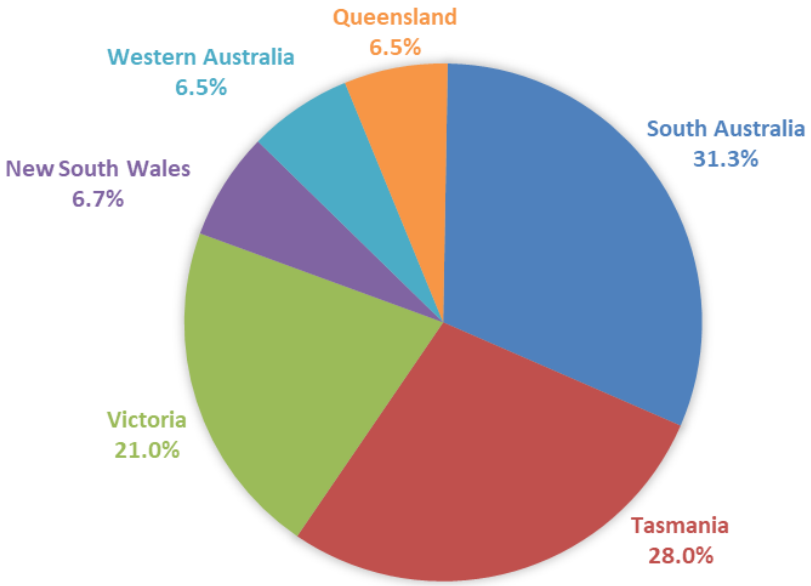



**Figure 1-2. Potato production in Australia by States in 2018-2019 (ABS, 2020)**

Fresh and frozen potatoes play a minor role in potato trading. In 2016, approximately 14 million tonnes of fresh potato, 7.8 million tonnes of frozen potato products and half a million tonnes of potato flour were exported (FAOSTAT, 2020a). More than 60 % of the potato produced went into processing in Australia, with the remaining going to the fresh market (ABS, 2020). In contrast, potatoes produced in WA are mainly for the fresh market; only 23 % are processed (ABS, 2020).

### 1.2.2 Consumption and nutritional value

According to the CIP, less than half of harvested potatoes are used as fresh vegetables for human consumption; the rest is processed into food products, food ingredients, industrial starch, livestock feeding or used as seed tubers (CIP, 2018). Potato starch is used intensively in some industries as a texture or adhesive agent, filler, or biodegradable plastic parts. Waste from potato processing can be fermented to make bioethanol. Global potato consumption is shifting from fresh to processed food, with more than seven million tons of potato made into French fries alone (CIP, 2018).

The potato tuber is not only a good source of dietary fibre and antioxidants, but it also has a relatively low level of fat (Table 1-1). The fat content in potato tubers is even lower than in cooked

rice and pasta (Camire et al., 2009). Vitamin C is the predominant vitamin in potato tubers; it can be as high as 145 mg per 100 g dry weight in some cultivars (Camire et al., 2009). Several B vitamins are also present in potato, with vitamin B6 being the most abundant. On the other hand, carbohydrates make up approximately 75 % of the total dry matter, where starch is the predominant carbohydrate (Camire et al., 2009). The presence of vitamins, minerals and antioxidants in the tuber make potato a good food source that can help prevent human malnutrition.

**Table 1-1. Nutritional components per 175 g of boiled potato with skin (McGregor, 2007)**

| | Per 175 g | % Daily value* |
|---|---|---|
| **Vitamin C** | 15.75 mg | 44 |
| **Vitamin B6** | 0.58 mg | 29 |
| **Potassium** | 753 mg | 22 |
| **Iron** | 2.8 mg | 20 |
| **Vitamin B1** | 0.23 mg | 16 |
| **Folate** | 33 µg | 16 |
| **Fibre** | 2.6 mg | 14 |
| **Magnesium** | 31.5 mg | 11 |
| **Calories** | 115.5 kcal | 6 |
| **Fat** | 0.5 g | 0.7 |

* Based on 2000 kilocalorie (kcal) controlled diet

Fried potato crisps are manufactured in more than 50 countries and are consumed extensively worldwide. The properties and quality of potato crisps have become a focus of interest for researchers (Yee and Bussell, 2007). One of the most significant features of processed potato is the colour of crisps, which plays an essential role in consumer acceptance of the product: crisps with light golden colour are considered to be of excellent quality, whereas brown or dark colours are not desirable because they are associated with burnt crisps. Processing companies are also concerned about acrylamide formation in crisps (Matthäus and Haase, 2014). This substance is a by-product of potato manufacturing processes and is a concern for human health due to its possible carcinogenic effect from dietary intake (Scientific Committee on Food, 2002). Its level in fried products is directly influenced by reducing sugars in potato tubers, which increase during cold storage (Malone et al., 2006).

There has long been an interest in refining potato characteristics to improve quality. Quality attributes related to production include improving resistance to pests and pathogens, tolerance to abiotic stress, enhancing nutritional value, and increasing longer-term storability (de Haan and Rodriguez, 2016). Nevertheless, commercial potato varieties suffer from a limited gene pool, and its expansion has been a subject of study for more than 50 years (Barrell et al., 2013). The conventional breeding method that has been used to generate new cultivars is by hybridizing parental clones and selecting superior individuals amongst large seedling populations (Barrell et al., 2013). However, the process is time-consuming because potato is heterozygous and polyploid, and a cultivar must be propagated vegetatively to maintain its unique trait combinations. The autotetraploidy of all commercial potato varieties makes it more challenging to combine desirable alleles through conventional hybridization. With emerging genetic technologies, it is becoming easier to directly manipulate the potato genome and introduce desirable properties into this economically important food crop.

## 1.3 Improving potato properties through genetic engineering

Potato is essential as a global food crop, yet conventional breeding of this heterozygous and polyploid crop is still challenging. Developments in biotechnology are helping to boost the breeding process and broaden the choices for generating elite potato characteristics. Many potato cultivars respond well to tissue culture, making it a suitable subject for genetic engineering studies. The 844-megabase genome of potato has been sequenced and assembled up to 86% from a homozygous doubled-monoploid clone (*S. tuberosum* group Phureja DM1-3 516 R44) and a heterozygous diploid breeding line (*S. tuberosum* group Tuberosum RH89-039-16) (Potato Genome Sequencing et al., 2011). A few years later, nine monoploid and three doubled monoploid clones derived from native potato landraces in South America were examined. The study focused on genome-wide structural variation, gene copy number variation, which impacted 30.2% of the potato genome, and explained

its highly heterozygous nature (Hardigan et al., 2016). These databases provide a knowledge platform and create more opportunities to modify the potato genome for elite traits.

### 1.3.1 Micropropagation of potato

The first *in vitro* culture of potato was done more than 60 years ago in 1951 by Stewart and Caplin (Vinterhater, 2008), and there have been numerous related studies done since then. Micropropagation involves multiplying nodal cuttings of surface-sterilized explants on standard culture media and regenerating whole plantlets from an explant. New shoots can proliferate from axillary buds on a stem segment. The process is used routinely for clonal propagation of virus-free potato material, maintaining genetic stocks and international transport of pathogen-free germplasm. Similar processes are used to generate transgenic potato plants. Although *in vitro* culture of potato has been studied for more than half a century, shoot regeneration from callus can still be problematic. Attention has moved towards direct shoot regeneration from explants, thus reducing or skipping a callus stage, and this can also help reduce tissue culture-induced somaclonal variation in regenerants (Vinterhater, 2008).

Different parts of potato plants, such as leaves, shoots and tubers, have been used as explants for direct shoot regeneration. Leaf explants are more likely to regenerate shoots with less chance of forming aneuploid plants compared to explants from tubers (Wheeler et al., 1985). Leaves also serve as a good source from which protoplasts can be isolated, while internodal segments are most responsive and offer a high regeneration rate (Millam, 2007). Media for potato micropropagation are highly cultivar dependent. In a study done by Shepard and Totten (1977), up to five different media were used in different stages of the culture process: protoplast isolation, protoplast cultivation, callus induction, shoot morphogenesis and root initiation. Most studies employ MS medium (Murashige and Skoog, 1962) as a base culture medium. Benzyladenine (BA) and naphthaleneacetic acid (NAA) are commonly used for shoot induction, gibberellic acid ($GA_3$) is added to promote the differentiation process (Peng et al., 2008, Webb et al., 1983, Wheeler et al., 1985).

Potato protoplasts have been isolated and studied since the 1970s. The culture of potato protoplasts has been considered a promising approach to addressing some problems associated with traditional breeding methods. The temporary absence of a cell wall makes protoplasts amenable for generating interspecies and intraspecies hybrids. However, protoplast culture is likely to induce somaclonal variation, which reduces the number of plants with normal phenotypes and desirable traits (Jones et al., 1983, Fossi et al., 2019). Besides, plant regeneration from protoplasts is also highly genotype-dependent, and thus there is a need to develop and optimise tissue culture and regeneration protocols for each potato genotype (Vinterhater, 2008).

### 1.3.2 Genetic modification

Potato was one of the first crops to be genetically transformed (Barrell et al., 2013): the initial attempts were made more than thirty years ago by infecting potato stems with *Agrobacterium rhizogenes* (Ooms et al., 1986). Since then, transformation technologies have been applied to this crop to develop resistance to viruses, insects, fungi, nematodes, and tolerance to herbicides and drought, and to improve its nutritional properties (Millam, 2004). *Agrobacterium*-mediated transformation is the preferred approach for genetically modifying potato, and transgenic potato plants have been regenerated in many different laboratories. The *nptII* gene conferring resistance to the antibiotic kanamycin has been the favoured selectable marker because it is highly effective and often leads to the recovery of many transformed plants. On the other hand, marker-free transformation approaches have also been applied to potato, in which transformed plants have been regenerated without antibiotic selection and transgenic events identified by PCR screening. However, this approach results in a very low recovery frequency and may result in chimeric plants among transformation events (Barrell et al., 2013).

Transformation of potato has also been achieved by direct DNA uptake by protoplasts through electroporation or in the presence of polyethylene glycol (PEG). Heat shock at 45°C for 5 min was first used in the transformation process, but soon it was detrimental to survival and decreased

protoplasts division (Fehér et al., 1991). PEG is added in the process to promote DNA uptake, with 25% and 40% are the commonly used concentrations (Clasen et al., 2016, Craig et al., 2005, Fehér et al., 1991). Selection agents such as kanamycin and hygromycin have also been used to screen and select transformants (Fehér et al., 1991, Craig et al., 2005).

Cold storage and processing traits are areas of research focus for potato, aiming to reduce the dark appearance of crisps and the synthesis of high acrylamide levels on frying. Studies in this area have focused on solving these problems, mainly by genetic engineering, and more recently, using gene editing. Although commercial growth of genetically modified (GM) plants is highly regulated, food products derived from transgenic Innate potato (developed by the Simplot company) have been accepted in eight countries, including Australia (ISAAA, 2020). Food Standards Australia New Zealand (FSANZ) has also granted permits for food derived from several GM potato lines since 2001, including potato lines that are resistant to potato leaf roll virus, potato virus Y or have reduced browning and polyphenol oxidase (FSANZ, 2019).

#### 1.3.2.1 Cold-induced sweetening (CIS) in potato tubers

In most potato cultivation regions, harvesting is only done once a year, and harvested tubers are stored with a tuber shelf-life of about six months. In such cases, it is essential to use cold storage to reduce sprouting, shrinkage, and disease loss of stored tubers. However, cold storage triggers the accumulation of glucose and fructose (hexose or reducing sugars), known as cold-induced sweetening (CIS). This phenomenon was first described in 1882 by Miller-Thurgau, and it was found that the level of accumulated reducing sugars in tubers is cultivar dependent (Sowokinos, 2001). CIS develops rapidly, within two weeks when tubers are stored at 4°C (Matsuura-Endo et al., 2006)

A carbohydrate metabolism pathway in potato tuber during cold storage was proposed by Malone et al. (2006) (Figure 1-3). In the tuber, starch is degraded into maltose, glucose, glucose 1-phosphate and glucose 6-phosphate, which are converted into sucrose in the cytosol. Acid invertase hydrolyses a portion of this sucrose to glucose and fructose in the vacuole. On the other hand, glucose 6-

phosphate is also converted into fructose 6-phosphate and then into fructose 1,6-bisphosphate, and this is an essential step of glycolysis. During cold storage, glycolysis is constrained because of the conversion of fructose 6-phosphate (Fru-6-P) to fructose 1,6-bisphosphate (Fru-1,6-$P_2$) by pyrophosphate: fructose 6-phosphate phosphotransferase (PFP) and phosphofructokinase (PFK) enzymes are suppressed. PFP and PFK are cold-labile enzymes, and their activities are negatively affected by cold treatments.

In contrast, other steps in the carbohydrate metabolism pathways are induced at low temperatures, such as the conversion of fructose-6-P to sucrose-P by sucrose phosphate synthase (SPS) or the breakdown of sucrose into fructose by invertase. Taken together, CIS occurs when starch is slowly hydrolysed to glucose and fructose, and the glycolytic pathway in which sugar products are normally metabolized is depressed, so the products of starch degradation are directed to the sucrose synthesis pathway with the resultant accumulation of reducing sugars. When processed at high temperature, reducing sugars in tubers create a dark colour associated with a bitter taste (Figure 1-4). The accumulation of reducing sugars is known to be significantly lower in tubers stored above 8°C (Matsuura-Endo et al., 2006). However, such temperatures favour sprouting of tubers, requiring the application of sprout suppressing chemicals, which may leave undesirable residues and affect food safety negatively (Paul et al., 2016).

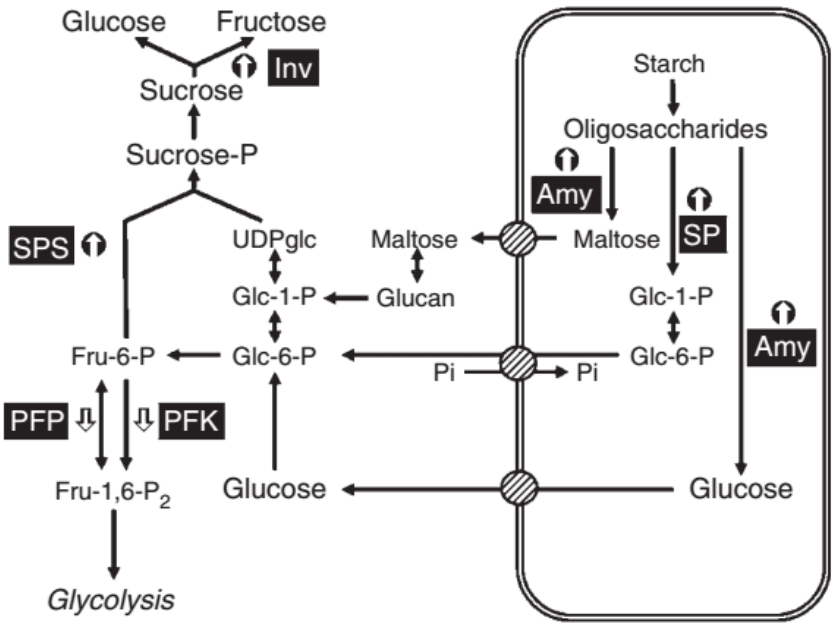



**Figure 1-3. Proposed pathways of carbohydrate metabolism in potato tubers during cold storage (Malone et al., 2006).** Up arrow in black circles: the increased catalytic capacity of enzymes at low temperature. Down arrows: steps catalyzed by enzymes that are inhibited or inactivated at low temperatures. Inv: invertase, SPS: sucrose phosphate synthase, PFP: pyrophosphate: fructose 6-phosphate phosphotransferase, PFK: phosphofructokinase, Amy: amylase, SP: starch phosphorylate.

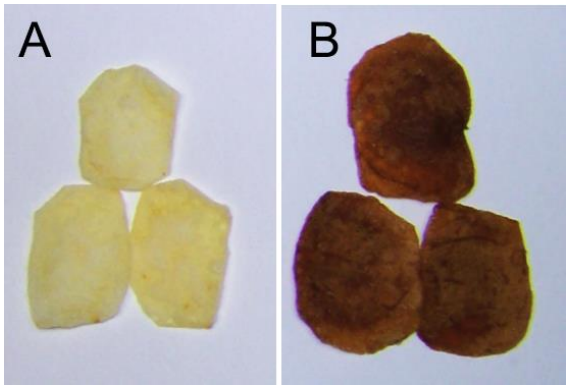



**Figure 1-4. Contrasting colours of potato crisps (Wu et al., 2011).**(A) standard-coloured crisps, (B) crisps produced from tubers cold-stored at 4°C for 30 days.

While the cold lability of PFP and PFK appears to be the main reason for CIS in tubers, it is not easy to manipulate these key enzymes without causing significant changes in potato carbohydrate metabolism, and so attention has shifted towards modifying other pathways. During cold storage at 4°C, sucrose is rapidly converted into hexoses, and invertase levels also increase rapidly (Pressey and Shaw, 1966). These findings indicate a direct role of invertase in the accumulation of reducing sugars in tubers when stored at low temperatures. The *VInv* gene, a member of the invertase gene

family, is a vital enzyme in converting sucrose into hexose sugars (Sowokinos, 2001). One reason is that invertases, and most of the reducing sugars generated during cold storage, are located in the vacuole (McKenzie et al., 2013). A strong correlation between hexose: sucrose ratio and acid invertase activity in cold-stored tubers from different cultivars has been established (McKenzie et al., 2013). Significantly, VINV does not appear to play a crucial role in tuber development, making it an ideal target for controlling the hexose content (Bhaskar et al., 2010).

There is evidence that silencing the *VInv* gene does indeed appear to be a promising strategy to prevent the accumulation of hexoses in tubers during cold storage (Bhaskar et al., 2010, Ye et al., 2010, Wu et al., 2011, Wiberley-Bradford et al., 2014, Zhu et al., 2014, Clasen et al., 2016, Zhu et al., 2016). Early studies were done using RNAi, which involves generating double-stranded RNA in tubers in which the antisense component induces degradation of target gene mRNA. Such RNAi lines exhibited a significant reduction of *VInv* expression, up to 98 %, accompanied by a decrease in fructose levels up to 50-fold compared to wildtype tubers (Wu et al., 2011). All cold-stored RNAi tubers produced lighter colour crisps on processing compared to those from wildtype potato. Field trials showed similar phenotypes and growth between wildtype and RNAi lines (Bhaskar et al., 2010). The tuber performance of both wildtype and RNAi lines were investigated by Wiberley-Bradford et al. (2014), who found a significant difference in invertase activities and hexoses content between wildtype and transgenic tubers. The accumulation of hexoses in tubers of transgenic lines was prevented after eight months in cold storage. Clasen et al. (2016) silenced the *VInv* using a different approach, transcription activator-like effector nucleases (TALENs), to generate mutants in potato after protoplast transfection. The results were comparable to those obtained using RNAi. Tubers from these plants had undetectable hexose sugars, and crisps produced from these tubers also had lighter colour than those of wildtype. The mutagenesis percentage was genotype-dependent and depended on the TALEN construct; it varied from 2.1% to 15.9% when using three different TALENs pairs for four different potato varieties (Clasen et al., 2016). Furthermore, this study demonstrated

that gene editing technology can facilitate full knockout of the target gene, which is difficult to achieve using RNAi.

These studies indicated the potential of reducing the accumulation of hexose sugars by suppressing the *VInv* gene without altering plant development and yield performance. Interestingly, the acrylamide level in crisps derived from *VInv*-silenced tubers was reduced by 4 to 15-fold (Bhaskar et al., 2010, Clasen et al., 2016, Ye et al., 2010). Nevertheless, the acrylamide content in fried potato is also controlled by the AS1 activity, another endogenous enzyme in potato tubers.

#### 1.3.2.2 The acrylamide-forming potential in fried potato

Acrylamide has been produced commercially for industrial uses, and its toxicological properties are well established. However, its presence in processed food was only recognized in 2002 after a Scientific Committee on Food meeting assessed the implications of a study by Tareke et al. (2000) showing the association of acrylamide with "a considerable cancer risk". Levels of acrylamide in different foods and food products were studied, and it was reported that fried potato has up to 3500 µg/kg of acrylamide, higher than in biscuits and breakfast cereals (SCF, 2002). In contrast, no acrylamide was detected in raw or boiled foods. Therefore, it was concluded that acrylamide is formed mainly in carbohydrate-rich foods processed at high temperatures (SCF, 2002). The mechanisms by which the acrylamide is formed following heat processing were unknown until Mottram et al. (2002) found that acrylamide is a product of the Maillard reaction between reducing sugars (hexose sugars) and free amino acids, such as asparagine which is abundant in potato and cereals (Mottram et al., 2002).

Since the undesirable effects of acrylamide on human health were recognized, research has focused on decreasing the level of this compound in foods. Acrylamide is viewed as a food-borne contaminant generated by the Maillard reaction, and thus factors that influence this reaction are considered keys for controlling acrylamide levels (Matthäus and Haase, 2014). The Maillard reaction is influenced by factors such as temperature, pH of the food and content of precursors, such as

reducing sugars and asparagine (Matthäus and Haase, 2014). At first, it was proposed to reduce acrylamide content in fried potato by changing the way it is prepared and processed (Pedreschi et al., 2006). These methods involve immersion in an acidic solution, using different oil types and changing the frying temperature (Rydberg et al., 2003, Pedreschi et al., 2006). However, these treatments were only partially effective, and they were difficult and costly to apply on a large scale (Rommens et al., 2008). Therefore, potatoes that contain a low level of acrylamide precursors are much preferred, but such varieties with high processing quality are not currently available. Given the complexity of the potato genome, genetic manipulation was a faster way to generate varieties with low acrylamide-forming potential. Targeting the acrylamide precursor, asparagine, was a promising approach for controlling the potential for acrylamide formation. Asparagine is synthesized from aspartate by *asparagine synthetase* (AS), and two cDNAs (*AS1* and *AS2*) were identified from the potato variety Ranger Russet *(*Rommens et al., 2008*).* Silencing both genes using RNAi resulted in decreased free asparagine in potato tubers by up to 95% with no adverse effect on tuber yield or development of glasshouse-grown transgenic plants (Chawla et al., 2012, Rommens et al., 2008). However, in field trials, transgenic plants produced small and cracking tubers with secondary growth (Chawla et al., 2012), suggesting that AS has an essential role in tubers, and low AS levels may be associated with stress which leads to deformed tubers. By individually targeting *AS1* and *AS2*, it was found that the physical changes in tubers were linked to down-regulation of *AS2,* and *AS2*-silenced plants did not show a decrease in AS level. Silencing *AS1* alone was sufficient for lowering acrylamide formation in tubers while maintaining the quality and yield of glasshouse-grown tubers (Chawla et al., 2012). The *AS2* is closely related to the *Arabidopsis AS2* gene, with 86% of identity, thus suggesting its role in ammonium metabolism (Chawla et al., 2012, Rommens et al., 2008). Further studies also indicated that *AS2* is mainly expressed in leaves and may be involved in the detoxification of ammonium ions ($NH4^+$) in this part of the plant, and so silencing *AS2* had negative impacts on plant growth (Chawla et al., 2012).

It is clear that reducing asparagine synthesis is necessary, but it does not always guarantee a low-acrylamide product (Rommens et al., 2008). Factors other than asparagine control the formation of acrylamide, and reducing sugars are the most important. Hence, the *AS1* genes could be silenced together with those corresponding to cold-induced sweetening to achieve better results. This idea was supported by a study by Shepherd et al. (2010) using trait segregation in a breeding population; their data showed that low-acrylamide crisps were derived from tubers of plants that had both low asparagine and reducing sugars. Zhu et al. (2016) generated transgenic Russet Burbank with three genes *VInv, AS1,* and *AS2* silenced through RNAi. Fried potatoes derived from tubers of *VInv/AS1/AS2*-triple silencing plants showed a reduction in acrylamide level when compared to controls. All tested RNAi lines exhibited normal phenotypes with a similar yield to controls when grown in the greenhouse. However, seed tubers from these plants developed into stunted and chlorotic plants.

RNAi has been used in several studies to engineer the potato genome. This technique is effective and considered specific, although it results in random integration of transgene(s), and off-target activity has also been documented (Jackson et al., 2003). The potato genome can be modified more precisely with the emerging gene-editing techniques such as TALENs and CRISPR/Cas9. An example is the complete knockout of the *VInv* gene by transient expressing TALENs constructs in potato protoplasts (Clasen et al., 2016).

## 1.4 Gene editing

Genome or gene editing is a new breeding technology with various applications in crop improvement. This rapidly developing suite of technologies includes different methods employing site-directed nucleases (SDNs) to create double-stranded breaks (DSBs) in the target gene sequence. Once the DSBs occur, they may be repaired before genome replication, either via the non-homologous end-joining (NHEJ) pathway or the homologous recombination (HR) pathway. NHEJ is an error-prone process and can result in mutations at break sites from random insertion or deletion

(indel) of bases. If mutations occur in a coding sequence or the promoter region, gene function may be disrupted (Voytas, 2013). The indel mutations can be a few edited nucleotides (nt) at one DSB or up to hundreds of kilobase pair deletions between two DSB sites (Hilscher et al., 2017). For instance, a large chromosomal deletion between two DSBs was achieved in rice, in which approximately 245 kb was removed (Zhou et al., 2014).

Gene editing started with the discovery of an intron-encoded protein, a meganuclease, in *Saccharomyces cerevisiae*, which was used to integrate an intron into a mitochondrial gene (Jacquier and Dujon, 1985). Later, Pavletich and Pabo (1991) introduced the first engineered nuclease by fusing a zinc finger DNA-binding protein to a bacterial restriction endonuclease *FokI*. Even though this technique has been applied effectively in plants for creating mutagenesis, the target range for zinc finger nucleases (ZFNs) is limited (Voytas, 2013). Furthermore, ZFNs must be designed and constructed specially for each target site. This process is time-consuming and requires expertise in molecular biology. There were limited approaches to the SDNs to create DSBs until the DNA-binding specificity of transcription activator-like effectors of phytopathogenic bacteria of the genus *Xanthomonas* was discovered (Boch et al., 2009, Moscou and Bogdanove, 2009): its use became a new way to manipulate target gene by fusing it with *FokI*, creating transcription activator-like effector nucleases (TALENs). TALENs also function in pairs like ZFNs, but they do not require re-engineering to construct TALENs arrays. The binding sites of TALENs must not start with a T base, which limits the target range of this technique (Gaj et al., 2013). The most recent tool is the CRISPR/Cas9 system, has proved to be an efficient and simpler alternative to ZFNs and TALENs for generating precisely targeted genetic alterations.

### 1.4.1 The CRISPR/Cas9 system

CRISPR was first discovered in *Escherichia coli* (*E.coli*) by Ishino et al. (1987), but its function was only understood and proved twenty years later by Barrangou et al. (2007). When adjacent to CRISPR-associated (Cas) proteins, CRISPR forms an RNA-mediated adaptive defence system against invading

genetic material such as viral DNA or plasmids. The natural CRISPR/Cas system consists of three components: Cas protein, CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA). When encountering foreign DNA, CRISPR is transcribed and processed into crRNAs which are approximately 40 nt in length. These crRNAs then combine with tracrRNA to activate and guide the Cas nuclease protein to make DSBs in the invading nucleic acid sequence (Barrangou et al., 2007). This defence strategy's specificity lies in the 20 nt upstream of the protospacer-adjacent motif (PAM) (Bortesi and Fischer, 2015).

The CRISPR/Cas system can be classified into two classes based on the number of proteins involved in the target degradation process (Makarova et al., 2015). Class 1 requires different multi-subunit crRNA-effector complexes, while class 2 only needs a single subunit crRNA-effector molecule. These two classes are further categorized into five types based on the genes encoding protein effectors: type I, type III and type IV belong to class 1; type II and type V belongs to class 2. Type II CRISPR is the most widely used system for gene editing because of its simplicity and ease of manipulating. Various type II systems which require different PAM sequences have also been discovered (Table 1-2). Among these, CRISPR/Cas9 from *Streptococcus pyogenes* is the most popular system due to the relatively small size of the Cas9 protein with a short PAM sequence (Bortesi and Fischer, 2015).

**Table 1-2. Summary of different nucleases associated with the CRISPR system (Nakade et al., 2017)**

| Nuclease type | Enzyme name | WT/mutants | PAM | Protein size | Typical protospacer length | DNA end | Pros/Cons |
|---|---|---|---|---|---|---|---|
| Cas9 | SpCas9 | WT[5] | 5′-NGG-3′ | 1,368 a.a. | 20 nt | blunt end | Most commonly used/Large protein size |
| | | VQR[25] | 5′-NGAN-3′ | | | | Different PAM specificities/Large protein size |
| | | EQR[25] | 5′-NGAG-3′ | | | | |
| | | VRER[25] | 5′-NGCG-3′ | | | | |
| | SaCas9 | WT[15] | 5′-NNGRRT-3′ | 1,053 a.a. | | | Small protein size/Relatively strict PAM |
| | | KKH[41] | 5′-NNNRRT-3′ | | | | |
| | FnCas9 | WT[43] | 5′-NGG-3′ | 1,629 a.a. | | | Less restrictive PAM/Large protein size, less application examples |
| | | RHA[43] | 5′-YG-3′ | | | | |
| | NmCas9 | WT[69] | 5′-NNNNGATT-3′ | 1,082 a.a. | 24 nt | | Small protein size/Strict PAM |
| | St1Cas9 | WT[70] | 5′-NNAGAAW-3′ | 1,121 a.a. | 20 nt | | Small protein size/Strict PAM |
| | BlatCas9 | WT[42] | 5′-NNNNCNDD-3′ | 1,092 a.a. | 21 nt | | Less restrictive PAM, small protein size/Less application examples |
| Cpf1 | AsCpf1 | WT[44] | 5′-TTTN-3′ | 1,307 a.a. | 23 nt | sticky end | Various unique characteristics (see main text)/Strict PAM |
| | LbCpf1 | WT[44] | 5′-TTTN-3′ | 1,228 a.a. | | | |

The significant advance in using the CRISPR/Cas9 system was its modification by combining the crRNA and tracrRNA to create a functional chimeric, single guide RNA (gRNA) molecule (Jinek et al., 2012). Its specificity still depends on the 20-nt guide sequence, and 12 nt near the PAM are called the seed sequence, which is crucial in the pairing between crRNA and the target DNA (Bortesi and Fischer, 2015). The role of the seed sequence was demonstrated by Zheng et al. (2017), and mismatches in this sequence are less likely to result in off-target effects. Compared to other gene-editing techniques, CRISPR/Cas9 offers significant advantages and better opportunities for specifically targeted mutations in plants. It is a much simpler system that still provides high efficiency and versatility. Instead of requiring synthetic peptides with the DNA-binding domain, the CRISPR/Cas9 system only needs a gRNA sequence to recognize a target sequence (Bortesi and Fischer, 2015). Therefore this system can be reprogrammed easily, and its range of target sites is nearly unlimited (Shan et al., 2014). Because targeting relies solely on the complementarity between the guide RNA and target sequence, the CRISPR/Cas9 system can also be used for multiplex gene editing (Hilscher et al., 2017). Besides, it is much easier to deliver the system into plant cells because the gRNA sequence is shorter than the long ZFN or TALEN-encoding vectors (Shan et al., 2014).

CRISPR-mediated targeted gene modification in plants was first successfully demonstrated in Arabidopsis, tobacco, sorghum and rice by Jiang et al. (2013). The Cas9 coding sequence and the gRNAs were assembled as plasmid DNA and delivered into plants via *Agrobacterium tumefaciens,* particle bombardment or PEG-mediated protoplast transfection. This system has been applied quickly to other plants, including wheat, maize, cotton, apple, citrus and potato (Li et al., 2013, Liang, 2013, Xie and Yang, 2013, Brooks et al., 2014, Shan et al., 2014, Butler et al., 2015, Weeks, 2017).

The integration of foreign DNA into the plant genome can result in insertional mutations and, occasionally, plant mosaicism (Svitashev et al., 2016). There has been an increased focus on research on DNA-free gene editing, in which there is no detectable transgene in gene-edited plants.

Transient expression of CRISPR/Cas9 has been shown to have the potential of producing transgene-free mutants. A DNA construct containing a Cas9 coding sequence and gRNA expression cassette can be coated on particles and bombarded into immature embryos (Zhang et al., 2016a). Although mutants with no detectable transgene can be obtained via this method, there still be a possibility that CRISPR/Cas9 DNA might be integrated into the plant genome. One way to remove an integrated editing cassette is to self-cross edited plants and select those lines with the desired edit but lack the expression cassette (null segregants).

Another approach is to deliver the CRISPR/Cas9 system as an RNP complex into protoplasts or cells. RNPs were successfully transfected into protoplasts from Arabidopsis, tobacco, lettuce and rice with mutation frequencies up to 46% in lettuce (Woo et al., 2015). No mosaicism or off-target mutations were detected in gene-edited lettuces, and the mutations were inherited in the next generation. Recently, the delivery of CRISPR/Cas9 RNP into potato protoplast was demonstrated by Andersson et al. (2018). This study targeted gene coding for granule-bound starch synthase (GBSS). All mutated lines were transgene-free, and a complete knockout of this gene was found in 2-3% of the regenerated shoots. An additional consideration is that, by using a DNA-free approach to edit plant genome, products from this process might be exempt from the regulations related to growth and use of genetically modified plants because of the absence of introduced DNA in plants cells.

### 1.4.2 Gene editing in potato with CRISPR/Cas9

The first application of CRISPR/Cas9 on potato was made using *Agrobacterium* to transfer a construct encoding Cas9 protein and gRNAs targeting the *acetolactate synthetase 1* gene in diploid and tetraploid cultivars (Butler et al., 2015). Up to 15% of mutations were detected in diploid X914-10 while 29% of transformed tetraploid Desiree carried mutations, with detected mutations ranged from 1 bp insertion to 38 bp deletion. Since then, a few studies have explored the potential of this technique to manipulate the potato genome (Table 1-3). The delivery methods were either via *Agrobacterium*-mediated transformation or protoplast transfection. The cultivar choices were not

diverse; Desiree and a few diploid varieties were mainly used. The targets ranged from herbicide tolerance (Butler et al., 2015, Butler et al., 2016, Nadakuduti et al., 2019a, Veillet et al., 2019b), removing self-incompatibility (Enciso-Rodriguez et al., 2019, Ye et al., 2018), virus resistance (Zhan et al., 2019), altering tuber chemical composition and nutritional value (Andersson et al., 2017, Andersson et al., 2018, Nakayasu et al., 2018, Yang and Xie, 2015) to modifying plant pathways (Wang et al., 2015, Zhou et al., 2017).

Most of the putative transformed events from these studies involved a selection process using either kanamycin or hygromycin. Veillet et al. (2019b) used both kanamycin and chlorsulfuron to screen for transformed events. This work resulted in a 100% mutation efficiency of the selected lines. The two-stage selection used in this study helped screen out all untransformed events, and all events that were resistant to both kanamycin and chlorsulfuron carried mutations in the target gene, *acetolactate synthase,* which can confer chlorsulfuron resistance. Apart from this study, mutation rates of other research activities varied from 2% (Andersson et al., 2018) to 81.8% (Zhou et al., 2017). Such differences in reported mutation rates result from differences in how mutation rates were calculated in each report. The 2% reported by Andersson et al. (2018) was calculated as the edited events that showed complete knockout of the target gene (and mutations in all alleles) by all regenerated shoots. In contrast, the 81.8% frequency from Zhou et al. (2017) only indicated the proportion of lines carrying mutations of total analysed lines, and not all alleles of the target gene were mutated. Similarly, Wang et al. (2015) tested six transgenic potato plants expressing Cas9 and found that five of them carried mutations, making the mutation rate 83.3%. In general, the mutation rate was lower when using protoplast transfection as a delivery method for the CRISPR/Cas9 system. This was highlighted in a study by Tuncel et al. (2019), in which a 6% mutation rate was achieved using *Agrobacterium* -mediated plant transformation, while it was only 1.5% when PEG-mediated protoplast transfection was used.

Multiallelic mutations appear to be a challenge, with limited successes reported. Andersson et al. (2018) identified mutations in 9% of regenerated shoots, with only 2% of them carrying mutations in all four alleles. Most of the mutagenesis reported in potato were heterozygous mutations with small indels (ranging from 1 bp to 50 bp), but in rare cases, up to a 175 bp deletion or 236 bp insertion has been found (Andersson et al., 2017, Kusano et al., 2018). Attempts have been made to increase Cas9-induced mutagenesis in potatoes, one of which used the endogenous potato *StU6* promoter in the expression vector of CRISPR components. The percentage of edited alleles was increased nine-fold (from 8% to 71%) by changing the *AtU6* promoter to the *StU6* promoter (Johansen et al., 2019). Another strategy is integrating the translation enhancer, *dMac3*, directly upstream of the Cas9 expressing sequence (Kusano et al., 2018). This method improved the mutation rate 2.7 times compared to a Cas9-expressing vector with no translation enhancer. The applications of CRISPR/Cas9 to edit the potato genome is still in the early stages of development, with induced mutagenesis varying among studies. Given the complexity of the potato genome and its well-known impact of genotype in both transformation and regeneration process, application of the CRISPR/Cas9 system needs to be programmed and modified depending on target genes of chosen potato varieties/genotypes.

**Table 1-3. Application of CRISPR/Cas9 in potato.** RNP: Cas9-gRNA ribonucleoprotein complex

| Variety | Ploidy | Delivery method | Explant type | Target gene | Enzyme/ trait associated with the gene | Number of gRNA | Selection agents | Mutation rate | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Desiree | 4x | *A.tumefaciens* | leaves | *acetolactate synthase1 (StALS1)* | herbicide tolerance | 2 | hygromycin | 5% to 60% | Butler et al. (2015) |
| X914-10 | 2x | | | | | | | 3% to 55% | |
| double haploid | 2s | *A.tumefaciens* | stem segments | *StIAA2* | Aux/IAA protein | 1 | kanamycin | 83.3% | Wang et al. (2015) |
| double haploid | 2x | protoplast transfection with a plasmid vector | protoplast | *asparagine synthetase 1 (StAS1)* | asparagine synthetase | 2 | | 3.6% and 4.6% | Yang and Xie (2015) |
| Desiree | 4x | *A.tumefaciens* | leaves | *acetolactate synthase1 (StALS1)* | herbicide tolerance | 1 | kanamycin | 12.5% | Butler et al. (2016) |
| X914-10 | 2x | | | | | | | | |
| Kuras | 4x | protoplast transfection with a plasmid vector | protoplasts | *granule-bound starch synthase (GBSS)* | granule-bound starch synthase | 4 | | 2.2% to 11.6% | Andersson et al. (2017) |
| Desiree | 4x | *A.tumefaciens* | internodes | *StMYB44* | phosphate transport via roots | 1 | kanamycin | 81.8% | Zhou et al. (2017) |
| Kuras | 4x | protoplast transfection with RNPs | protoplasts | *granule-bound starch synthase (GBSS)* | granule-bound starch synthase | 1 | | 9% to 25% | Andersson et al. (2018) |
| Sayaka | 4x | *A.tumefaciens* | internodes | *granule-bound starch synthase 1 (GBSS1)* | granule-bound starch synthase | 3 | hygromycin | 7% to 79% | (Kusano et al., 2018) |
| Mayqueen | 4x | *A.rhizogenes* | *in vitro* shoots | *St16DOX* | a steroid 16α-hydroxylase | 2 or 3 | | 24% | Nakayasu et al. (2018) |
| *S.phureja* S15-65 clone | 2x | *A.tumefaciens* | internodes | *Stylar ribonuclease gene (S-Rnase)* | self-incompatibility | 1 | kanamycin | 5.2% | Ye et al. (2018) |

| Variety | Ploidy | Delivery method | Explant type | Target gene | Enzyme/ trait associated with the gene | Number of gRNA | Selection agents | Mutation rate | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Desiree | 4x | *A.tumefaciens* | stem and petiole | *acetolactate synthase (ALS)* | chlorsulfuron resistance | 1 | kanamycin and chlorsulfuron | 100% | Veillet et al. (2019b) |
| Desiree | 4x | *A.tumefaciens* | leaves | the genome of *potato virus Y* | virus resistant | 1 | kanamycin | not available | Zhan et al. (2019) |
| DMRH-S5 28-5 | 2x | protoplast transfection with a plasmid vector | protoplast | *acetolactate synthase1 (ALS1), 5-Enolpyruvylshikimate-3-phosphate synthase1 (EPSPS1)* | resistance to the imidazolinone group of herbicides and glyphosate | not available | | 11% to 47% | Nadakuduti et al. (2019a) |
| DRH-195 and DRH-310 F1 | 2x | *A.tumefaciens* | leaves | *Stylar ribonuclease gene (S-Rnase)* | self-incompatibility | 2 | hygromycin | not available | Enciso-Rodriguez et al. (2019) |
| Desiree | 4x | protoplast transfection with a plasmid vector | protoplasts | *starch-branching enzyme* | starch-branching enzyme | 2 | | 60.8% | Johansen et al. (2019) |
| Wotan | | | | | | | | | |
| Desiree | 4x | *A.tumefaciens* | stems | *starch-branching enzyme 1* and *2 (SBE1, SBE2)* | starch-branching enzymes | 4 for *SBE1* and 6 for *SBE2* | kanamycin | 6% | Tuncel et al. (2019) |
| | | protoplast transfection with a plasmid vector | protoplasts | | | | | 1.5% | |

## 1.5 Project aim and objectives

The aim of this project was to develop healthier potato lines for human consumption with low acrylamide-forming potential and low cold-induced sweetening. The strategy was to apply the CRISPR/Cas9 system to alter the *VInv* and *AS 1* genes in potato. The project had five specific objectives:

Objective 1: Optimisation of a micropropagation system for potato (*Solanum tuberosum L.*) cultivars Atlantic and Desiree.

Objective 2: Identification of gRNAs targeting *VInv* and *AS1* genes and *in vitro* evaluation of gRNA efficiency.

Objective 3: Delivery of the CRISPR/Cas9 system into plant cells via *Agrobacterium*-mediated plant transformation and obtain modified plantlets.

Objective 4: Direct delivery of the CRISPR/Cas9 into plants via protoplast transfection or particle bombardment for generating transgene-free gene-edited plants.

Objective 5: Biochemical analysis of potato tubers to assess their acrylamide-forming potential and accumulation of hexose sugars after cold storage.

# Chapter 2

# Optimisation of tissue culture regeneration for the potato cultivars Atlantic and Desiree

## 2.1 Introduction

The *in vitro* regeneration of potato has been studied and developed for various cultivars, and it is well established that this process is strongly genotype dependent. Potato cultivars respond differently to the same culture medium and growing conditions, with variation in the number of shoots produced or in some cases, there may be no shoot formation (Ghosh et al., 2014, Webb et al., 1983, Wheeler et al., 1985). An efficient *in vitro* regeneration protocol is indispensable to facilitate plant research, especially for plant transformation.

Two potato cultivars, Atlantic and Desiree, were chosen as subjects for this study. Desiree has yellow flesh with red skin and has been widely studied in laboratory conditions because it is highly responsive to *in vitro* culture. Thus, it can be used as a model for developing new micropropagation or transformation system. On the other hand, Atlantic is a standard chipping variety and has creamy-white flesh with yellow skin (Webb et al., 1978). Desiree has been used in numerous studies on micropropagation, microtuberisation and plant transformation of potato (Wheeler et al., 1985, Butler et al., 2015, Butler et al., 2016, Zhou et al., 2017, Veillet et al., 2019b, Zhan et al., 2019). In an early study on shoot formation of potato explants, Desiree exhibited the best performance among 14 test varieties for tissue culture media response and shoot regeneration rate (Wheeler et al., 1985). Desiree has also been used to study the effectiveness of gene delivery systems due to its high transformation efficiency, such as in geminivirus-mediated transformation (Butler et al., 2016), particle bombardment (Veillet et al., 2019b) or gene-editing abilities of CRISPR/Cas9 (Butler et al., 2015) and CRISPR/Cas13a (Zhan et al., 2019).

In contrast, Atlantic has only been used in a limited number of studies with mixed results on regeneration and transformation efficiency (Peng et al., 2008, Wu et al., 2011, Han and Lee, 2015). In comparison with other potato cultivars, Atlantic often ranked the lowest in shoot induction and transformation frequency. When Atlantic was transformed with an RNAi construct targeting the *VInv* gene, it yielded about half of the transgenic lines produced by the varieties Snowden and

MegaChip (Wu et al., 2011). Atlantic was also reported to be more recalcitrant *in vitro* than cultivar Jowon during the regeneration stage (Han and Lee, 2015). Its regeneration rate was 10.1%, and transformation efficiency was 0.5%, much lower than 119% and 18.4% of Jowon (Han and Lee, 2015). Exceptionally, up to a 100% shoot induction rate has been reported for Atlantic (Peng et al., 2008), but the reproducibility of this micropropagation system remains to be determined. However, it is well established that potato micropropagation is highly genotype-dependent (Webb et al., 1983, Wheeler et al., 1985, Ghosh et al., 2014), thus a more optimum *in vitro* shoot regeneration system for Atlantic can still be developed.

Microtuberisation in cultured potato shoots has been developed for a wide range of cultivars. The production of microtubers helps facilitate tuber analysis in laboratory conditions without the requirement of glasshouse or field experiments and shortens the time needed to obtain potato tubers. Microtubers can be induced under aseptic conditions and have similar morphology to tubers produced in the field (Barker, 1953, Estrada et al., 1986). However, the induction of microtubers is also partly genotype-dependent (Gopal et al., 1998) and requires optimisation when working with new cultivars.

The aim of the work in this chapter was to establish efficient protocols for micropropagation of potato cultivars Atlantic and Desiree. Established media systems from previous studies were tested for shoot regeneration from leaf explants and microtuberisation of both cultivars. The effect of different combinations of 6-benzylaminopurine (BAP), NAA and gibberellic acid ($GA_3$) in shooting media was also assessed to improve shoot induction of Atlantic.

## 2.2 Methodology

### 2.2.1 Preparation of media for in vitro culture of potato explants

Murashige & Skoog Basal Medium (MS medium, Cat. No. M5519, Sigma – Aldrich, Inc.) was used as a basal nutrient medium. It was prepared by dissolving 4.4 g of MS in 800 mL of distilled water, with

the addition of sucrose or glucose depending on different treatments, and the volume was adjusted to one litre with distilled water. The pH was adjusted to 5.6 with 1 M KOH, and 2.8 g/L Gelrite (Sigma-Aldrich, Inc) was added to the medium prior to sterilisation by autoclaving at 121°C for 16 min. The medium was cooled to about 60°C before adding plant growth regulators (PGRs) or antibiotics. Murashige & Skoog Modified Basal Medium with Gamborg Vitamins (MSGV) (Phytotech Labs) was used as the basal medium in shoot induction experiments for Atlantic. This medium was prepared as for MS medium. All PGRs and antibiotics were prepared as stock solutions, filter-sterilised through 0.22 µm sterile filter units (Merck) and added to media.

### 2.2.2 Regeneration of potato plants from leaf explants

Potato shoot cultures of Atlantic and Desiree were maintained *in vitro* by subculturing nodal explants every four to six weeks on MS medium with 20 g/L sucrose (MS20 medium) and 2.8 g/L Gelrite in 250-mL clear plastic sterile containers with screw caps (Labserv). All cultures were kept in a growth room under a 16 h light/8 h dark photoperiod at 22°C. In the first experiment, different PGR combinations and carbohydrate sources (sucrose and glucose) from previously published studies were tested for shoot regeneration (Table 2-1). *In vitro* leaves from four-week-old plantlets were cut into squares (1 x 1 cm) and embedded with the abaxial surface in direct contact with the culture medium. A total of 40 to 48 explants were divided into three replicates for each treatment. Treatments included direct shoot regeneration (T1 and T2) in which explants were sub-cultured bi-weekly on the same type of medium and two-step regeneration (T3, T4, T5 and T6) in which explants were cultured on callus induction medium first and then transferred to shoot induction medium. In treatment T3, explants were kept on the callus induction medium for two weeks before transferring to the shoot induction medium (Peng et al., 2008). The callus induction phase in the treatments T4, T5 and T6 was one week, and then explants were transferred to shooting media (Kumlay and Ercisli, 2015, Banerjee et al., 2006, Nadakuduti et al., 2019b). The treatment T6 involved two shooting media: explants were cultured for two weeks on SIM1 medium (Table 2-1) and then moved to SIM2

medium (Table 2-1). The treatment T7 was based on the shoot regeneration protocol developed for Atlantic by Han and Lee (2015), using four media types (M1 to M4). Explants were cultured in liquid M1 ($NH_4NO_3$ and $CaCl_2$ were added to the medium before autoclaving) for one day, followed by culture on solid medium M2 for two days, four weeks on M3 and finally to M4 until shoot regeneration.

**Table 2-1. Media composition for shoot regeneration from leaf explants of potato cultivars Altlantic and Desiree.** DSR: direct shoot regeneration media, CIM: callus induction media, SIM: shoot induction media. NAA: 1-naphthaleneacetic acid, BAP: 6-benzylaminopurine, $GA_3$: gibberellic acid, ZR: trans-zeatin-riboside, 2,4-D: 2,4-dichlorophenoxyacetic acid. Murashige & Skoog Basal Medium (MS medium) (Sigma – Aldrich, Inc.) was used as a basal nutrient medium. Media were adjusted to pH 5.6 with 1M KOH. Gelrite (Sigma-Aldrich, Inc) was used as the gelling agent.

| Treatment | Glucose (g/L) | Sucrose (g/L) | NAA (mg/L) | BAP (mg/L) | $GA_3$ (mg/L) | ZR (mg/L) | 2,4-D (mg/L) | $NH_4NO_3$ (mg/L) | $CaCl_2$ (mg/L) | Thiamine -HCl (mg/L) | Gelrite (g/L) | Type | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T1 | - | 20 | 0.02 | 3.0 | 10 | - | - | - | - | - | 2.8 | DSR | - |
| T2 | - | 30 | 2.5 | 2.0 | - | - | - | - | - | - | 2.8 | DSR | Yasmin et al. (2003) |
| T3 | - | 30 | 0.3 | 2.5 | - | - | - | - | - | - | 2.8 | CIM | Peng et al. (2008) |
| | - | 30 | 0.1 | 3.0 | 5.0 | - | - | - | - | - | 2.8 | SIM | |
| T4 | - | 30 | 2.0 | 3.0 | | - | - | - | - | - | 2.8 | CIM | Kumlay and Ercisli (2015) |
| | - | 30 | - | 2.0 | 0.25 | - | - | - | - | - | 2.8 | SIM | |
| T5 | 16 | - | 5.0 | 0.1 | - | - | - | - | - | - | 2.8 | CIM | Banerjee et al. (2006) |
| | 16 | - | 0.02 | - | 0.15 | 2.2 | - | - | - | - | 2.8 | SIM | |
| T6 | - | 30 | - | - | | 0.8 | 2.0 | - | - | 0.9 | 2.8 | CIM | Nadakuduti et al. (2019b) |
| | - | 30 | - | - | 4.0 | 0.8 | - | - | - | 0.9 | 2.8 | SIM1 | |
| | - | 30 | - | - | 2.0 | 0.8 | - | - | - | 0.9 | 2.8 | SIM2 | |
| T7 | - | 30 | 10.0 | 10.0 | - | - | - | 147 | 80 | - | - | M1 | Han and Lee (2015) |
| | - | 30 | - | - | - | - | 2.0 | - | - | - | 2.8 | M2 | |
| | - | 30 | 0.01 | - | 0.1 | 2.0 | - | - | - | - | 2.8 | M3 | |
| | 16 | - | 0.02 | - | 0.15 | | | | | | 2.8 | M4 | |

The second experiment was set up to improve the shoot induction rate of Atlantic using a two-step regeneration process. First, leaves were detached from 4- to 6-week-old *in vitro* plantlets, and leaf discs (0.7 cm) were made using a cork borer. For each treatment, explants were cultured for five days on MSGV supplemented with 16 g/L glucose, 5 mg/L NAA and 2 mg/L or 0.1 mg/L BAP, pH 5.6 before transferring to eight different shoot induction media with varying concentrations of BAP (2 mg/L to 8 mg/L) with or without 0.1 mg/L NAA supplemented with 5 mg/L of $GA_3$ and 16 g/L glucose, pH 5.6 (Table 2-2). Explants were sub-cultured weekly, and the number of induced shoots was recorded after four and eight weeks on shoot induction media. Experiments were replicated three times with a total of 42 explants for each treatment.

**Table 2-2. Media composition for *in vitro* shoot regeneration optimisation of potato cultivar Atlantic.** Modified Murashige & Skoog Basal medium with Gamborg vitamins (Phytotech Labs) was used as a basal nutrient medium. Media were adjusted to pH 5.6 with 1M KOH. Gelrite (Sigma-Aldrich, Inc) was used as gelling agent at 2.8 g/L. Explants were cultured on callus induction media (16 g/L glucose, 5 mg/L NAA and 2 mg/L BAP, pH 5.6) for five days before transfer to the shoot induction treatments.

| Treatment | Glucose (g/L) | NAA (mg/L) | BAP (mg/L) | $GA_3$ (mg/L) |
|---|---|---|---|---|
| S1 | 16 | - | 2.0 | 5.0 |
| S2 | 16 | - | 4.0 | 5.0 |
| S3 | 16 | - | 6.0 | 5.0 |
| S4 | 16 | - | 8.0 | 5.0 |
| S5 | 16 | 0.1 | 2.0 | 5.0 |
| S6 | 16 | 0.1 | 4.0 | 5.0 |
| S7 | 16 | 0.1 | 6.0 | 5.0 |
| S8 | 16 | 0.1 | 8.0 | 5.0 |

### 2.2.3 Induction of microtubers in culture

Multiple-nodal explants of Atlantic and Desiree were cultured on MS medium supplemented with 80 g/L sucrose and 2.8 g/L Gelrite, pH 5.6 (MS80) to induce microtubers (Gopal et al., 1998). In addition, single-nodal explants of Atlantic were also cultured on MS20 medium for up to seven weeks without sub-culturing (Han and Lee, 2015). Twelve explants were used for each cultivar in each treatment. All cultures were kept under a 16 h photoperiod at 22°C. After 60 days of culture in 16 hr light, Atlantic explants on MS80 were transferred to the dark until microtuber formation.

## 2.3 Results

### 2.3.1 Response of Atlantic and Desiree leaf explants to tissue culture regeneration

In the first experiment, the treatments T1 to T5 were tested for both Atlantic and Desiree leaf explants. Treatments T6 and T7 were tested only with Atlantic. Forty explants on treatment T1 (0.02 mg/L NAA, 3 mg/L BAP, 10 mg/L $GA_3$) did not show significant development after one week and later displayed necrosis with no further differentiation until week three. All explants on treatments T3 to T7 (45 explants each) thickened around the cut edges of leaves and exhibited callus induction signs after one week. Similar signs were observed in the direct shoot regeneration medium for treatment T2 (Figure 2-1 A and C). Callus continued to grow on leaves of both cultivars on the direct shoot regeneration medium treatment T2, and roots developed after six weeks without any shoot formation (Figure 2-1 B and D). After two weeks on shoot induction medium, treatment T3, necrosis occurred on Desiree explants while Atlantic explants developed light green, friable calli (Figure 2-2 A and 2B). These calli continued to increase in size for a further four weeks and eventually turned brown. Explants on the treatment T4 expanded in size but had friable calli and developed roots after two weeks on shooting medium (Figure 2-2 C and D). Their colour gradually turned brown, and no further development was observed in the explants of both cultivars.

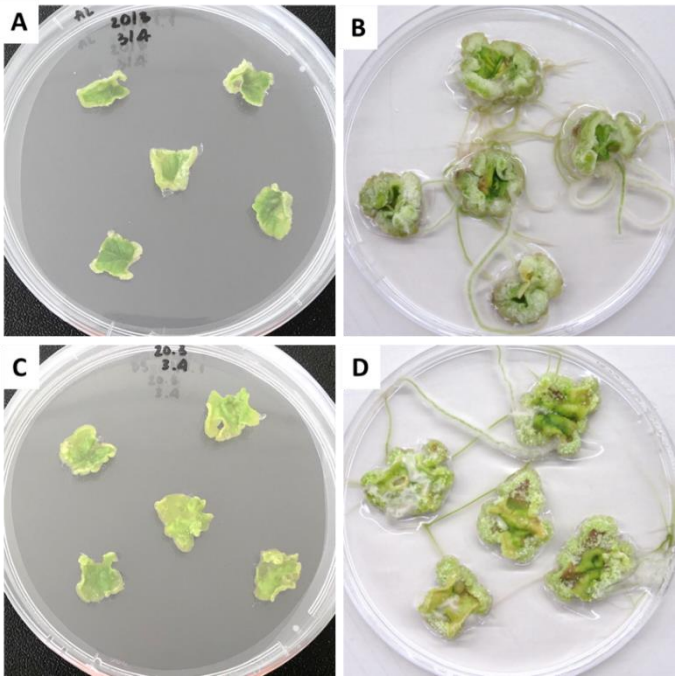



**Figure 2-1. Response of Atlantic and Desiree leaf explants on shoot induction treatment T2.** Treatment T2: MS medium with 30 g/L sucrose, 2.5 mg/L NAA, 3 mg/L BAP, pH 5.6. (A) and (B): Atlantic leaf explants after one and six weeks of culture, respectively. (C) and (D): Desiree leaf explants after one and six weeks of culture, respectively.

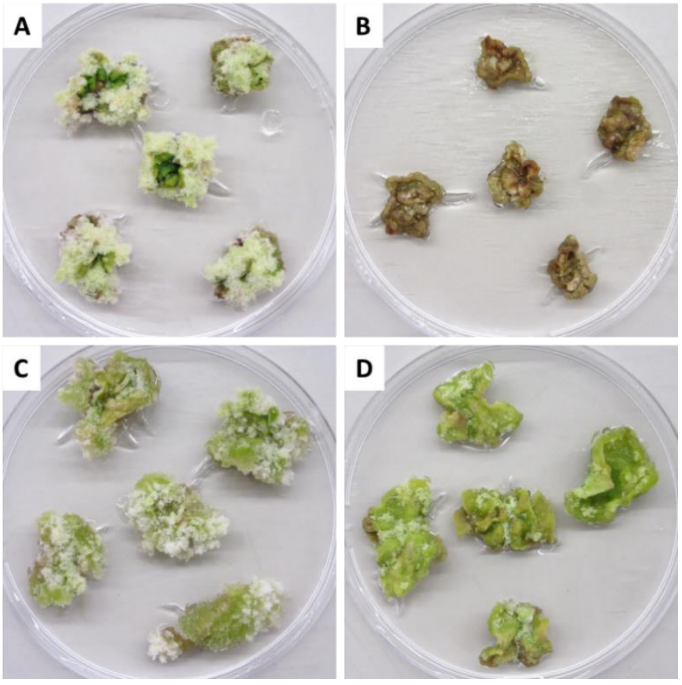



**Figure 2-2. Atlantic and Desiree leaf explants after two weeks on shoot induction medium of the treatments T3 and T4. (A)** and **(B)**: Atlantic and Desiree explants on the treatment T3 (MS + 30 g/L sucrose + 0.1 mg/L NAA + 3.0 mg/L BAP + 5.0 mg/L $GA_3$, pH 5.6), respectively. **(C)** and **(D)**: Atlantic and Desiree explants on the treatment T4 (MS + 30 g/L sucrose + 2.0 mg/L BAP + 0.25 mg/L $GA_3$, pH 5.6), respectively.

**Table 2-3. Shoot regeneration rate of Desiree and Atlantic leaf explants after eight weeks of culture on treatments T1 to T7**. Data are presented as mean ± standard deviation. Desiree leaf explants were not tested on treatment T6 and T7. N/A: not applicable

| Treatment | Desiree | | Atlantic | |
|---|---|---|---|---|
| | Total explants | Percentage of shoot producing explant (%) | Total explants | Percentage of shoot producing explant (%) |
| **T1** | 40 | 0.00 ± 0.00 | 40 | 0.00 ± 0.00 |
| **T2** | 40 | 0.00 ± 0.00 | 40 | 0.00 ± 1.00 |
| **T3** | 40 | 0.00 ± 0.00 | 40 | 0.00 ± 2.00 |
| **T4** | 40 | 0.00 ± 0.00 | 40 | 0.00 ± 3.00 |
| **T5** | 45 | 100.00 ± 0.00 | 39 | 12.82 ± 4.44 |
| **T6** | N/A | N/A | 40 | 27.08 ± 9.08 |
| **T7** | N/A | N/A | 48 | 10.42 ± 3.61 |

For the treatment T5, glucose was used instead of sucrose as the carbohydrate source, and ZR was the cytokinin instead of BAP (Table 2-1). It was found that both Atlantic and Desiree explants were greener and displayed better vigour on this treatment than those on T3 and T4 (Figure 2-2, Figure 2-3 A and B). During the first week of transfer on shooting medium, shoot initiation was observed for Desiree and healthy shoots developed from all explants by the eighth week (Figure 2.3 C, Table 2-3). Atlantic shoots were also induced in this treatment, but only a 12.8% shoot regeneration rate was obtained after eight weeks of culture.

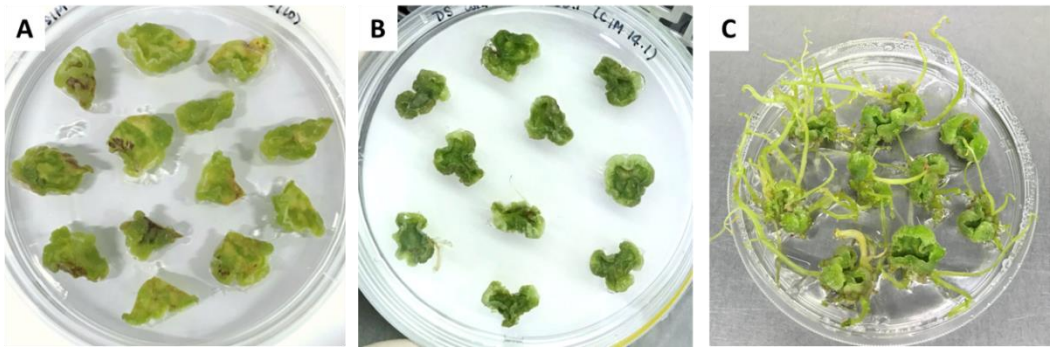



**Figure 2-3. Atlantic and Desiree leaf explants on shoot induction medium of the treatment T5**. Medium contains MS + 16 g/L glucose + 0.02 mg/L NAA, 0.15 mg/L $GA_3$ and 2.2 mg/L ZR, pH 5.6. **(A)**: Atlantic leaf explants after four weeks of culture. **(B)** and **(C)**: Desiree leaf explants after two and eight weeks of culture, respectively.

Because the treatment T5 resulted in the highest regeneration rate for Desiree, T6 and T7 were only used for Atlantic. Treatment T6 induced shoots in 27.08% for 40 Atlantic leaf explants after eight weeks (Table 2-3). A much lower shoot regeneration rate, 10.42%, was obtained from the treatment T7, with only five out of 48 explants generated shoots (Table 2-3).

In the second experiment, MSGV was used as the basal medium for the regeneration of Atlantic leaf explants. The explants exhibited better vigour on the MSGV medium than the MS medium (Figure 2-4 A and B). The callus induction medium was the same as in treatment T5, which was supplemented with 16 g/L glucose, 5 mg/L NAA and 0.1 mg/L BAP. After five days, explants thickened around their edges without much callus induction. The amount of BAP in the callus induction medium was later increased to 2 mg/L, which resulted in better callus formation after the same period. Also, explants cultured on callus induction medium with 2 mg/L BAP had a healthier appearance with greener colour than those on medium with 0.1 mg/L BAP (Figure 2-4 C and D). The treatment S3 is given as an example in Figure 2.4, indicating the better performance of explants on medium with MSGV as the basal medium and 2 mg/L BAP than MS as the basal medium and 0.1 mg/L BAP (Figure 2-4).

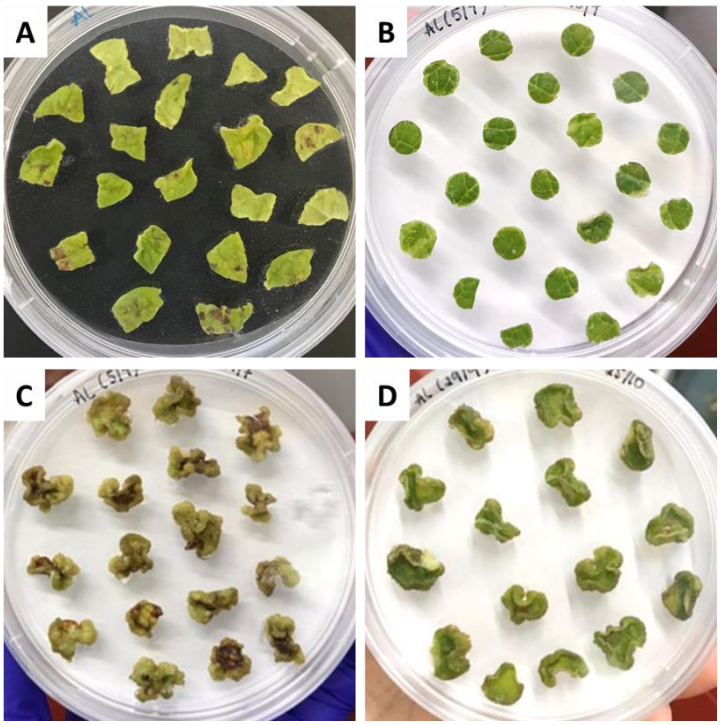



**Figure 2-4. Atlantic leaf explants after five days on callus induction medium and after three weeks on shoot induction medium of the treatment S3.** Medium treatment S3: modified MS medium with Gamborg vitamins (MSGV) + 6 mg/L BAP + 5 mg/L $GA_3$. **(A)**: explants after five days on MS medium + 5 mg/L NAA + 0.1 mg/L BAP. **(B)**: explants after five days on MSGV + 5 mg/L NAA + 0.1 mg/L BAP. **(C)**: explants were transferred from MSGV + 5 mg/L NAA + 0.1 mg/L BAP and cultured for three weeks on treatment S3. **(D)**: explants were transferred from MSGV + 5 mg/L NAA + 2 mg/L BAP and cultured for three weeks on treatment S3. All media were supplemented with 16 g/L glucose, pH 5.6.

Shoot induction was recorded for all treatments S1 to S8 (Table 2-2) after four weeks. After eight weeks, more shoots regenerated in shooting medium without NAA (treatments S1 to S4), ranging from 7.14% in S4 to 64.29% in S3, compared to media with 0.1 mg/L NAA, which gave only 4.76% (in S5 and S6) to 28.57% (in S8) (Table 2-4). Interestingly, root formation occurred in all media with 0.1 mg/L NAA (treatments S5 to S8). An increasing trend in regeneration rate over time was observed for most treatments (S1, S2, S3, S4, S7 and S8) from four to eight weeks of culture, except for treatments S5 and S6 (Table 2-4). The treatment S3 induced the most shoots from leaf-derived calli of Atlantic after eight weeks (64.29%) with an average of five shoots per explant (Table 2-4).

**Table 2-4. Shoot regeneration rate of Atlantic leaf explants after four and eight weeks on shoot induction medium for treatments S1 to S8.** Modified Murashige & Skoog Basal medium with Gamborg vitamins (Phytotech Labs) was used as a basal nutrient medium. Media were adjusted to pH 5.6 with 1M KOH. Gelrite (Sigma-Aldrich, Inc) was used as gelling agent at 2.8 g/L. Explants were cultured on callus induction medium (16 g/L glucose, 5 mg/L NAA and 2 mg/L BAP, pH 5.6) for five days before being transferred to shoot induction medium of each treatment. Data are presented as mean ± standard deviation.

| Treatment | Total explants | Shoot regeneration rate (%) | | Average number of shoots per explant | |
|---|---|---|---|---|---|
| | | 4 weeks | 8 weeks | 4 weeks | 8 weeks |
| **S1** | 42 | 21.43 ± 10.10 | 30.95 ± 23.57 | 0.52 ± 1.27 | 0.83 ± 1.72 |
| **S2** | 42 | 9.52 ± 13.47 | 19.05 ± 26.94 | 0.12 ± 0.40 | 0.57 ± 1.45 |
| **S3** | 42 | 47.62 ± 13.47 | 64.29 ± 37.04 | 1.74 ± 2.87 | 5.00 ± 7.69 |
| **S4** | 42 | 4.76 ± 6.73 | 7.14 ± 3.37 | 0.05 ± 0.22 | 0.10 ± 0.37 |
| **S5** | 42 | 4.76 ± 6.73 | 4.76 ± 6.73 | 0.05 ± 0.22 | 0.05 ± 0.22 |
| **S6** | 42 | 4.76 ± 6.73 | 4.76 ± 6.73 | 0.05 ± 0.22 | 0.05 ± 0.22 |
| **S7** | 42 | 7.14 ± 10.10 | 7.14 ± 10.10 | 0.14 ± 0.57 | 0.17 ± 0.70 |
| **S8** | 42 | 26.19 ± 30.30 | 28.57 ± 33.67 | 0.69 ± 1.66 | 1.17 ± 2.30 |

### 2.3.2 Microtuber induction

Microtubers were produced successfully from all 12 multiple-nodal explants of each cultivar, Atlantic and Desiree, on MS80 medium. Desiree explants initiated microtuber formation after four weeks under a 16 h photoperiod. At least one microtuber formed on each explant and reached approximately 7 mm in diameter after six weeks in culture (Figure 2-5A). Atlantic explants did not show any sign of microtuberisation after 60 days of culture with a 16h light photoperiod. However, within one week of culture in the dark, white stolons developed and elongated, followed by the formation of microtubers. Atlantic microtuber size was approximately 10 mm after 30 days (Figure 2-5B). No microtubers were formed on single-nodal explants of Atlantic after seven weeks on MS20 medium without subculturing (Table 2-5).

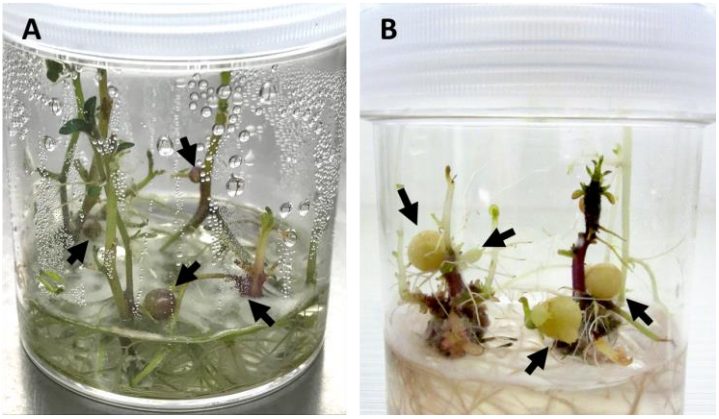



**Figure 2-5. Microtuberisation of Desiree and Atlantic from multi-nodal explants on MS medium with 80 g/L sucrose. (A)**: microtubers initiated from multi-nodal explants of Desiree after four weeks of culture with 16h light/ 8h dark photoperiod at 22°C. **(B)**: Microtubers developed from multi-nodal explants of Atlantic after 30-day culture in the dark. Explants were initially kept at 16h light/ 8h dark photoperiod at 22°C for 60 days before transferring to the dark. Black arrows point to the microtubers.

**Table 2-5. Microtuberisation of potato cultivars Atlantic and Desiree from multiple-nodal explants under different culture conditions.** Modified Murashige & Skoog Basal medium with Gamborg vitamins (Phytotech Labs) was used as a basal nutrient medium. Media were supplemented with either 20 g/L sucrose (MS20) or 80 g/L sucrose (MS80) and adjusted to pH 5.6 with 1M KOH. Gelrite (Sigma-Aldrich, Inc) was used as gelling agent at 2.8 g/L.

| Media | Total explants | Culture conditions | Variety | Percentage of explants produced microtubers |
|---|---|---|---|---|
| MS20 | 12 | 16 h photoperiod at 22°C | Atlantic | 0% |
| MS80 | 12 | For initial 60 days: 16 h photoperiod at 22°C<br>For remaining period: continuous dark, 16 h photoperiod at 22°C | Atlantic | 100% |
| MS80 | 12 | 16 h photoperiod at 22°C | Desiree | 100% |

## 2.4 Discussion

The results obtained in this chapter agree with previous findings that the regeneration process of potato is highly genotype-dependent (Gopal et al., 1998, Ghosh et al., 2014, Webb et al., 1983, Wheeler et al., 1985). Optimum shoot regeneration and microtuberisation in Atlantic and Desiree were obtained by testing different media formulations and culture conditions. The use of different PGRs was shown to have varied effects on the differentiation and regeneration of plant tissues. In

addition, modifying the media components (such as adding vitamins) and the carbohydrate source in the culture medium also impacted plant tissue vigour.

A combination of auxin and cytokinin is vital for the regeneration of shoots in plant tissue culture, as their interaction governs cell differentiation and subsequent shoot initiation (Schaller et al., 2015). A high concentration of auxin is required for callus induction, and it must be accompanied by an optimal addition of cytokinin. The contribution of cytokinin in callus formation is believed to promote the periclinal divisions after primordia are induced due to the effect of auxin (Schaller et al., 2015). Furthermore, cytokinin acts as an auxin antagonist to inhibit root formation (Schaller et al., 2015). The use of auxin and cytokinin alone has previously been reported to induce low or no callus in potato variety Cardinal (Yasmin et al., 2003). However, combining NAA and BAP in callus induction media raised the callus induction rate of Cardinal leaf explants to 95% (Yasmin et al., 2003). Additionally, it appears that a moderate ratio of auxin to cytokinin is preferred for callus formation rather than a high ratio of the two PGRs. In this study, leaf explants displayed better vigour on callus induction medium with increased cytokinin (2 mg/L of BAP) than the one with a lower level of cytokinin (0.1 mg/L BAP) given the same concentration of auxin (5 mg/L NAA, Figure 2-4). Hence, the 2.5 times higher ratio of auxin (5 mg/L NAA) to cytokinin (2 mg/L BAP) was preferable to the 50 times higher concentration of auxin (5 mg/L NAA) to cytokinin (0.1 mg/L BAP).

In the shoot induction stage, the auxin concentration in the medium must be limited as a continuous supplement of high auxin tends to promote root growth (Christianson and Warnick, 1983, Webb et al., 1983). For example, the cultivar Superior produced roots when cultured on a direct shoot regeneration medium with either NAA or IAA (Dhital et al., 2011). This root-formation effect of auxin was observed in the direct regeneration medium of the T2 treatment with 2.5 mg/L NAA resulting in explants forming roots without any sign of shoot induction after six weeks. Root formation also occurred in the shoot induction medium of treatments S5 to S8, which contained only 0.1 mg/L NAA. The high level of NAA (5 mg/L) in the callus induction stage of these treatments S5 to

S8 may have accumulated in the cells and thus promoted root initiation in the presence of the same auxin, even at low concentration, in shoot induction medium.

In the second experiment on leaf explant shoot regeneration, the changes in basal medium components and the carbohydrate source positively affected the vitality of Atlantic explants. The MS medium was replaced by MSGV containing the Gamborg vitamins, which improved the shoot induction rate for this cultivar. The addition of Gamborg vitamins probably promoted cell growth and division due to the high level of thiamine-HCl (10 mg/L), together with double the concentration of nicotinic acid and pyridoxine HCl (1 mg/L each) compared to MS medium (0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl and 0.1 mg/L thiamine-HCl) (Gamborg et al., 1968). A similar effect of thiamine on regeneration was also found for the treatment T6, which had 0.9 mg/L thiamine-HCl in the shooting medium resulting in a shoot induction rate of 32.5 % for Atlantic. Apart from Gamborg vitamins, the replacement of sucrose by glucose also contributed to better shoot regeneration from leaf explants, as seen in treatments T5 and S3. Sucrose is a common carbon source in plant tissue culture media, and the use of sucrose at 20 g/L concentration is optimum for culturing both Atlantic and Desiree nodal segments for plant micropropagation. Differences in the regeneration of plants *in vitro* on using glucose or sucrose as the carbohydrate source have not been investigated thoroughly. Sucrose, glucose and fructose have similar effects in culture on potato plant height, internode length, number of nodes, number of leaves and plantlet fresh mass (Rahman et al., 2010). However, how these sugars affect explant regeneration is still unexplored. In other plants, for example, *Capsicum annum* and *Alnus crispa*, it has been speculated that glucose might support callus growth and morphogenesis and promote better *in vitro* growth than sucrose (George et al., 2008), while in common bean (*Phaseolus vulgaris*), glucose is used faster than sucrose in tissue culture (Wolff and Price, 1960). Glucose has been used as a substitute for sucrose in regeneration media resulting in an improved regeneration rate (potato variety Atlantic), but no clear mechanism of how glucose contributes to the plant regeneration process has been established. Additional experiments are

necessary to address the effect of glucose compared to sucrose as a carbon source for potato explants for *in vitro* shoot regeneration.

Direct shoot regeneration was not achieved when leaf explants of Atlantic and Desiree were cultured on media containing NAA and BAP (treatment T1) or NAA, BAP and $GA_3$ (treatment T2). The combination of plant growth regulators used, and explant type was thus not ideal for direct organogenesis. Switching BAP in the NAA-BAP-$GA_3$ combination to ZR can facilitate direct shoot regeneration - ZR is believed to induce plant cell division, shorten the callus phase, and promote shoot formation. It has been used in shoot production from leaf and internodal explants of different potato cultivars such as Berolina, Russet Burbank, Bintje and Kaptah Vande (De Block, 1988, Beaujean et al., 1998, Anjum and Ali, 2004, Banerjee et al., 2006, Rezende et al., 2013, Nadakuduti et al., 2019b). Previously, the potato cultivars Superior and Monalisa produced shoots (up to 75% shoot induction rate) on media supplemented with NAA-$GA_3$-ZR combinations without going through the callus induction stage (Dhital et al., 2011, Campos et al., 2016). In this chapter, shoot induction from Desiree leaf explants was obtained from a shooting medium containing the NAA-$GA_3$-ZR combination in treatment T5. Another factor that influences direct shoot organogenesis could be the explant type. Leaf and nodal segments are suitable as explants for direct shoot formation, but their regeneration ability is cultivar dependent. For example, leaf explants were best for potato cultivar Cardinal (Yasmin et al., 2003), while nodal explants worked better in cultivars Gui valley and Bora valley (Dhital et al., 2011). Future experiments on direct shoot organogenesis of Desiree and Atlantic can explore the effect of different plant growth hormones combination such as NAA-$GA_3$-ZR or utilise the nodal segments as explants. Because the callus and shoot induction system had successfully facilitated the highest regeneration rate in Desiree, and Atlantic also responded to the process, direct shoot regeneration of these cultivars was not investigated further.

The induction of microtubers was achieved for Desiree and Atlantic under surprisingly different culture conditions. Normal light at 16-h photoperiod was sufficient for microtuber formation in

Desiree, and this is similar to the cultivar Pentland Javelin (Garner and Blake, 1989). In contrast, microtuberisation of Atlantic required culture in the dark after initial culture in light. Likewise, the absence of light is a requirement for microtuberisation of some other potato cultivars, including Kennebec, Russet Burbank and Shepody (Seabrook et al., 2004). Switching to dark culture after long-term culture in 16-hrs photoperiod can trigger leaves to synthesise systemic signals to shift growth from above to below-ground parts of the plant as a response to change in day-length, stimulating the formation of stolons and tubers (Jackson, 1999, Ewing and Wareing, 1978, Rodríguez-Falcón et al., 2006). This study confirmed that the interaction between genotype and culture conditions influences the microtuberisation process (Seabrook et al., 2004, Garner and Blake, 1989, Gopal et al., 1998).

Based on the results in this chapter, the callus and shoot induction medium in the treatment T5 was optimum for regenerating leaf explants from potato cultivar Desiree. MSGV medium supplemented with 16 g/L glucose, 5 mg/L NAA, 2 mg/L BAP, 2.8 g/L Gelrite, pH 5.6, and shoot induction media of the treatment S3 were suitable for callus formation and shoot regeneration from leaf explants for Atlantic. These media conditions were then used to induce shoot regeneration after *Agrobacterium*-mediated transformation (Chapter 4) and particle bombardment of potato leaf tissues (Chapter 5). Because the experiments in this chapter and the *Agrobacterium*-mediated transformation in chapter 4 were undertaken simultaneously, the M1 to M4 medium in treatment T7 were used first for shoot regeneration of Atlantic. With the results from this chapter, the regeneration media for this cultivar were switched to MSGV with 5 mg/L NAA, 2 mg/L BAP for callus induction, and media of treatment S3 for shoot induction.

# Chapter 3

# *In silico* identification and *in vitro* testing the efficiency of gRNAs to target Cas9 cleavage of the *VInv* and *AS1* genes

## 3.1 Introduction

In the CRISPR/Cas9 system, gRNA is the vital component that defines the location and efficiency of creating DSBs in target gene DNA. The specificity of gRNA is directly linked to the uniqueness of the target site, which is 20 nt directly upstream of a PAM sequence. In a polyploid plant, like potato, it is crucial to select target sites in conserved regions of the gene, so that gRNA can guide Cas9 to cleave all alleles to achieve complete gene knockout if required. Cultivated potatoes are autotetraploid and highly heterozygous. Their complex genomes are affected by structural genome variations such as single nucleotide polymorphisms (SNPs) and copy number variations (Kyriakidou et al., 2020). High allelic variation in the *VInv* gene has been reported with an average of one SNP per 24 bp (Draffehn et al., 2010). The allelic polymorphism must be identified before selecting target sites. Sequencing PCR amplicons of the gene or its clones is the most common method to screen and identify DNA variations in target genes.

Multiplexing gene editing can be achieved with the CRISPR/Cas9 system because it can be designed to cleave two or more sites on a gene or different genes simultaneously (Cong et al., 2013, Li et al., 2013, Xie et al., 2015). The effectiveness of the dual gRNA system in creating large deletions has been shown in model and crop plants. Early work was reported by Li et al. (2013), in which two targets were designed 24 bp apart on the *AtPDS3* (*Arabidopsis thaliana phytoene desaturase*) gene, leading to a 48 bp deletion of the genomic segment in between, including a part of the target sequence. The mutation frequency was 7.7% higher than the 5.6% frequency when only one gRNA was used targeting the same gene. Dual gRNAs have also been used successfully to generate 247 bp and 459 bp deletion in *WRKY20* and *VQ33* genes of Arabidopsis, respectively (Pauwels et al., 2018). The ability to generate large chromosomal deletions of the CRISPR/Cas9 dual gRNA system was also demonstrated in rice, with approximately 245 kb deleted from chromosome 2 (Zhou et al., 2014). In tomato, 90 bp and 140 bp were deleted in the *slago7* (*Solanum lycopersicum argonaute 7*) gene with

two gRNAs (Brooks et al., 2014), while in the *granule bound starch synthase I* (*GBSSI*) gene of potato, two gRNAs targeting exon 1 and 2 resulted in a 135-bp deletion (Veillet et al., 2019a).

In this chapter, the *VInv* and *AS1* genes of potato cultivars Atlantic and Desiree were amplified and sequenced to provide data for *in silico* identification of target sites. Two gRNAs were selected for each gene using the Cas-Designer web tool. The targets were in the form of 5'-N(8)S(12)NGG-3', where N(8) is the eight nucleotides directly upstream of 12-nt seed sequence S(12), and NGG is the PAM sequence for *Streptococcus pyogenes* Cas9. The uniqueness of the gRNA was assessed using a nucleotide BLAST search against the nucleotide collection of *Solanum tuberosum* on NCBI (National Centre for Biotechnology Information, USA) website (https://www.ncbi.nlm.nih.gov) to avoid potential off-targets. Selected gRNAs were synthesised *in vitro*, and their efficiency to create DSBs in the target DNA was assessed *in vitro* with the Cas9 cleavage assay.

## 3.2 Methodology

### 3.2.1 Identification of target gene sequences and primer design

The complete coding sequence (cds) of the *VInv* gene was published by Draffehn et al. (2010) (accession no. HQ110080.1). The full-length gene is 6,097 bp, including two untranslated regions (UTRs) at the 5' and 3'-end, seven exons and six introns (Figure 3-1 A). The mRNA sequence of this gene is also available on NCBI at accession no. NM_001288064.1. For the *AS1* gene, only its mRNA sequence is available on the NCBI database, with accession no. XM_006343993.2. This mRNA sequence was mentioned by Zhu et al. (2016) and Rommens et al. (2008), in which its cDNA clone (GenBank: CK278037.1) was used to design primers to amplify the *AS1* gene. The NCBI information indicates a predicted mRNA (5,045 bp in length) derived from a genomic sequence (accession no. NW_006238977.1, region 1,560,303 to 1,565,347). An alignment between the mRNA (XM_006343993.2) and DNA (NW_006238977.1) sequences reveals that the *AS1* gene has 13 exons (Figure 3-1 B).

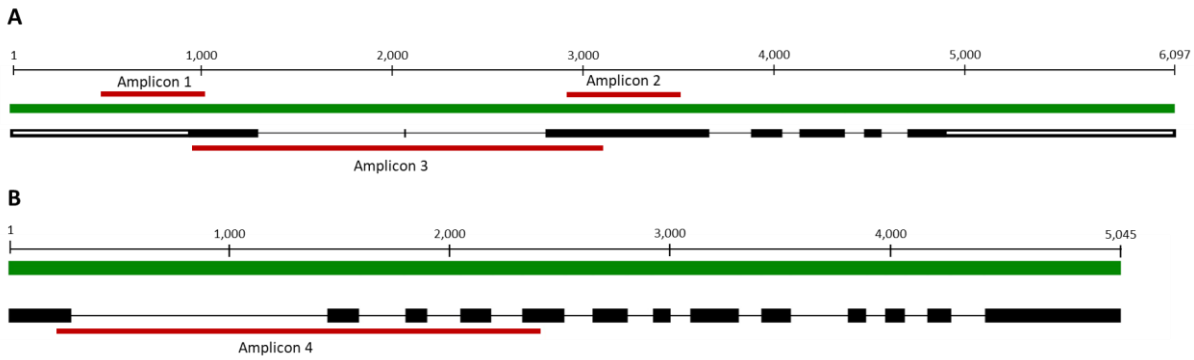



**Figure 3-1. Structures and primer flanking regions (A) *vacuolar invertase* and (B) *asparagine synthetase 1* genes.** Green lines represent the length of target genes; white boxes represent untranslated regions; thick black lines represent exons; thin black lines represent introns; red lines represent amplicons of corresponding primer pairs, cds: complete coding sequence. Amplicon 1 to 4: corresponding amplified products of primer pairs named VInv1-F & VInv1-R, VInv2-F & VInv2-R, VInv3-F & VInv3-R and AS1-F & AS1-R.

Primers were designed using the Primer-BLAST tool (Ye et al., 2012) with default settings. The cds of the *VInv* gene (accession no. HQ110080.1) was used to design two pairs of primers, VInv1 and VInv2, amplifying the first and third exons, respectively. Another primer, VInv3, was designed to amplify exon 1 to exon 3 of the *VInv* gene using the mRNA sequence (accession no. NM_001288064.1) as reference. Similarly, the mRNA sequence of the *AS1* gene (accession no. XM_006343993.2) was used for designing primers amplifying exon 1 to exon 5. All primer sequences and their expected amplicon sizes are presented in Table 3-1.

**Table 3-1. Primers for *vacuolar invertase* and *asparagine synthetase 1* genes**

| Target gene | Primer name | Primer sequence (5'-3') | Expected amplicon (bp) | Accession no. of the reference sequence |
|---|---|---|---|---|
| *vacuolar invertase* | VInv1-F | GGTTGGCAAGTTCGGGATAA | 543 | HQ110080.1 |
| | VInv1-R | GATCCGGGAGGAATGTGTAATG | | |
| | VInv2-F | CACTGGCTCTACTTGCCTTT | 593 | |
| | VInv2-R | GGGTTATCGGGTGTCCATTTAT | | |
| | VInv3-F | CACGCAGTACCACTCAAGTTA | 664 | NM_001288064.1 |
| | VInv3-R | CCAGTCTAGAAGGAGAGGATCA | | |
| *asparagine synthetase 1* | AS1-F | GGCTTTGTTGGGTTGTTCGG | 530 | XM_006343993.2 |
| | AS1-R | TAGAGTACAAGTGCCCCGGA | | |

### 3.2.2 Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from leaves of *in vitro* plantlets of Atlantic and Desiree using the hexadecyltrimethylammonium bromide (CTAB) method described by Doyle (1991) with minor modifications. Briefly, CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1% polyvinyl pyrrolidone) was pre-warmed in a water bath at 65°C. Leaf tissue (approximately 1.5 cm in size) was collected from *in vitro* plantlets and cut into small pieces (0.5 x 0.5 cm) before putting into a 1.5 mL centrifuge tube. The leaf pieces were ground into a fine powder in liquid nitrogen using a plastic pestle, and 500 µL of warm CTAB buffer was added to the tube. The tube was incubated at 65°C for 30 minutes with occasional mixing by inversion. After incubation, 500 µL of chloroform: isoamyl alcohol (24:1) solution was added and mixed thoroughly with the sample by pipetting. The mixture was centrifuged at 10,000 rpm (Microcentrifuge 5425, Eppendorf) for 5 minutes at room temperature for phase separation, followed by transferring 200 µL of supernatant to a new tube with 300 µL of ice-cold isopropanol. The solution was mixed gently by inverting and then incubated at 4°C for 30 minutes. The nucleic acid pellet was collected by centrifugation at 4°C, 5000 rpm for 5 minutes, and washed with 500 µL of 70 % ethanol. The pellet was air-dried at room temperature for 15 minutes before resuspending in 100 µL of nuclease-free water. The concentration and purity of the extracted DNA were measured with a spectrophotometer (NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer, Thermo Scientific™). All DNA samples were stored at -80°C for future experiments.

Parts of the *VInv* gene were amplified from extracted DNA from Atlantic and Desiree using primers VInv1, VInv2 and VInv3 (Table 3-1). All PCR reactions were done using the commercially available PCR master mix (GoTaq® Green Master Mix, Promega Corporation, Table 3-2).

**Table 3-2. PCR reaction composition.**

| Reagent | Volume | Final concentration |
|---|---|---|
| 2X GoTaq Green Master Mix | 12.5 µL | 1 X |
| Forward primer | 1.25 µL | 0.5 µM |
| Reverse primer | 1.25 µL | 0.5 µM |
| DNA template | 1 µL | 100 ng |
| Nuclease-free water | 10.5 µL | |
| Total volume | 25 µL | |

The PCR programs were set up in a thermal cycler (Applied Biosystems Veriti Thermal Cycler, Thermo Fisher Scientific) with initial denaturation at 95°C for 5 minutes; followed by 30 cycles of 95°C for 30 s, 65°C (for primer VInV1) or 55°C (for primer VInV2) for 30 s, 72°C for 40 s; and with a final extension at 72°C for 7 minutes. The results were analysed on a 1 % agarose gel (Agarose, Fisher Biotech) in 1 X tris-acetate-EDTA (TAE) buffer alongside a 100 bp – 3000 bp DNA marker (100 bp Ladder DNA marker, Axygen Biosciences). Gels were stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific). Gel electrophoresis was done in the same 1 x TAE buffer at 70 V for 70 minutes. The gel was visualised using a UV transilluminator, and the amplified DNA bands were isolated and cleaned up using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation) for sequencing.

The Atlantic *VInv* gene fragment was also amplified with the VInv1 primer pair and high fidelity Taq polymerase (Phusion® High-Fidelity DNA Polymerase, New England Biolabs-NEB). A 20-µL PCR reaction was set up with 4µL of 5X Phusion buffer, 0.4 µL of 10mM dNTPs, 1 µL of 10mM primer each of VInv1-F and VInv1-R, 0.2 µL of high-fidelity DNA polymerase (x units), 1 µL of genomic DNA (100 ng) and 12.4 µL of nuclease-free water. The PCR program was set up at 98°C for 5 minutes; followed by 30 cycles of 95°C (30 s), 55°C (30 s), 72°C (40 s); and a final extension at 72°C for 7 minutes. Gel electrophoresis and DNA clean-up were done as described in the previous paragraph. The PCR product was A-tailed to facilitate TA cloning in a 10-µL reaction of 5 µL of 2X GoTaq Green Master Mix (Promega Corporation), 0.2 µL of 10mM dATP and 4.8 µL of DNA cleaned-up from

agarose gel. The reaction was incubated for 30 minutes at 70°C in a thermal cycler and was cleaned using the Wizard® SV Gel and PCR Clean-Up System.

### 3.2.3 Plant RNA extraction and PCR amplification

RNA was extracted from *in vitro* leaves of Atlantic and Desiree using the TRIzol method as described by Rio et al. (2010) with minor modifications. Leaf material (approximately 1.5 cm in length) was cut into small pieces (0.5 x 0.5 cm) and put in a 1.5 mL centrifuge tube. The sample was powdered in liquid nitrogen with a plastic pestle, and 1 mL of TRIzol was added and mixed thoroughly by vortexing, followed by incubation at room temperature for 10 minutes and the addition of 200 µL of chloroform. The mixture was vigorously vortexed for 15 seconds and incubated at room temperature for 3 minutes. The tube was then centrifuged at 13,000 rpm, 4°C for 15 minutes, and the aqueous upper phase was transferred to a new tube. Then, 500 µL of ice-cold isopropanol was added and mixed thoroughly with the sample by shaking, followed by overnight incubation at -80°C to maximise the RNA precipitation. RNA was collected by centrifugation at 13,000 rpm for 10 minutes at 4°C. The supernatant was removed, and the pellet was washed by adding 1 mL of cold freshly prepared 75% ethanol and centrifugation at 7,500 rpm for 5 minutes at 4°C. The liquid was removed, and the pellet was air-dried at room temperature for 15 minutes before resuspension in nuclease-free water. The RNA concentration was measured using a spectrophotometer, and the sample was stored at -80°C for future experiments.

First-strand cDNA was synthesised from the extracted RNA using a reverse transcriptase enzyme (GoScript™ Reverse Transcriptase, Promega Corporation). First, a mixture of 80 ng of RNA, 0.5 µg of random primers and nuclease-free water to make up a volume of 5 µL was prepared on ice and heated at 70°C for five minutes, followed by incubation on ice for five minutes. After that, the mixture was added to 15 µL of reverse transcription mix (Table 3-3), mixed and incubated at 25°C for five minutes, 42°C for one hour, and 70°C for 15 minutes. All the heating steps were done in a thermal cycler.

**Table 3-3. Reaction composition for first-strand cDNA synthesis.**

| Reagent | Volume | Final concentration/ Amount |
|---|---|---|
| **For combining RNA and primer** | | |
| RNA sample | 1 µL | 80 ng |
| Random primer | 1 µL | 0.5 µg |
| Nuclease-free water | 3 µL | |
| Total volume | 5 µL | |
| **Reverse transcription reaction mix** | | |
| 5X reaction buffer | 4 µL | 1 X |
| MgCl2 (25 mM) | 2.4 µL | 3 mM |
| dNTP (40 mM) | 1 µL | 2 mM |
| Reverse transcriptase | 1 µL | |
| Nuclease-free water | 6.6 µL | |
| Total volume | 15 µL | |

The synthesised cDNA was tested to confirm the successful transcription with no genomic DNA contamination using primers StGAPDH-F (5'- GTTTGGTTGCTAGAGTTGCTCTG-3') and StGAPDH-R (5'-GTTGACACCCACAACAAACATGG-3'), which amplify *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (accession no. NW_006238953.1), a housekeeping gene. Leaf DNA was used as a positive control with the expected amplicon sizes 775 bp for DNA and 368 bp for cDNA. The cDNA was later amplified with AS1 primers (Table 3-1), and all PCR reactions were prepared as described in Table 3-2. The PCR conditions were 95°C for 5 minutes, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 40 s and a final extension step at 72°C for 7 minutes. Gel electrophoresis and DNA fragment/amplicon clean-up for sequencing were done as described in section 3.2.2.

### 3.2.4 Cloning of PCR products of VInv1 and VInv3 primers

#### 3.2.4.1 Ligation and transformation

The PCR products of VInv 1 and VInv3 primers were cloned into *Escherichia coli* (*E. coli*) JM109 competent cells using the pGEM®-T easy vector, following the manufacturer's instructions (Promega

Corporation). The DNA samples cleaned from the agarose gel were quantified with a spectrophotometer, and they were ligated into the pGEM®-T easy vector at the ratio of 5:1 (Table 3-4). The amount of DNA insert for ligation was calculated according to the equation provided in the Promega technical manual:

$$\frac{ng\ of\ vector\ x\ size\ of\ insert\ (kb)}{kb\ size\ of\ vector}\ x\ insert{:}\ vector\ molar\ ratio = ng\ of\ insert$$

**Table 3-4. Reagents of a ligation reaction**

| Reagent | Amount | Final concentration | DNA size |
|---|---|---|---|
| 2X Rapid ligation buffer | 5 µL | 1 X | |
| pGEM®-T easy vector | 50 ng | 50 ng | 3015 bp |
| T4 DNA ligase | 1 µL | 3 Weiss units | |
| DNA insert | 45 ng (PCR product of primer VInv1) | | 543 bp |
| | 33.2 ng (PCR product of primer VInv3) | | 664 bp |
| Nuclease-free water | Makeup final volume to 10 µL | | |

The ligation reaction was incubated overnight at 4°C to maximise the number of transformants. After that, the vector containing insert was transformed into *E. coli* JM109 competent cells by adding 2 µL of the ligation reaction in 25 µL of *E. coli* competent cells, which were left on ice until just thawed. The mixture was incubated on ice for 20 minutes, followed by heat shock at 42°C for 50 s and then immediately chilled on ice for 2 minutes. The cells were grown in 500 µL LB broth at 37°C in the dark on a rotary shaker at 225 rpm for 1.5 hrs and collected by centrifugation at 1,000 rpm (Microcentrifuge 5425, Eppendorf) for 10 minutes before being resuspended in 200 µL of LB broth. Then 100 µL of resuspended cells was plated on LB agar containing 100 mg/L ampicillin, 80 mg/L X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) and 0.5 mM of IPTG (isopropyl β-D-1-thiogalactopyranoside). The plate was incubated overnight in the dark at 37°C to facilitate bacterial colony growth.

#### 3.2.4.2 Colony PCR and extraction of DNA plasmid

The transformants were screened for DNA insert using blue-white screening and colony PCR methods. At least six white colonies were picked for each ligation and were resuspended separately in 20 µL of nuclease-free water, from which 5 µL was used as the template in a PCR reaction with M13-F (5'-GTAAAACGACGGCCAGTG-3') and M13-R (5'-CAGGAAACAGCTATGACCAT-3') primers (Table 3-2). The PCR program was set up on a thermal cycler, starting at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 40 s; ending with a final extension at 72°C for 7 minutes. The results were analysed on an agarose gel, as described in section 3.2.2. The PCR-positive colonies were grown overnight in 5 mL of LB medium supplemented with 100 mg/L ampicillin, in the dark at 37°C on a rotary shaker at 225 rpm. Plasmid DNA was extracted from 4.5 mL of the culture grown overnight using a commercial kit following the manufacturer's protocol (QIAprep Spin Miniprep Kit, QIAGEN). Extracted DNA was quantified using a spectrophotometer. The remaining 0.5 mL of the bacterial culture was used to make glycerol stock by mixing it with 0.5 mL of 50 % sterile glycerol and stored at -80°C for future use.

### 3.2.5 Sequencing reactions

The nucleotide sequences of the desired DNA fragments and plasmid DNAs were obtained from Sanger sequencing using BigDye® Terminator v3.2 Cycle Sequencing Kit (Applied Biosystems). The reaction was set up in a total volume of 10 µL, including 1.75 µL of 5X sequencing buffer, 0.5 µL of Dye terminator mixture, 3.2 pmol of forward or reverse primer, 20 ng of DNA template (for PCR product from 500 bp to 1000 bp) or 250 ng of plasmid DNA (for plasmid less than 3 kb) and nuclease-free water. The reverse primers were used in sequencing reactions for DNA fragments to include sequencing of the 5'-end. The M13 forward primer was used for sequencing plasmid DNA after cloning. The sequencing program was set up on a thermal cycler, starting at 96°C for 2 minutes, 25 cycles of 96°C for 10 s, 55°C for 5 s and 60°C for 4 minutes. The product was purified using the ethanol/EDTA precipitation method described in BigDye® Terminator v3.1 Cycle Sequencing Kit

protocol (Applied Biosystems) with modifications. Briefly, the following reagents were added (in order as mentioned) to the reaction after thermal cycling: 1 µL of 125 mM EDTA, 1 µL of 3 M sodium acetate (pH 5.2) and 25 µL of 100 % ethanol, after which the reaction was mixed thoroughly by pipetting and incubated at room temperature for 20 minutes in the dark. The DNA pellets were collected by centrifugation at maximum speed 21,330 *x g* (Microcentrifuge 5425, Eppendorf) for 30 minutes. The supernatant was discarded, and the pellets were washed with 125 µL of freshly prepared 70% ethanol, followed by centrifugation at 21,330 *x g* for 5 minutes. All the liquid was removed, and the DNA was air-dried at room temperature, in the dark, for 15 minutes. Nucleotide analyses using Sanger sequencing were performed at the Western Australian State Agricultural Biotechnology Centre (SABC) sequencing facility at Murdoch University. The results were analysed on Geneious Prime software (Biomatters) and aligned with the reference sequences for *VInv* (accession no. HQ110080.1) and *AS1* (accession no. XM_006343993.2) genes from NCBI for confirmation of the correct gene amplification and cloning. The conserved regions of the gene were identified by aligning the different alleles cloned and later used for selecting gRNA target sites. Multiple alignments of sequences were done using the Multalin web tool with standard settings (Corpet, 1988).

### 3.2.6 *In silico* identification of gRNA target sites for *VInv* and *AS1* genes

The consensus sequences from the gene sequence alignments were used for identifying target sites for CRISPR/Cas9 using a web-based tool named Cas-Designer (http://www.rgenome.net/cas-designer) (Park et al., 2015). All potential target sites containing a TTTT stretch were excluded to avoid transcription termination by RNA polymerase III. The remaining sequences were filtered based on their positions on the multiple sequence alignments of the target gene. Those located in the conserved regions of target genes were searched against the nucleotide collection of *Solanum tuberosum* on the NCBI website using nucleotide BLAST. Two guide RNA target sequences for which the seed sequence S(12) had no potential off-targets which contained up to six mismatches in the

potato sequence database were chosen for each target gene. The first 20-nt sequence directly upstream PAM of each target site was used for assembling transcription template and construct of gRNA.

### 3.2.7 *In vitro* Cas9 cleavage assay

Two pairs of target sites, g67 and g10, g4 and g7, were selected for *VInv* and *AS1* genes. The efficiency of gRNAs was tested using the *in vitro* Cas9 cleavage assay. The transcription templates of gRNA were prepared by amplifying the gRNA construct ligated to a pGEM-T easy vector (referred to in section 4.3.1 of chapter 4) with the gRNA forward primer appended with T7 promoter sequence and reverse primer complementary to the 3'end of gRNA scaffold sequence (gRNA-R) (Table 3-5). The forward primers were designed to have the T7 promoter sequence ending with two Gs (guanines) before the guide sequences (Table 3-5), and the PCR was performed as described in section 3.2.2 of this chapter.

**Table 3-5. Primers for assembling gRNA transcription templates.** T7 promoters are in bold and guides are shaded in blue

| Primer name | Corresponding gRNA | Sequence (5'-3') |
|---|---|---|
| T7g67 | g67 | AAGCTAATACGACTCACTATAGGATCAATGGTACGATATTAA |
| T7g10 | g10 | AAGCTAATACGACTCACTATAGGTCAAGTACAAAGGCAACC |
| T7g4 | g4 | AAGCTAATACGACTCACTATAGGAGTTCTTGAGCTTTCTCGC |
| T7g7 | g7 | AAGCTAATACGACTCACTATAGGTGAAGCATCGTGGACCGGAT |
| gRNA-R | | GCCAACTTTGTACAAGAAAGCTGG |

The gRNAs were transcribed *in vitro* using T7 RNA polymerase (HiScribe™ Quick T7 High Yield RNA Synthesis Kit, NEB) following the manufacturer's protocol. Briefly, a 20 µL reaction was assembled by mixing 1.5 µL of 10X reaction buffer (0.75X final concentration) with 1.5 µL each of ATP, GTP, CTP, UTP (7.5 mM final concentration for each), 1.5 µL of T7 RNA polymerase mix and 1 µg of template DNA. The reaction was incubated at 37°C for 14 hrs in a thermal cycler. Template DNA was removed from the reaction by DNase treatment using TURBO DNase I (Invitrogen™) by adding 70 µL of nuclease-free water, 10 µL 10X TURBO DNase buffer, 1 µL of TURBO DNase I (Invitrogen™) to the

transcription reaction after incubation for 14 hrs, followed by incubation at 37°C for 30 min. Synthesised gRNA was purified following the purification protocol of synthesised RNA by NEB (New England Biolabs ®). The reaction was adjusted to 180 µL by adding 79 µL of nuclease-free water followed by the addition of 20 µL of 3M sodium acetate (pH 5.2) and 200 µL of chloroform. The solution was mixed thoroughly and then centrifuged at 10,000 rpm (Microcentrifuge 5425, Eppendorf) for 5 min at room temperature. The aqueous phase was transferred to a new 1.5 ml tube, and two volumes of ice-cold 96% ethanol were added. The sample was incubated at -80°C overnight to maximise the precipitation of gRNA. The gRNA was then collected by centrifugation at 5,000 rpm (Microcentrifuge 5425, Eppendorf) for 5 min at 4°C. The resulting pellet was rinsed with 500 µL ice-cold 70% ethanol, centrifuged at 5,000 rpm (Microcentrifuge 5425, Eppendorf) for 2 min at 4°C. Ethanol was removed, and the pellet was air-dried before resuspending in nuclease-free water. The gRNA was quantified using a spectrophotometer, denatured at 70°C for 5 min and analysed on a 2 % agarose gel (70 V for 30 to 60 min).

The Cas9 cleavage assay was initially performed *in vitro* at a mass ratio 2:2:1 of Cas9:gRNA:target DNA by incubating in a 10 µL reaction containing 1 µg (0.0062 nM) of Cas9 protein (ProteoWA), 1 µg (0.031 nM) of gRNA, 1X NEBuffer 3.1 and nuclease-free water for 10 min at room temperature. After that, 10 µL of nuclease-free water, 1X NEBuffer 3.1 and 500 ng (0.0018 nM) target DNA (530 bp) was added, and the reaction was incubated at 37°C for 15 min. The cleavage assay was also performed with 10:10:1 molar ratio of Cas9:gRNA:target DNA in a 10 µL reaction containing 0.01 nM (1600 ng) of Cas9, 0.01 nM (322.09 ng) of gRNA and 1X NEBuffer 3.1, incubation at room temperature for 10 min followed by addition of 10 µL of 0.001 nM (400 ng) target DNA (593 bp) and 1X NEBuffer 3.1. The reaction was again incubated at 37°C for 15 min. The amount of Cas9 protein was calculated using the web tool Weight to Molar Quantity (for proteins) (Bioline, https://www.bioline.com/media/calculator/01_04.html). The amount of gRNA (100 nt in length) was calculated using the formula: mass of ssRNA (g) = moles of ssRNA (mol) x ((length of ssRNA (nt) x 321.47 g/mol/nt) + 18.02 g/mol) (NEBioCalculator®, NEB, https://nebiocalculator.neb.com/). The

amount of target DNA was calculated using the formula: mass of dsDNA (g) = moles of dsDNA (mol) x ((length of dsDNA (bp) x 617.96 g/mol/bp) + 36.04 g/mol) (NEBioCalculator®, NEB). After incubation, the reaction was mixed with 6X loading dye (NEB) and analysed on a 2% agarose gel. Control DNA template was prepared by mixing 500 ng (for 2:2:1 assay) or 400 ng (for 10:10:1 assay) of target DNA with nuclease-free water and 6X loading dye. The Cas9 cleavage assay with a ratio of 2:2:1 of Cas9: gRNA: target DNA was done for g4 and g7, and the target DNA was the 530 bp amplicon of the AS1 gene amplified from cDNA of Desiree. The assay with a 10:10:1 ratio of molecular weight of Cas9: gRNA: target DNA was done for g67 and g10, and the target DNA was the 593-bp amplicon of the VInv gene from DNA of Desiree.

## 3.3 Results

### 3.3.1 PCR amplification of target genes

All primers were initially tested at 55°C annealing temperature. The VInv2 primers worked efficiently with a 593-bp PCR product amplified (Figure 3-2), but there was no product for the VInv1 primer pair at this annealing temperature. A gradient PCR with VInv1 primers was performed with annealing temperatures ranging from 48°C to 66°C to find the optimal temperature. The desired 543-bp DNA fragment was successfully amplified from Desiree at an annealing temperature of 65°C, and a slightly larger DNA band from Atlantic was observed at the same annealing temperature on agarose gel (Figure 3-2).

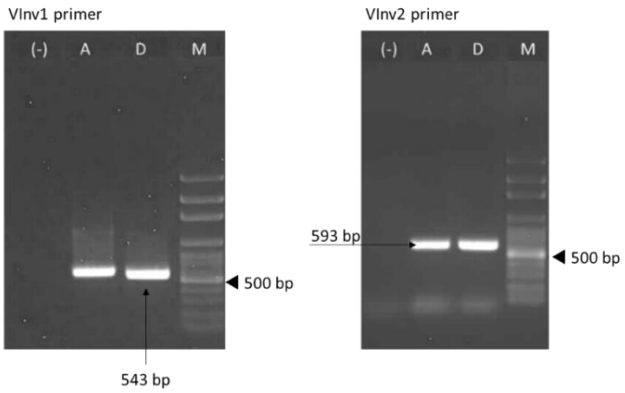



**Figure 3-2. Gel electrophoresis of PCR products using VInv1 and VInv2 primers.** Lane (-): negative control, lane A: Atlantic DNA, lane D: Desiree DNA, lane M: 100 bp – 3000 bp DNA marker.

The cDNA synthesised from RNA of Atlantic and Desiree was tested with primers for the endogenous *GAPDH* gene in a PCR reaction. Gel electrophoresis results showed the primers could amplify a 368-bp amplicon from cDNA samples, verifying that the cDNAs were successfully synthesised from the extracted RNAs and there was no DNA contamination in the total RNA extracted (Figure 3-3). This cDNA was used as a template for PCR with VInv3 and AS1 primers, and their expected amplicons, 664 bp and 530 bp, respectively, were amplified at 55°C annealing temperature (Figure 3-4).

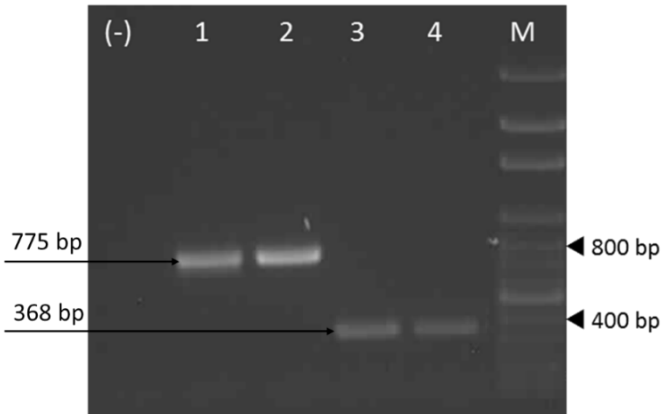



**Figure 3-3. Gel electrophoresis of the PCR amplifying a *GAPDH* gene fragment from cDNA of potato.** Lane (-): negative control, lanes (1) and (2): amplicons from the DNAs of Atlantic and Desiree, respectively; lanes (3) and (4) synthesised cDNA from RNA of Atlantic and Desiree, respectively; lane M: 100 bp – 3000 bp DNA marker.

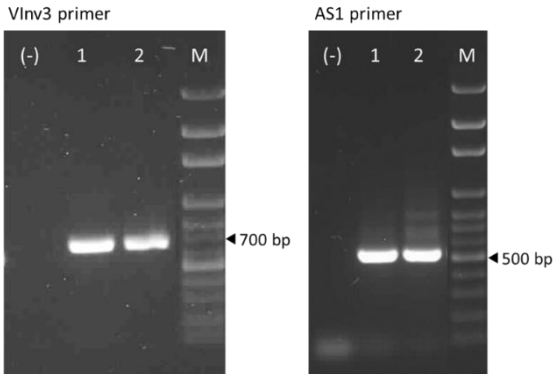



**Figure 3-4. Gel electrophoresis of RT-PCR using VInv3 and AS1 primers.** Lane (-) negative control; lanes (1) and (2): amplicons from synthesised cDNA from RNA of Atlantic and Desiree, respectively; lane M: 100 bp – 3000 bp DNA marker.

### 3.3.2 Sequencing the PCR products and identification of target sites

The PCR and RT-PCR products of VInv2 and AS1 primers were sequenced with corresponding reverse primers, and the sequencing chromatograms showed clear and evenly spaced nucleotide peaks. The multiple sequence alignments revealed that Atlantic and Desiree are highly conserved when compared with the reference sequence. Several double peaks or overlapping peaks were found in the chromatograms, indicating potential SNPs, and they are labelled as black 'N' in the alignment (Figure 3-5 and Figure 3-6).

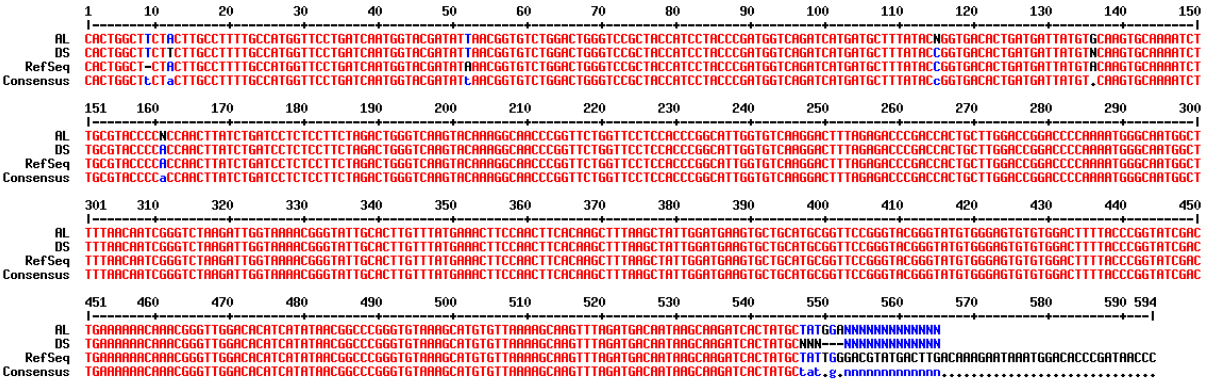



**Figure 3-5. Multiple sequence alignment of PCR products amplified with the VInv2 primer pair from Atlantic and Desiree.** AL: DNA sample of Atlantic, DS: DNA sample from Desiree, RefSeq: reference DNA sequence of *vacuolar invertase* gene (accession no. HQ110080.1).

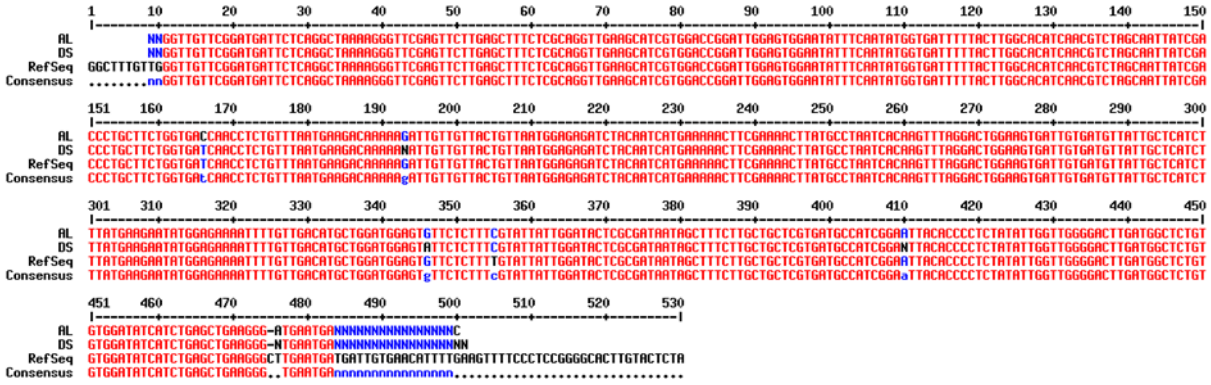



**Figure 3-6. Multiple sequence alignment of RT-PCR products amplified with AS1 primer pair from Atlantic and Desiree.** AL: synthesised cDNA from RNA sample of Atlantic, DS: synthesised cDNA from RNA sample of Desiree, RefSeq: mRNA sequence of *asparagine synthetase 1* gene (accession no. XM_006343993.2)

The PCR products of VInv1 primers from both cultivars showed mixed signals in the chromatograms. These products were cloned into *E. coli,* and six individual clones of each PCR product were used for sequencing. Three alleles (D1, D2 and D4) with 97.6% of identical sites were detected from multiple sequence alignment of Desiree, as shown in Figure 3-7 A (D1, D3, D5 and D6 are the same). Variations found among sequences were mostly single-base changes and a one-base insertion at position 453 of D4 (Figure 3-7A). In contrast, the alignment of the cloned sequences of Atlantic showed only 90.3% of identical sites, and all six sequences were different. Besides single-base changes, there were four insertions of 1-bp, 14-bp and 15-bp found among the six clones (Figure 3-7B).

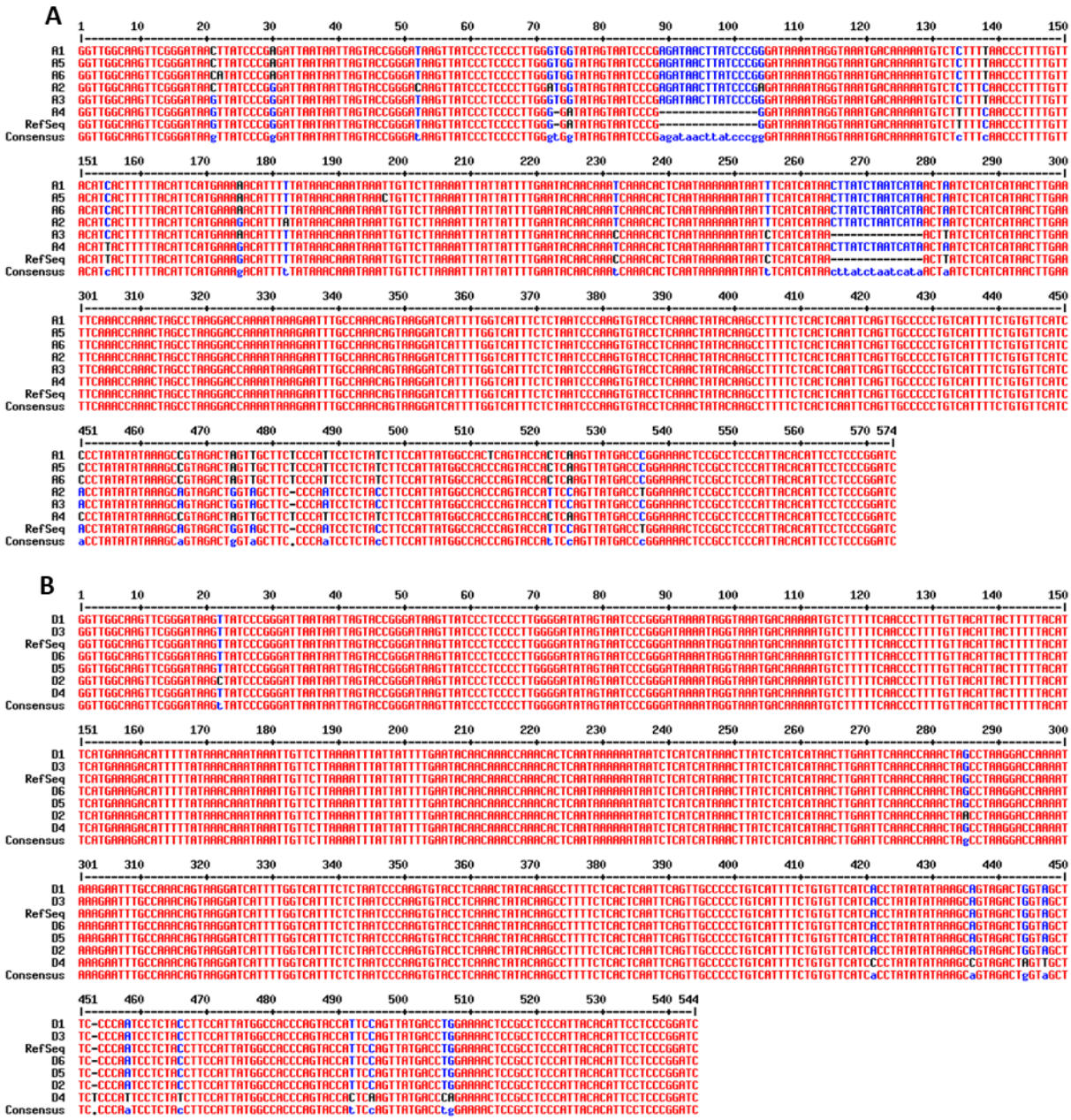



**Figure 3-7. Multiple sequence alignments of cloned PCR amplicons of the Vlnv1 primer pair from Atlantic and Desiree.** A: alignment of clone sequences from Atlantic. B: alignment of clone sequences from Desiree. A1 to A6: individual clones from Atlantic, D1 to D6: individual clones from Desiree, RefSeq: DNA sequence of *vacuolar invertase* gene (accession no. HQ110080.1).

The *VInv* gene from Atlantic was PCR amplified again with the same VInv1 primer pair and high-fidelity DNA polymerase, and the product was cloned for sequencing. The alignment from eight clones revealed two alleles, and they differed only at the 5'-end of primer binding region where there was a 3-bp deletion (Figure 3-8, clone A6, A7 and A8). Apart from the two insertions of 14-bp and 15-bp compared with the reference sequence (accession no. HQ110080.1), the eight clones

amplified with the proof-reading Taq enzyme had different variations than the previous six clones from PCR product with normal Taq enzyme. As shown previously (Figure 3-7 A), there were other variations among the eight sequences.

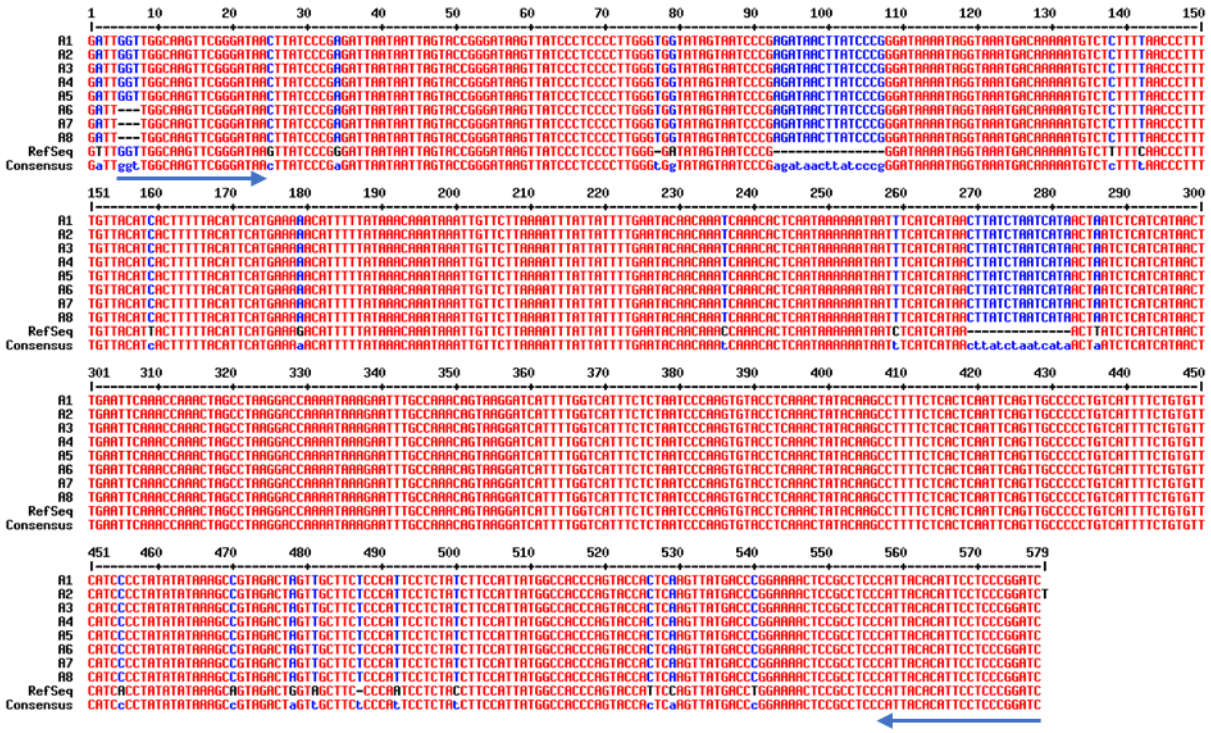



**Figure 3-8. Multiple sequence alignment of cloned PCR products of Atlantic using VInv1 primers and high-fidelity DNA polymerase.** A1 to A8: individual clones of PCR amplicon of VInv1 primer from Atlantic, RefSeq: reference DNA sequence of *vacuolar invertase* gene (accession no. HQ110080.1). Blue arrows indicate the position and direction of primers.

Mixed signals were also observed in the chromatograms results of direct amplicon sequencing from VInv3 amplicons of both cultivars. These amplicons were cloned and sequenced as done previously for amplicons of VInv1 primers. Alignments of clone sequences revealed five different sequences among six clones of Atlantic (Figure 3-9 A, clone A4 and A6 are the same), and all six clones of Desiree were different from each other (Figure 3-9 B).

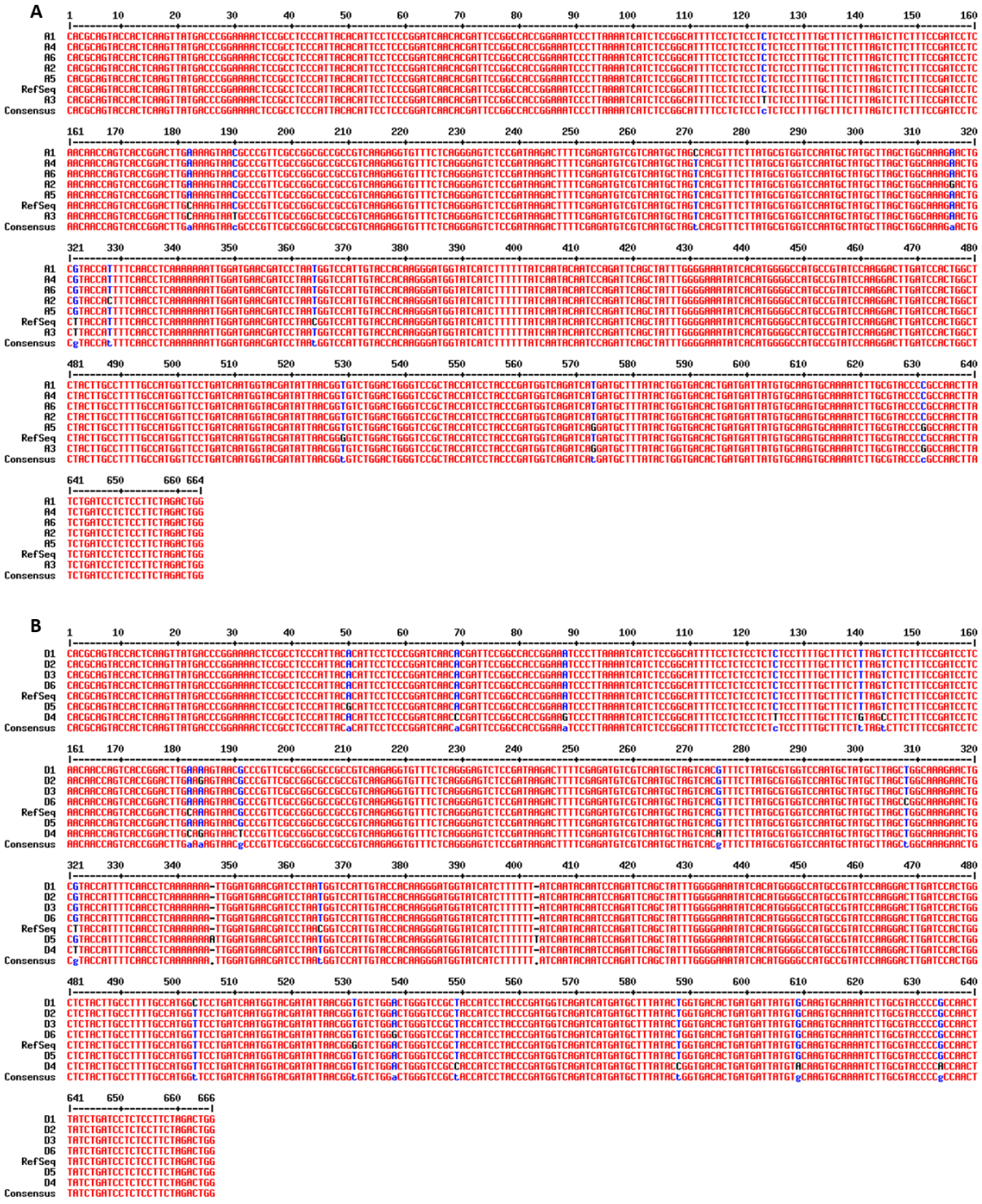



**Figure 3-9. Multiple sequence alignments of cloned RT-PCR products of the Vlnv3 primer pair from Desiree and Atlantic.** A1 to A6: individual clones from Atlantic, D1 to D6: individual clones from Desiree. RefSeq: reference mRNA sequence of *vacuolar invertase* gene (accession no. NM_001288064.1)

The consensus sequences generated from multiple sequence alignments were submitted to the Cas Designer web tool to search for potential target sites. There were approximately 100 potential

targets found on both forward and reverse strands for each target gene. After excluding those containing a TTTT stretch or hypervariable regions, the remaining sequences were searched on the potato genome to filter out those with potential off-targets. Target sites that were close to the 5'-end with no similar sequences containing up to six mismatches in the seed sequence were chosen. Three suitable targets were selected for the *VInv* gene and two targets for the *AS1* gene (Figure 3-10). One suitable target (g5) was found on exon 1 of the *VInv* gene, but it was specific only to Atlantic, while the others (g67, g10 on *VInv* gene, g4 and g7 on *AS1* gene) were specific to both Atlantic and Desiree.

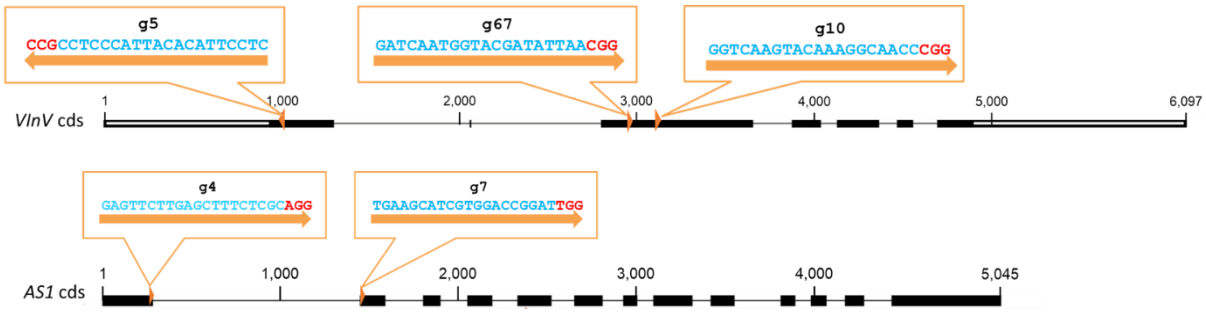



**Figure 3-10. Target sites for gRNAs for *vacuolar invertase* and *asparagine synthetase 1* genes.** White boxes represent untranslated regions; thick black lines represent exons; thin black lines represent introns; orange arrows represent the direction of selected target sites; the blue text indicates guide sequences, and protospacer-adjacent motifs (PAMs) are indicated by red text. The targets were named according to the list regenerated by the Cas-Designer tool.

### 3.3.3 *In vitro* Cas9 cleavage assay

The assembly of gRNA with scaffold construct is described in section 4.3.1 of chapter 4, and this construct was used to make the DNA template for gRNA transcription. The templates were successfully assembled by PCR, amplifying a product at approximately 150 bp (Figure 3-11). After *in vitro* transcription, RNA was purified and resuspended in 0.1 mM EDTA before gel electrophoresis analysis. The RNA samples bands were expected to be 133 bp, and they were between the 100 bp and 200 bp of DNA marker on the gel. The denatured RNA appeared as a slightly smaller band compared to non-denatured RNA (Figure 3-12).

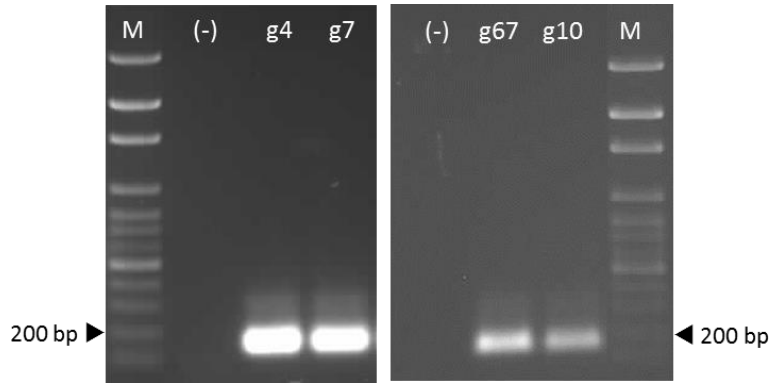



**Figure 3-11. Gel electrophoresis of PCR for generating DNA templates for gRNA transcription.** Lane (-): negative control with primers and nuclease-free water; lanes g4, g7, g67 and g10: gRNA transcription templates of corresponding gRNAs; lane M: 100 bp – 3000 bp DNA marker.

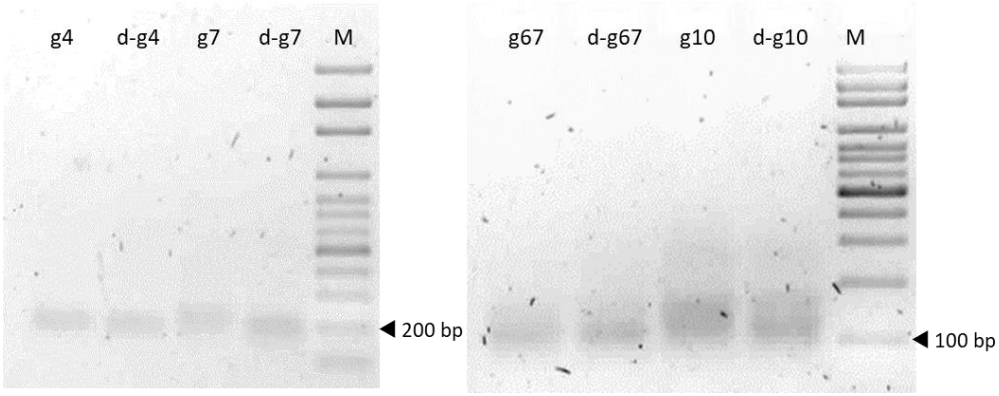



**Figure 3-12. Gel electrophoresis analysing gRNAs.** Lanes g4, g7, g67, g10: gRNAs of corresponding targets; lanes d-g4, d-g7, d-g67, d-g10: denatured gRNAs of corresponding targets; lane M: 100 bp – 3000 bp DNA marker.

The Cas9 cleavage assays were performed with the expected cleavage sites on the respective PCR products illustrated in Figure 3-13. The results showed that at the 2:2:1 ratio of Cas9:gRNA:target DNA used for g4 and g7, not all target DNA was cleaved, as uncut DNA can be seen on the gel (Figure 3-14 A). There were redundant gRNAs observed on the gel, between the 100 bp and 200 bp sizes DNA markers. The two bands visible near the 400 bp size marker in lane g4 and g7 were similar to the expected 466-bp and 442-bp cut products; the smaller products (44 bp of g4 and 68 bp of g7) were not visible (Figure 3-14 A). In contrast, no uncut DNA and no residual gRNA was detected on the gel of the 10:10:1 (molecular weight ratio) assay, indicating that all gRNAs are bound to Cas9, and all DNA was cleaved. The cut products were of the expected size: 540 bp for g67, 382 bp and

191 bp for g10, the smaller product (33 bp) of g67 was not visible (Figure 3-14B). These results established that all four gRNAs efficiently created DSBs in DNA by guiding Cas9 to the intended target sites.

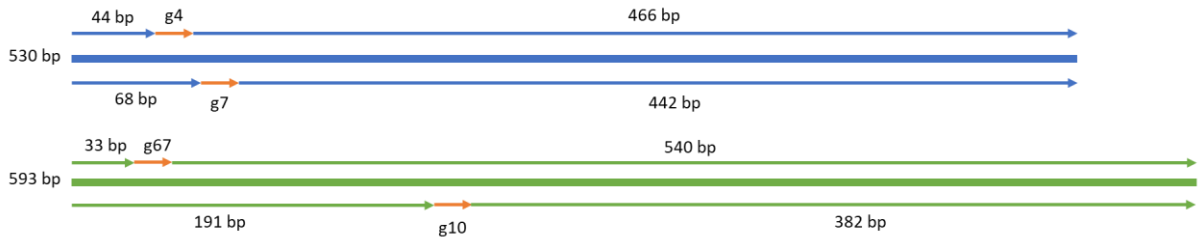



**Figure 3-13. Expected DNA products of Cas9 cleavage assay.** The thick lines represent target DNA (AS1 amplicon = 530 bp, Vlnv2 amplicon = 593 bp), orange arrows represent gRNA target sequences, thin arrows represent digested amplicons.

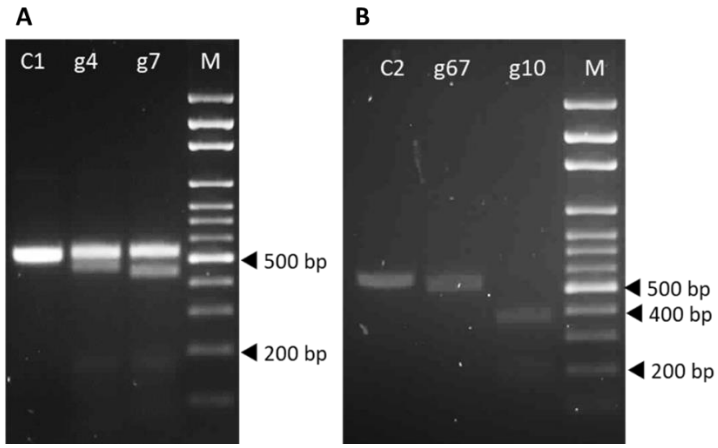



**Figure 3-14. Gel electrophoresis of Cas9 cleavage assay.** Lane C1: 530-bp amplicon of *AS1* gene from cDNA of Atlantic; lane C2: 593-bp amplicon of *VInv* gene from DNA of Atlantic; lanes g4, g7, g67, g10: corresponding gRNAs in Cas9 assay; lane M: 100 bp – 3000 bp DNA marker.

## 3.4 Discussion

In this chapter, *VInv* and *AS1* genes from potato cultivars Atlantic and Desiree were amplified and sequenced to provide data for designing gRNAs. Sequencing results revealed that amplified fragments of the *AS1* gene and exon 3 of the *VInv* gene from Atlantic and Desiree were highly conserved. In contrast, exon 1 of the *VInv* gene had overlapping peaks in sequencing chromatograms of PCR products indicating multiple PCR products. Cloning and sequencing identified different alleles for the *VInv* gene. The cultivated potatoes are well-known for their

tetraploid and heterozygous characteristics. For example, approximately half a million SNPs and indels were identified in the DM1-3 reference genome (Kyriakidou et al., 2020). These genetic variations can create uninterpretable sequence reads from amplicon sequencing, as seen in VInv1 and VInv3 primer cases. The tetraploid characteristic of potato allows a maximum of four alleles of a given gene which is present as only one copy in the genome. The presence of six different *VInv* gene sequences from PCR clones of Atlantic (Figure 3-7B) obtained in this study did not fit this pattern. The genomic structure of the *VInv* gene has been studied before, and there were no duplicated fragments of this gene found in the potato genome (Draffehn et al., 2010). Thus, the variations in cloned sequences were probably due to Taq polymerase errors. This hypothesis was confirmed when repeating PCRs with VInv1 primers and high-fidelity DNA polymerase followed by cloning and sequencing of individual clones indicated only two alleles, and no variation found previously were repeated (Figure 3-8). This result could also explain the sequence variants in the case of VInv3 primers. However, since the conserved regions found among cDNA clones from VInv3 primers were sufficient for identifying target sites, these amplicons were not double-checked by using proof-reading DNA polymerase.

The error-prone repair of DSBs via the NHEJ pathway can lead to indels which may initiate frameshift and produce premature stop codons (Ueta et al., 2017, Nishitani et al., 2016, Tuncel et al., 2019). If DNA mutations are induced in early exons, it will trigger nonsense-mediated mRNA decay, leading to mRNA degradation (Popp and Maquat, 2016). Thus, the exons near the 5' end of the target genes were ideal for targeting as they should maximise the chance of knocking out or down-regulating gene expression. In addition, the use of multiple gRNAs for one target gene would also increase the gene-editing efficiency as reported in Arabidopsis (Li et al., 2013, Pauwels et al., 2018), rice (Zhou et al., 2014), tomato (Brooks et al., 2014) and potato (Veillet et al., 2019a). This strategy has been applied in several crop plants, such as tomato, in which the seven-exon *SlIAA9* gene involved in leaf morphology was targeted in exon 2 with three gRNAs (Ueta et al., 2017). Premature stop codons were generated with a 1-bp and 73-bp deletion, resulting in abnormal leaf morphology, indicating

knockdown or even knockout of the *SlIAA9* gene. Similarly, four gRNAs were designed to target regions in the first seven exons of the 15-exons *phytoene desaturase* (*PDS*) gene in apple, resulting in premature stop codons and eventually gene knockout (Nishitani et al., 2016). In potato, the loss of function of the GBSSI protein was successfully achieved using two gRNAs targeting the first two exons of the gene (Veillet et al., 2019a). These reports confirmed that the gene knockout could be achieved using multiple gRNAs targeting early exons of the desired gene.

In the *in vitro* Cas9 cleavage assays, the 2:2:1 amount ratio of Cas9: gRNA: target DNA was 1 µg Cas9: 1 µg gRNA: 0.5 µg target DNA resulted in uncut target DNA and redundant gRNA after the reaction. Given that the Cas9 protein is 160 kDa, there was 0.0063 nM in 1 µg, but gRNA was 100 nt in length, making it 0.031 nM in 1 µg, five times more than Cas9 protein in the reaction. An excess amount of gRNA over Cas9 explains the redundant gRNA after the reaction, as only one gRNA is required for a Cas9 protein. On the other hand, 1 µg (0.0063 nM) of Cas9 was approximately triple the amount of 0.0018 nM in 0.5 µg of target DNA, but it was not sufficient for complete cleavage of the target DNA. A molar ratio of 5:5:1 or higher of Cas9:gRNA:target DNA was recommended (Anders and Jinek, 2014), and the 10:10:1 molar ratio of Cas9:gRNA:target DNA resulted in a complete cutting of the target DNA without redundant gRNAs. Cas9 is a single-turnover enzyme; thus it is crucial to have excess Cas9 protein-gRNA complex over the target DNA to ensure complete cleavage (Sternberg et al., 2014).

In conclusion, two gRNAs were designed to target two sites of each gene, *VInv* and *AS1,* to improve editing efficiency and the potential to delete a large fragment between two sites. The efficiency of the designed gRNAs was demonstrated in an *in vitro* cleavage assay, and they performed as expected. All four gRNAs used to make transformation vectors were also used to assemble ribonucleoprotein complexes with the Cas9 protein for the direct delivery of the CRISPR/Cas9 system into plant cells (see chapter 5).

# Chapter 4

# Generation of transgenic potato events expressing Cas9 protein and gRNAs targeting *vacuolar invertase* and *asparagine synthetase 1* genes

## 4.1 Introduction

In this chapter, binary vectors harbouring the Cas9 protein and gRNA(s) coding sequences were assembled and then delivered to plant cells using *Agrobacterium tumefaciens*. The gRNAs complementary to the target site (20 nt in length) appended to a gRNA scaffold and driven by a promoter of choice were assembled into a construct. One gRNA construct may be sufficient to induce gene editing by CRISPR/Cas9, but multiple gRNAs are needed for multiplexing or to increase editing efficiency (Li et al., 2013, Pauwels et al., 2018). For example, two gRNAs targeting the same gene (dual-gRNA system) can generate a DNA fragment deletion between two gRNA sites (Mao et al., 2013, Brooks et al., 2014, Zhou et al., 2014, Veillet et al., 2019a). This concept has been applied successfully both in model plants and commercial crops, including tobacco (Gao et al., 2015), tomato (Pan et al., 2016), Arabidopsis and rice (Feng et al., 2013, Li et al., 2013, Zhang et al., 2014). In principle, multiplexing can be achieved when using a CRISPR/Cas9 system with multiple gRNAs. For plant transformation with plasmid DNA, multiple gRNA constructs can be stacked in one vector, but this process is often challenging and time-consuming. In addition, due to the length of each gRNA construct, which is typically 400 bp to 500 bp (depending on the promoter size), and the plasmid vector size, the number of gRNA constructs that can be integrated into a vector may be limited. Examples of multiplexing include six gRNA-expressing cassettes integrated into a single plasmid vector (Xing et al., 2014, Zhang et al., 2016b). However, up to nine gRNAs or more can be delivered from a single gene cassette, for example, using endogenous tRNA processing (Xie et al., 2015). This tRNA strategy enables more gRNAs to be combined in a single vector, increasing the potential for multiple gene editing using CRISPR/Cas9. Interestingly, gRNA constructs with tRNA had increased transcription and a slightly higher mutation rate than those without tRNAs when tested in rice protoplasts (Xie et al., 2015). Hence, assembling a transformation vector with a tRNA processing system and multiple gRNAs is ideal for combining multiple individual gRNAs.

In this chapter, the DNA templates of gRNAs targeting the *VInv* and *AS1* genes were assembled using overlapping PCR. Two gRNA expression cassettes targeting each gene at two sites were joined and integrated into the pFGC-pcoCas9 vector via restriction sites. In addition, a construct was designed and synthesised commercially, with an expression cassette of four gRNAs complementary to two target sites each on the *VInv* and *AS1* genes, in which each gRNA was followed by a gRNA scaffold and tRNA processing system. This expression cassette was ligated into the vectors pFGC-pcoCas9 and pFN117-Cas9 (kindly provided by Dr Fatima Naim). Vectors with Cas9 and gRNA expressing sequences were delivered into leaf discs of potato cultivars Atlantic and Desiree using *Agrobacterium*-mediated plant transformation, followed by tissue culture and shoot regeneration. Recovered plantlets from selection media were screened by PCR and sequenced to identify transformants and putative mutants with edited *VInv* and *AS1* genes.

## 4.2 Methodology

### 4.2.1 Assembly of gRNA constructs

The DNA constructs of gRNA were assembled following the protocol of Li et al. (2013). The pUC119-gRNA vector (a gift from Jen Sheen, Addgene plasmid # 52255; http://n2t.net/addgene:52255 ; RRID:Addgene_52255) was used as a PCR template. This vector contains an Arabidopsis RNA polymerase III (Pol III) gene promoter U6 (AtU6-1) promoter driving a 20-nt guide RNA targeting Arabidopsis *phytoene desaturase 3* (*AtPDS3*) gene expression with a gRNA scaffold and a TTTTTT as the terminator (Figure 4-1). The 20-nt sequence was replaced by the target sequences for the *VInv* and *AS1* genes using overlapping PCR.

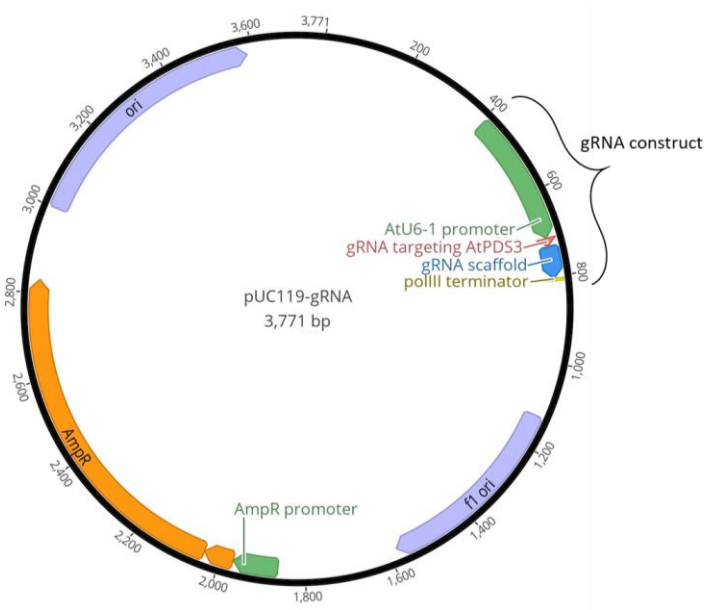



**Figure 4-1. Map of the pUC119-gRNA vector.** (Addgene plasmid # 52255; https://www.addgene.org/52255/). The gRNA construct region from 407 to 812 contains an Arabidopsis U6 (AtU6-1) promoter driving the expression of 20-nt gRNA targeting Arabidopsis *phytoene desaturase 3* (*AtPDS3*) gene with gRNA scaffold and polymerase III (Pol III) terminator. This plasmid was used as a DNA template in overlapping PCRs to introduce the selected target site into the new gRNA expression cassette.

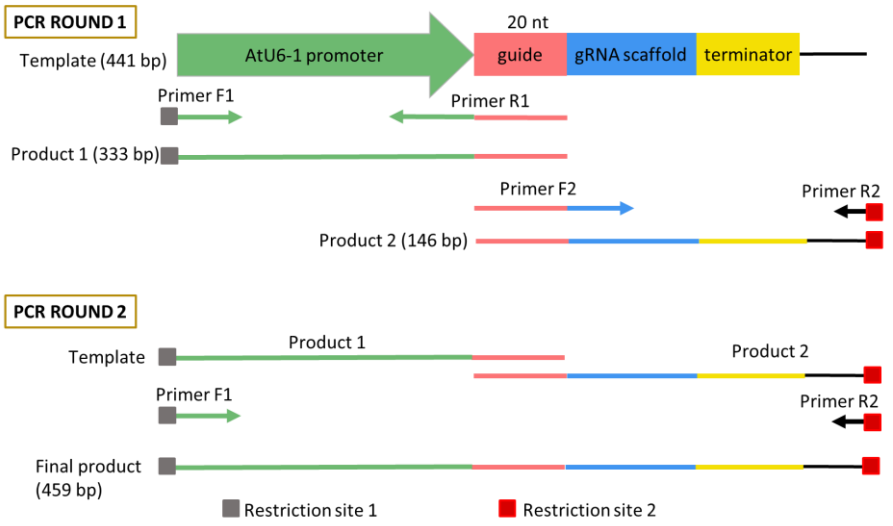



**Figure 4-2. Diagram of the overlapping PCR method for assembling gRNA expression cassettes.** In PCR round 1, the selected target site was introduced into the new gRNA construct using primers F2 and R1. The primers F1 and R1 yielded product 1 (333 bp) containing restriction site 1, AtU6-1 promoter and the target site. The primers F2 and R2 yielded product 2 (146 bp) containing the target site, gRNA scaffold, terminator, and restriction site 2. In PCR round 2, two products from PCR round 1 were joined into the final product (459 bp) using primers F1 and R2. Restriction sites 1 and 2 facilitate the assembly of dual-gRNA constructs and the integration of gRNA expression cassettes into a plasmid vector. Arrows indicate directions and positions of primers.

The overlapping PCR method consisted of two rounds. In the first PCR round, to make the g4RNA expression cassette, pUC119-gRNA plasmid DNA was amplified with the gRNAEcoRI-F and g4-AS1-R primers to amplify product 1 g4-AS1-F, and gRNAKpnI-R primers to amplify product 2 (Figure 4-2, Table 4-1). PCR reactions were set up with 5 µL of 2X GoTag Green Master Mix (Promega® Corporation), 1 µL of 10 µM forward and reverse primer each, 1 µL of 100 ng/µL pUC119-gRNA plasmid DNA and 2 µL of nuclease-free water. The PCR program was set up in a thermal cycler (Applied Biosystems Veriti Thermal Cycler, Thermo Fisher Scientific) with initial denaturation at 95°C for 5 minutes; followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 40 s; and a final extension at 72°C for 7 minutes. PCR amplicons were analysed on 1 % agarose gel (Agarose, Fisher Biotech) stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) in 1X tris-acetate-EDTA (TAE) buffer alongside a 100 bp – 3000 bp DNA marker (100 bp Ladder DNA marker, Axygen Biosciences). Gel electrophoresis was done at 70 V for 70 minutes. The gel was visualised using a UV transilluminator, and the DNA bands of products 1 and 2 from the gel were pooled and purified together using a Wizard® SV Gel and PCR Clean-Up System (Promega® Corporation).

In the second PCR round for overlapping the two products, the pooled and purified DNA of products 1 and 2 was amplified with the gRNAEcoRI-F and gRNAKpnI-R primers in a reaction with 10 µL of 2X GreenTaq Master Mix, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 1 µL of 100 ng of the pooled DNA and 7 µL of nuclease-free water. PCR was done with the same cycling conditions as round 1 in the thermal cycler; products were run on 1 % agarose gel and extracted with the Wizard clean-up kit. The g7RNA expression cassettes were assembled using the same method, with gRNAKpnI-F and g7-AS1-R, g7-AS1-F and gRNAPacI-R primers, respectively for product 1 and product 2 in the first PCR round; gRNAKpnI-F and gRNAPacI-R primers were used in the second round of PCR (Table 4-1). The g4RNA expression cassette consisted of EcoRI-AtU6 promoter-g4-gRNA scaffold-KpnI, and g7RNA expression cassette as KpnI-AtU6 promoter-g7-gRNA scaffold-PacI. This assembly method was also used to make the g67RNA and g10RNA expression cassettes in the format of EcoRI-

AtU6 promoter-g67-gRNA scaffold-XhoI and XhoI-AtU6 promoter-g10-gRNA scaffold-PacI, respectively. All primers used are listed in Table 1.

**Table 4-1. Primers for assembly of gRNA constructs by overlapping PCR.** Target regions are in blue, restriction sites are in red.

| Primer name | Primer sequence (5' to 3') |
|---|---|
| gRNAEcoRI-F | TCAGAATTCAGAAATCTCAAAATTCCG |
| gRNAKpnI-F | CATGTGGTACCAGAAATCTCAAAATTCCG |
| gRNAKpnI-R | TCAGGTACCTAATGCCAACTTTGTACA |
| gRNAPacI-R | GTGCTTAATTAATAATGCCAACTTTGTACA |
| gRNAXhoI-F | GCACTCGAGAGAAATCTCAAAATTCCG |
| gRNAXhoI-R | TCACTCGAGTAATGCCAACTTTGTACA |
| g4-AS1-R | GCGAGAAAGCTCAAGAACTCAATCACTACTTCGTCTCT |
| g4-AS1-F | GAGTTCTTGAGCTTTCTCGCGTTTTAGAGCTAGAAATAGC |
| g7-AS1-R | ATCCGGTCCACGATGCTTCAAATCACTACTTCGTCTCT |
| g7-AS1-F | TGAAGCATCGTGGACCGGATGTTTTAGAGCTAGAAATAGC |
| g67-VInV-R | TTAATATCGTACCATTGATCAATCACTACTTCGTCTCT |
| g67-VInV-F | GATCAATGGTACGATATTAAGTTTTAGAGCTAGAAATAGC |
| g10-VInV-R | GGTTGCCTTTGTACTTGACCAATCACTACTTCGTCTCT |
| g10-VInV-F | GGTCAAGTACAAAGGCAACCGTTTTAGAGCTAGAAATAGC |

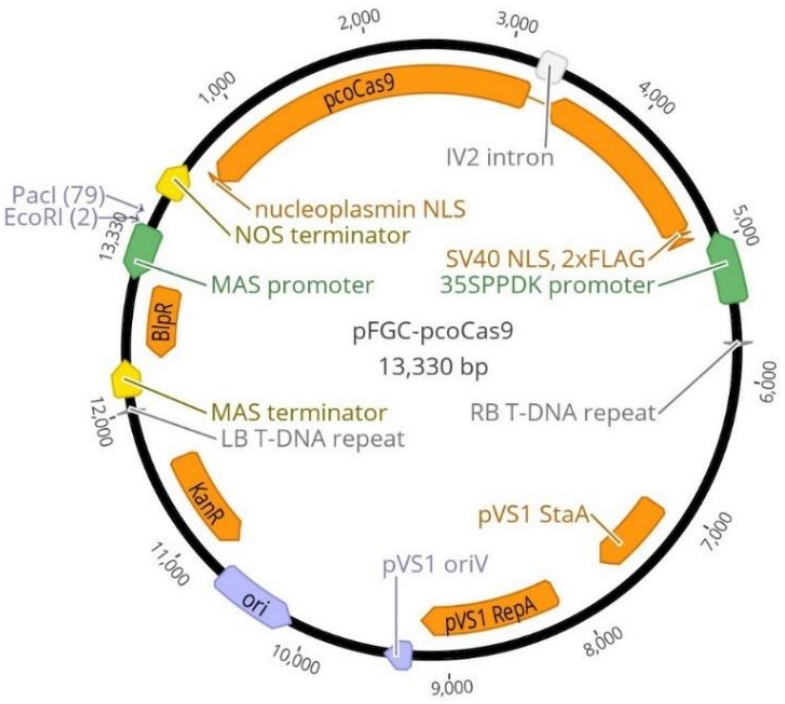
### 4.2.2 Assembly of the CRISPR/Cas9 transformation vector

#### 4.2.2.1 Constructing plant transformation vectors with dual-gRNA system targeting one gene

The g4RNA and g7RNA expression cassetees were joined via the KpnI restriction site, while g67RNA and g10RNA cassettes were joined via the XhoI restriction site to make the g4g7 dual-gRNA expression system (g4g7 cassette) and g67g10 dual-gRNA expression system (g67g10 cassette), respectively. Briefly, for each dual-gRNA assembly, a combination of 350 ng of each gRNA expression cassette purified after the second round of overlapping PCR was mixed with 3 µL of 10X CutSmart® Buffer (New England Biolabs ®), 1 µL of KpnI-HF (g4RNA and g7RNA) or XhoI (g67RNA and g10RNA) (New England Biolabs ®) and nuclease-free water to make up the final volume to 30 µL; the mixture was incubated at 37°C in a thermal cycler for two hours. The product was visualised on a 1%

agarose gel, and the DNA band was extracted from the agarose gel and cloned into the pGEM-T® easy vector (Promega®) for sequencing. Cloning was carried out as described in section 3.2.4.1 of Chapter 3, and the sequencing was done according to section 3.2.5 of Chapter 3. The correct dual-gRNA construct was selected after analysing the sequencing results and ligated to pFGC-pcoCas9 via EcoRI and PacI restriction sites (Figure 4-3).

Double restriction digestion of pGEMT-easy vector (to release the dual gRNA expression cassette) and of the pFGC-pcoCas9 (to linearise the vector for use as a backbone) was done in a 30 µL reaction by mixing 3 µL of 10X CutSmart® Buffer, 1 µL of EcoRI-HF (New England Biolabs ®), 1 µL of PacI (New England Biolabs®), 1 µL of 800 ng of plasmid DNA and 24 µL of nuclease-free water. The mixture was incubated at 37°C for four hours; the digested products were run on an agarose gel and purified for ligation at 5:1 ratio of linearised pFGC-pcoCas9 vector and the two digested dual-gRNA cassettes (g4g7 or g67g10) in each reaction. The vector was transformed into *E.coli* JM109 competent cells as described in section 3.2.4.1 of chapter 3, and bacterial colonies were screened by PCR using the primers FGC-F and FGC-R with an expected amplicon size of 1,134 bp–(Table 4-2). The thermal cycling program was set at 95°C for 5 min; 30 cycles of 95°C (30 s), 55°C (30 s), 72°C (1 min); and a final extension at 72°C for 7 minutes. Plasmid DNA of PCR-positive bacterial colonies was sequenced in both directions using the primers FGC-F2 and FGC-R to confirm the construct assembly (Table 4-2).




a

**Figure 4-3. Map of the pFGC-pcoCas9 vector.** (Addgene plasmid #52256, https://www.addgene.org/52256/). The vector contains a plant codon-optimised Cas9 expressing sequence (pcoCas9) with a potato IV2 intron. The pcoCas9 expression is driven by a 35SPPDK promoter (hybrid promoter consisting of the cauliflower mosaic virus 35S enhancer fused to the maize C4PPDK basal promoter). Selection marker for plants is the *BlpR* gene which confers resistance to bialaphos or phosphinothricin (glufosinate). The gRNA expression cassettes were inserted into the vector via ligation at the PacI and EcoRI restriction sites.

**Table 4-2. Primers used in PCR screening for gRNA constructs in the transformation vectors**

| Primer name | Primer sequence (5' to 3') | Corresponding vector |
|---|---|---|
| FGC-F | ACGTCACGTCTTGCGCACTG | pFGC-pcoCas9 |
| FGC-R | CTAGGATAAATTATCGCGCGCGGTG | |
| FGC-F2 | GAAATTCAGGCCCGGTTGCC | |
| FN117-F | CATAACGTGACTCCCTTAATTCTCC | pFN117-Cas9 |
| FN117-R | CATGTTGACCTCCAAGCTTGAATTC | |

#### 4.2.2.2 Constructing plant transformation vectors with four gRNA targeting two genes simultaneously

A construct expression cassette, named ASVI, was designed with AtU6-1 promoter, the four gRNAs separated by the tRNA (transfer RNA) processing sequences in between, XhoI and EcoRI restriction

sites upstream of the gRNA promoter and PacI and KpnI restriction sites downstream the terminator (Figure 4-4). The sequence was then sent to GENEWIZ® for synthesis. This expression cassette was inserted into the pFGC-pcoCas9 vector via EcoRI and PacI restriction sites to make the pFGC-ASVI vector and into the pFN117-Cas9 vector (Figure 4-5) via XhoI and KpnI restriction sites to make pFN117-ASVI vector following the method described in section 4.2.2.1. After ligation of the digested ASVI expression cassette and the vector (pFGC-pcoCas9 or pFN117-Cas9) digested with the same restriction enzymes, the vector was transformed into *E.coli* JM109 competent cells as described in section 3.2.4.1 of chapter 3. PCR colony screening for the ASVI cassette in pFGC-pcoCas9 was done using the primer pair FGC-F2 and FGC-R (amplicon size 1,342 bp, Table 4-2). Primers FN117-F and FN117-R (amplicon size 1,120 bp, Table 4-2) were used in PCR colony screening for ASVI-containing pFN117-Cas9 vector. Plasmid DNA of a colony with positive PCR screening result was sequenced in both directions using the same primer pairs respectively.

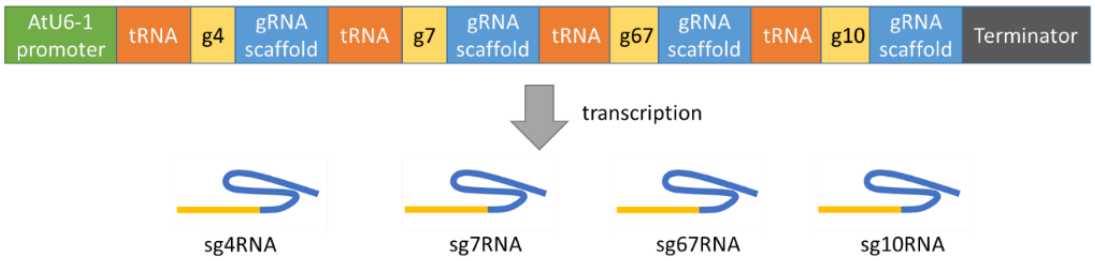



**Figure 4-4. The ASVI expression cassette contains four gRNAs appended to individual gRNA scaffolds and the tRNA (transfer RNA) processing system.** sg4RNA and sg7RNA: single guide-RNAs g4 and g7 target the potato *asparagine synthetase 1* gene, sg67RNA and sg10RNA: single guide-RNAs g67 and g10 target the potato *vacuolar invertase* gene.

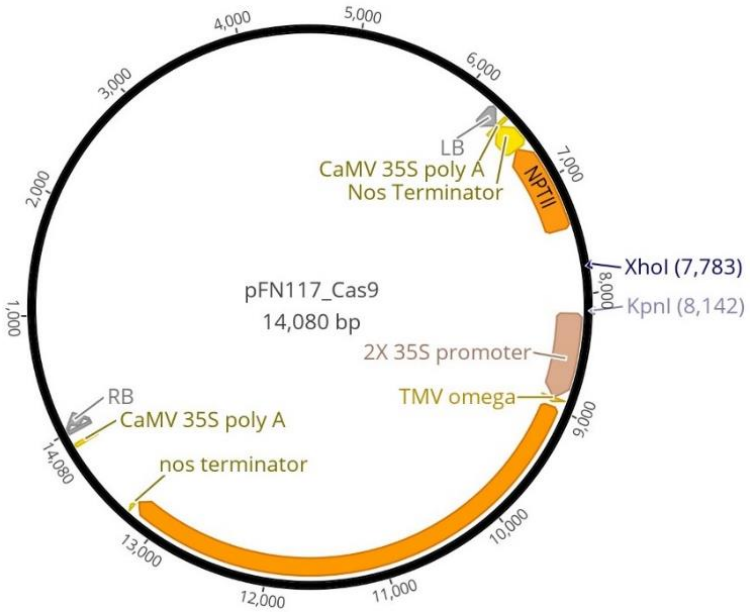



**Figure 4-5. Map of the pFN117-Cas9 vector.** The Cas9 coding sequence is shown in orange which is expressed by a 2X 35S promoter. TMV omega: a translation enhancer from tobacco mosaic virus. NPTII: *neomycin phosphotransferase II* (*nptII*) gene provides resistance to the antibiotic kanamycin. LB and RB: left border and right border, respectively. The gRNA expression cassettes were inserted into this vector via XhoI and KpnI restriction sites.

### 4.2.3 Transformation of *A. tumefaciens* competent cells with the assembled CRISPR/Cas9 vectors

#### 4.2.3.1 Preparation of freeze-thaw *A. tumefaciens* GV3101 competent cells

Bacterial glycerol stock was streaked on a solid LB medium (10 g/L bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl, 10 g/L bacto agar) containing 25 mg/L rifampicin and incubated overnight at 28°C in the dark. A single colony was cultured overnight in 10 mL of LB supplemented with 25 mg/L rifampicin in a 50-mL conical centrifuge tube with a screw cap on a rotary shaker at 180 rpm, at 28°C in the dark. The next day, 250 µL of the overnight culture was grown in 100 mL of LB with 25 mg/L rifampicin, in a 1 L Erlenmeyer flask, on a rotary shaker at 180 rpm, 28°C in the dark until $OD_{600}$ reached approximately 0.8. The culture was then transferred to two 250-mL centrifuge bottles, chilled on ice for 10 minutes and centrifuged at 4°C, 3000 x g for 30 minutes (Avanti J-30 I centrifuge, Beckman Coulter). The resulting bacterial pellets were gently resuspended in 1 mL of chilled filter-

sterilised 20 mM $CaCl_2$, and 50 µL aliquots were quickly dispensed into pre-chilled sterile 1.5-mL tubes. The tubes were snap-frozen in liquid nitrogen and then stored at -80°C.

#### 4.2.3.2 Delivery of the CRISPR/Cas9 vectors into competent cells of *A. tumefaciens*

A 50 µL aliquot of competent cells stored at -80°C was thawed on ice, and 350 ng of the CRISPR/Cas9 vector was added and mixed by gently stirring with a pipette tip. The mixture was incubated on ice for 20 minutes, then at 37°C for 5 minutes and back on ice for another 2 min. The cells were then grown in 700 µL LB medium in a sterile 10 mL tube on a rotary shaker at 180 rpm, 28°C in the dark. After three hours, 200 µL of culture was plated on LB agar supplemented with 25 mg/L rifampicin and 50 mg/L kanamycin. The plates were kept in the dark at 28°C for two to three days to facilitate the growth of bacterial colonies. Individual colonies were picked and screened for the transformed vector using colony PCR according to section 3.2.4.2, with the primer pair FGC-F and FGC-R or FN117-F and FN117-R (Table 4-2). The PCR-positive colonies were grown overnight in 5 mL LB medium supplemented with 25 mg/L rifampicin and 50 mg/L kanamycin at 180 rpm and 28°C in the dark. A 500 µL aliquot of this culture was mixed with 500 µL of sterile 50% glycerol in a 2-mL tube and stored at -80°C for future use.

### 4.2.4 Transformation of leaf discs with *A.tumefaciens* harbouring the CRISPR/Cas9 vector

#### 4.2.4.1 Preparation of leaf discs

Leaf disc explants used for plant transformation were obtained from potato plants growing in soil in a growth chamber. The potting mix was prepared by mixing 40 kg of soil, 20 g of dolomite, 15 g of $CaCO_3$, 40 g of wetting agent, 40 g of Osmocote® all-purpose fertiliser with 5 L of water and steam-pasteurised before use. Two-week-old *in vitro* potato plantlets were transferred to soil and covered with clear plastic for five days for acclimatisation. The plantlets were grown in a growth chamber at 30°C and under a 16h light/ 8h dark photoperiod for at least four weeks before leaf harvesting. Approximately 10 to 15 fully expanded leaves were detached from plants and left under running

water in a container covered with a muslin cloth for 15 minutes. Finally, all the water was drained, and leaves were transferred into a sterile glass beaker (1 L) in a laminar flow cabinet.

The leaves were decontaminated by surface sterilisation by incubating in 70% ethanol for 30 seconds with gentle swirling. Ethanol solution was decanted, and leaves were washed with sterile water, followed by a 40-s incubation in 250 mL of 1 % sodium hypochlorite solution containing three drops of Tween-20. The solution was decanted, and leaves were washed with 500 mL of sterile water with gentle swirling. The leaves were washed four times with sterile water with two-minute incubation and occasional swirling during each wash. After the final wash, water was decanted, and the leaves were kept in sterile water until further processing. Leaf discs were prepared by placing them in a stack and punching with a sterile cork borer (0.7 cm in diameter). Leaf discs were kept in liquid MS until used for *Agrobacterium*-mediated transformation.

#### 4.2.4.2 *A. tumefaciens*-mediated plant transformation

*A. tumefaciens* harbouring the CRISPR/Cas9 vector was prepared a few days before plant transformation. Bacterial glycerol stock was streaked on solid LB medium supplemented with 25 mg/L rifampicin and 50 mg/L kanamycin and incubated in the dark at 28°C for three days. Two days before transformation, a single colony was grown overnight in 5 mL of LB broth with the same antibiotics, in a 28-mL glass bottle, at 28°C while shaking at 250 rpm. The second culture was started by adding 100 µL of the first culture in 60 mL of LB broth, 100 µM of acetosyringone, 25 mg/L rifampicin and 50 mg/L kanamycin, in a 250-mL glass bottle with a screw cap (Schott Duran), at 28°C, 250 rpm, in the dark overnight until $OD_{600}$ reached 0.8. The bacterial culture was transferred to two 50-mL conical centrifuge tubes, and pellets were collected by centrifugation at 3000 x g for 15 minutes. The pellets were gently resuspended in 60 mL of liquid MS with 30 g/L sucrose, pH 5.6 and 100 µM acetosyringone.

During the transformation process using pcoCas9-g4g7 and pcoCas9-g67g10 vectors, leaf discs of each cultivar were immersed in bacterial suspension for 15 to 30 min with gentle shaking. Leaf discs

were then blotted on sterile filter paper and embedded on co-cultivation media with the abaxial surface directly in contact with the culture medium in a 90-mm sterile plastic petri dish. Desiree explants were cultured on callus induction medium (CIM) (treatment T5 in chapter 2), while Atlantic explants were cultured on M2 medium (treatment T7 in chapter 2). After two days of co-cultivation, explants were washed once with 200 mg/L timentin in sterile water and rinsed several times with sterile water to remove residual bacteria. Next, explants were blotted on sterile filter paper, and Desiree explants were cultured on CIM supplemented with 200 mg/L timentin and 0.5 mg/L glufosinate-ammonium. Atlantic explants were cultured on M3 medium (treatment T7 in chapter 2) supplemented with 200 mg/L timentin and 0.5 mg/L glufosinate-ammonium. Atlantic explants were sub-cultured bi-weekly until shoots appeared. Desiree explants were transferred after five days to shoot induction media (SIM) (treatment T5 in chapter 2) and subcultured bi-weekly for shoot induction. Individual shoots were then transferred to the selection medium: Murashige and Skoog basal medium (MS) supplemented with 20 g/L sucrose, 0.5 mg/L glufosinate-ammonium, and solidified with 2.8 g/L Gelrite, pH 5.6. A total of 1,093 Desiree leaf discs were used, and they were divided into four transformation batches with *A. tumefaciens* harbouring pcoCas9-g4g7 vector and four batches with *A. tumefaciens* harbouring pcoCas9-g67g10 vector. Atlantic leaf discs were transformed with pcoCas9-g4g7 in 11 transformation batches and pcoCas9-g67g10 in five batches with a total of 2,031 leaf discs. All transformation batches had 15 explants as the control for each batch, which were incubated in MS liquid medium, pH 5.6 instead of the bacterial suspension.

Transformation with *A. tumefaciens* harbouring pFGC-ASVI and pFN117-ASVI vectors was done with a few modifications. Leaf discs were immersed in bacterial suspension for one hour in the dark on a rocker at the lowest speed, then blotted on sterile filter paper and cultured on Murashige and Skoog basal medium with Gamborg vitamins (MSGV, Phytotech Labs) supplemented with 16 g/L glucose, 5 mg/L NAA and 2 mg/L BAP, pH 5.6 (C52 medium). Leaf discs and bacteria were co-cultivated for two days in the dark, and then explants were washed once with 200 mg/L timentin in sterile water, followed by several additional washes with sterile water (until the water became clear). Next,

explants were blotted on sterile filter paper, and those that were transformed with pFGC-ASVI vector were cultured on C52 medium supplemented with 200 mg/L timentin and 0.5 mg/L glufosinate-ammonium. Explants transformed with the pFN117-ASVI vector were cultured on C52 medium supplemented with 200 mg/L timentin and 50 mg/L kanamycin. After five days, explants were transferred to shoot induction media. Desiree explants were transferred to SIM (treatment T5, see chapter 2) supplemented with 200 mg/L timentin and 0.5 mg/L glufosinate-ammonium (pFGC-ASVI vector) or with 200 mg/L timentin and 50 mg/L kanamycin (pFN117-ASVI vector). Atlantic explants were transferred to petri dishes containing shoot induction medium of treatment S3 (see chapter 2), which was MSGV supplemented with 200 mg/L timentin and 0.5 mg/L glufosinate-ammonium (pFGC-ASVI vector) or with 200 mg/L timentin and 50 mg/L kanamycin (pFN117-ASVI vector). All explants were subcultured every two weeks until shoots appeared. For root induction, single shoots were transferred to selection media in 250 mL tissue culture containers with MS, 20 g/L sucrose and corresponding selection agent. These were allowed to grow into plantlets before leaf samples were taken for mutation screening. Regenerated shoots from transformation with pFGC-ASVI vector were cultured on MS supplemented with 20 g/L sucrose and 0.5 mg/L glufosinate-ammonium. Regenerated shoots from transformation with pFN117-ASVI were cultured on MS supplemented with 20 g/L sucrose and 50 mg/L kanamycin. All media were solidified with 2.8 g/L Gelrite (Sigma-Aldrich, Inc) and adjusted to pH 5.6 with 1 M KOH before autoclaving.

Transformation with *A. tumefaciens* harbouring pFGC-ASVI vector was done for 156 Atlantic leaf discs and 294 Desiree leaf discs. A total of 925 Atlantic leaf discs and 403 Desiree leaf discs were transformed with *A. tumefaciens* harbouring pFN117-ASVI vector. Sterile plastic petri dishes (90-mm in diameter) were used during co-cultivation, callus induction and shoot regeneration stages. Shoots were cultured in 250-mL sterile, clear round plastic containers with clear screw caps (Labserv).

### 4.2.5 Screening for putative transformants and mutants

Putative transgenic events that survived on selection media after two months were transferred to the PC2 glasshouse for tuber formation (see section 6.2.1 of Chapter 6) and subjected to mutation analysis. Leaves from *in vitro* plantlets of corresponding events were used for DNA extraction according to section 3.2.2 of Chapter 3. The integration of the *BlpR* gene in plantlets regenerated from transformation with the pFGC-pcoCas9 vectors (pcoCas9-g67g10, pcoCas9-g4g7, pFGC-ASVI vectors) was confirmed with PCR using BAR-F (5'-CAGATAAAGCCACGCACATTTAGG-3') and BAR-R (5'-CACGGTCAACTTCCGTACC-3') primers (654 bp amplicon). Regenerated plantlets from transformation with pFN117-ASVI vector were screened using the primers FN117-F and FN117-R (Table 4-2, section 4.2.2.1 of Chapter 4); the expected amplicon size was 1,120 bp. Three regions of *VInv* and *AS1* genes containing four target sites in the PCR-positive transformed plantlets were amplified using corresponding primers listed in Table 4-3. PCR amplification was done in a 10-µL reaction mix containing 5 µL of 2X GoTaq® Green Master Mix (Promega® Corporation), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primers, 300 ng of genomic DNA and nuclease-free water. The PCR program and gel electrophoresis were done as described in section 3.2.2 of Chapter 3. Amplified DNA fragments of the target regions were used for sequencing to detect putative mutations. The DNA was extracted from agarose gel for sequencing using the centrifugation method. Briefly, the DNA band was cut from the agarose gel and placed on top of the filter inside a 200-µL filter tip. The tip below the filter was cut off, and the remaining tip with filter and the gel piece was placed in a 1.5-mL tube. The tube was centrifuged at maximum speed for 1 min, the filter tip was removed, and the eluate from the filter tip was retained. Four µL of this DNA eluate was used for sequencing, with the reaction set-up described in section 3.2.5 of Chapter 3. Sequencing results were analysed using Geneious Prime software (Biomatters). PCR products with multiple peaks in the sequencing chromatogram were cloned according to section 3.2.4 of Chapter 3. At least 20 clones were sequenced for each PCR product, and results were analysed on Geneious Prime to assess mutation

types. The mutation rate was calculated as the number of plants with gene mutations divided by the number of plants with the integrated transgene.

**Table 4-3. Primers for amplifying guide RNA target regions and amplicon sequencing**

| Primer name | Primer sequence (5' to 3') | Target site | Amplicon size | Note |
|---|---|---|---|---|
| 67-F | GGGGAAATATCACATGGGGC | g67 and g10 | 385 bp | for sequencing |
| 10-R | AGTGCAATACCCGTTTTACCAA | | | |
| AS1-F | GGCTTTGTTGGGTTGTTCGG | g4 | 191 bp | |
| 4-R | AACCCTTCAATGCACAGACAG | | | for sequencing |
| 7-F | CCTAACGTGGGATAAGAAATCTCT | g7 | 407 bp | for sequencing |
| 7-R | GATGTGCCAAGTAAAAATCACCA | | | |

### 4.2.6 *In silico* protein translation of edited gene sequences

Ten transgenic edited events: ALpFN1, ALpFN2, DSpco3, DSpco4, DSpco5, DSpco7, DSpco8, DSpco9, DSpco12 and DSpFN4 were subjected to *in silico* protein translation to assess putative changes in their protein sequences. The g67 and g10 target sites on the *VInv* gene reference sequence (accession no. HQ110080.1) were replaced with detected mutations found at corresponding sites in edited events to generate a complete coding sequence of the mutated allele. Likewise, the g7 target site on the *AS1* gene reference sequence (accession no. NW_006238977.1:c1565347-1560303) was replaced with mutations found at this site in edited events. The untranslated regions and introns were removed from both reference and mutant sequences before *in silico* translation using Geneious Prime software (Biomatters).

## 4.3 Results

### 4.3.1 Transformation vectors expressing Cas9 and gRNAs were assembled successfully

After the first round of overlapping PCR, products 1 and 2 of each gRNA expression cassette were amplified successfully, with the expected amplicon sizes of 333 bp for product 1 and 146 bp for product 2 (Figure 4-6). Two products were joined in the second PCR round, and the expected 459 bp

amplicon size was obtained (Figure 4-7). Dual-gRNA expression cassettes were made by joining two gRNA cassettes targeting the same gene (g4g7 targeting *AS1* gene and g67g10 targeting *VInv* gene). The insertion and correct sequences of the dual-gRNA cassette in the pFGC-pcoCas9 vector were confirmed by colony PCR (Figure 4-8) and multiple sequence alignment after sequencing. Similarly, the insertion of the ASVI construct into pFGC-pcoCas9 and pFN117-Cas9 was also confirmed via colony PCRs (Figure 4-9), and the sequences were confirmed by sequencing in both directions.

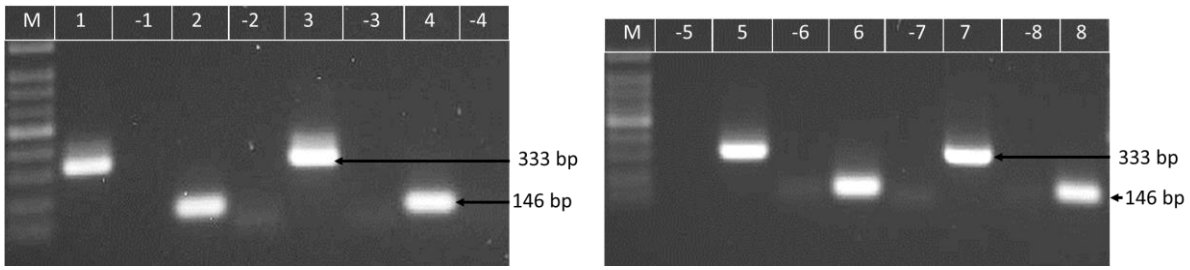



**Figure 4-6. Agarose gel electrophoresis image of amplified products from the first PCR round for assembling gRNA expression cassettes.** Lanes 1 and 2: product 1 and 2 of first-round PCR for assembling g4RNA cassette, respectively. Lanes 3 and 4: product 1 and 2 of first-round PCR for assembling g7RNA cassette, respectively. Lanes 5 and 6: product 1 and 2 of first-round PCR for assembling g67RNA cassette, respectively. Lanes 7 and 8: product 1 and 2 of first-round PCR for assembling g10RNA cassette, respectively. Lanes -1 and -8: negative controls of corresponding PCR products. M: 100 bp to 3000 bp DNA ladder.

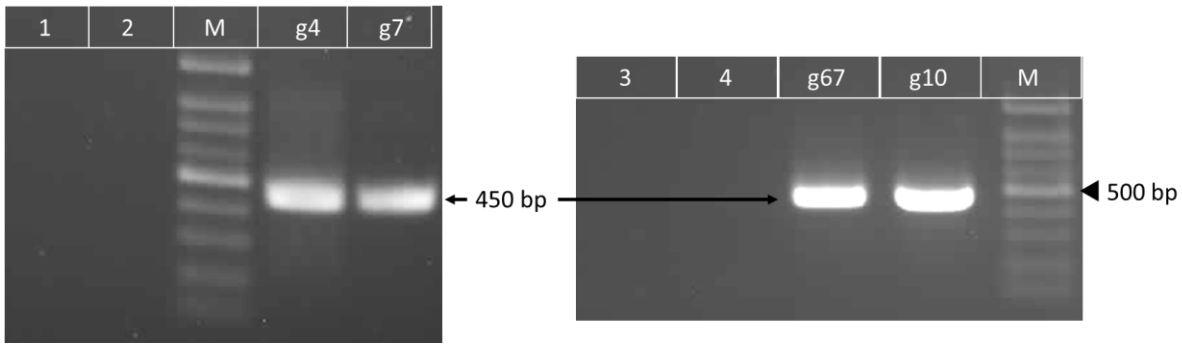



**Figure 4-7. Agarose gel electrophoresis image of amplified products from the second PCR round for assembling gRNA expression cassettes.** Lanes g4, g7, g67, g10: assembled g4RNA, g7RNA, g67RNA and g10RNA cassettes, respectively, with AtU6-1 promoter, 20-nt of the target sequence and gRNA scaffold in each DNA amplicon. Lanes 1, 2, 3 and 4: negative control for PCR assembling g4RNA, g7RNA, g67RNA and g10RNA, respectively. M: 100 bp to 3000 bp DNA ladder.

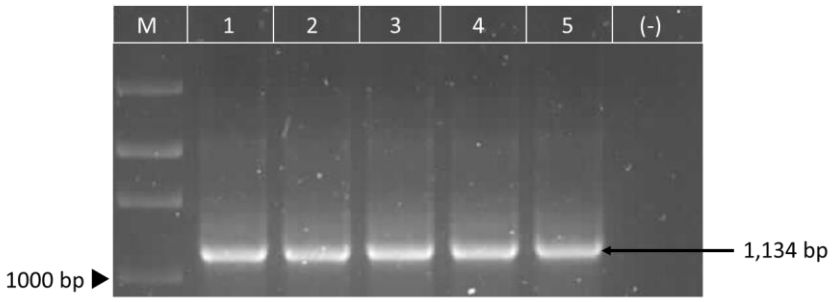



**Figure 4-8. Agarose gel electrophoresis image of amplified products from colony screening PCR for the insertion of the dual-gRNA expression cassette in the vector pFGC-pcoCas9** Lanes 1 and 2: amplified PCR products from colonies containing g4g7 dual-gRNA cassette. Lanes 3 to 5: amplified PCR products from colonies containing g67g10 dual-gRNA cassette. Lane (-): negative control; M: 100 bp to 3000 bp DNA ladder.

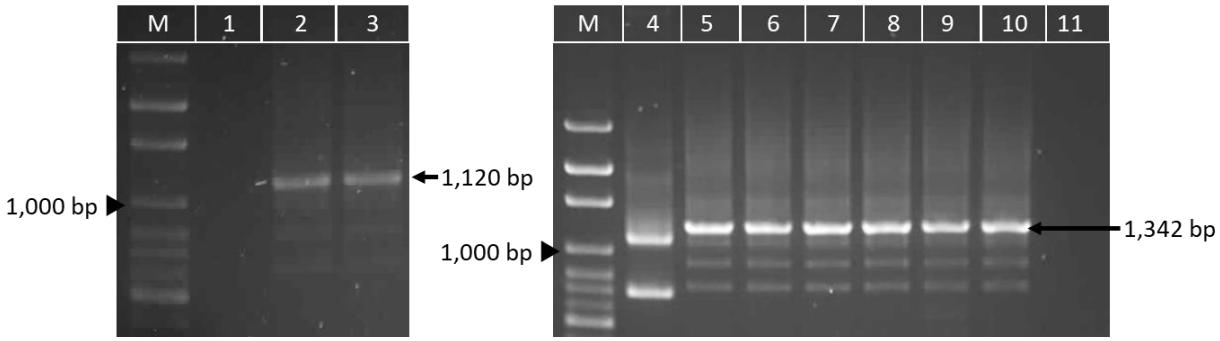



**Figure 4-9. Agarose gel electrophoresis images amplified products from colony screening PCR for the insertion of the ASVI construct in pFN117-Cas9 and pFGC-pcoCas9 vectors.** Lanes 1 and 11: negative controls. Lanes 2 and 3: amplified PCR products from colonies containing pFN117-Cas9 vector harbouring ASVI expression cassette. Lane 4: amplified PCR products from pFGC-g4g7 plasmid DNA as a positive control. Lanes 5 to 10: amplified PCR products from colonies containing pFGC-pcoCas9 vector harbouring ASVI expression cassette. M: 100 bp to 3000 bp DNA ladder.

### 4.3.2 CRISPR/Cas9-induced mutations in transgenic potato plants

Shoot formation was not achieved for Atlantic explants that were transformed with pcoCas9-g4g7 and pcoCas9-g67g10. Atlantic leaf discs formed callus with no further development or differentiation, and they gradually turned yellow with partial necrosis after two months. In contrast, Desiree explants displayed better vigour and shoot induction after six weeks of culture on shoot induction medium with the selection agent. All control samples died within two months on the selection medium. Nine Desiree shoots were obtained from transformation with pcoCas9-g4g7 (DSAS1 to DSAS9) and four shoots from transformation with pcoCas9-g67g10 (DSVI1 to DSVI4).

These shoots were grown to plantlets on the selection medium, and after two months, DNA was extracted from leaf tissues. PCR screening for the *BlpR* gene resulted in amplification from only two out of 13 shoots (Figure 4-10). Target gene sites in all 13 shoots were sequenced: no sequence variations were detected in the multiple sequence alignments with the wildtype sequences.

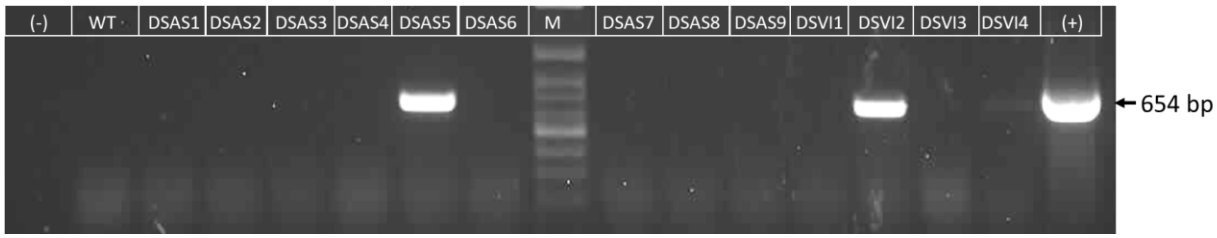



**Figure 4-10. Agarose gel electrophoresis images of amplified products from PCR screening for *BlpR* gene in Desiree wildtype and putative transgenic shoots.** Lane (-): negative control, lane WT: amplified PCR products from genomic DNA of wildtype Desiree. Lanes DSAS1 to DSAS9: amplified PCR products from genomic DNA of regenerated shoots from transformation with pcoCas9-g4g7 vector. Lanes DSVI1 to DSVI4: amplified PCR products from genomic DNA of regenerated shoots from transformation with pcoCas9-g67g10 vector. Lane (+): amplified PCR products from plasmid DNA of pcoCas9-g4g7 as a positive control, M: 100bp to 3000 bp DNA ladder.

The transformation with *A. tumefaciens* harbouring pFGC-ASVI was done once for each potato cultivar Atlantic and Desiree. After two months on shoot induction medium, shoots were not obtained for Atlantic, whereas 12 shoots were recovered for Desiree, named as events DSpco1 to DSpco12. PCR screening for the *BlpR* gene confirmed its presence in 11 out of 12 recovered shoots (Figure 4-11). Overlapping signals were observed in amplicon sequencing chromatograms of g67, g10 and g7 target sites of ten out of the 12 shoots, except DSpco1 and DSpco6 (Figure 4-12), indicating some of the alleles in regenerated events were mutated. Intriguingly, a SNP was detected at the third base directly upstream of PAM of the g67 target site on the *VInv* gene of Desiree (Figure 4-12).

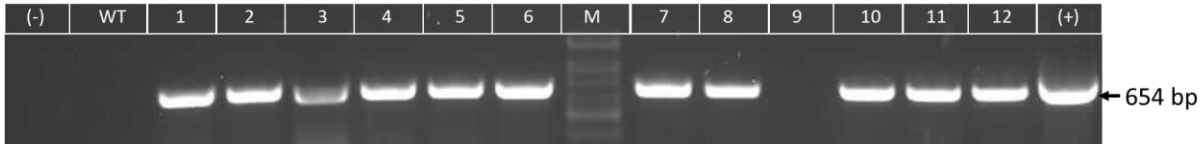



**Figure 4-11. Agarose gel electrophoresis image of products from PCR screening for the *BlpR* gene in putative transgenic Desiree shoots transformed with the pFGC-ASVI vector.** Lane (-): negative control, lane WT: amplified PCR products from genomic DNA of wildtype Desiree. Lanes 1 to 12: amplified PCR products from genomic DNA of DSpco1 to DSpco12, respectively. Lane (+): amplified PCR products from plasmid DNA of pFGC-ASVI as a positive control, M: 100 bp to 3000 bp DNA ladder.

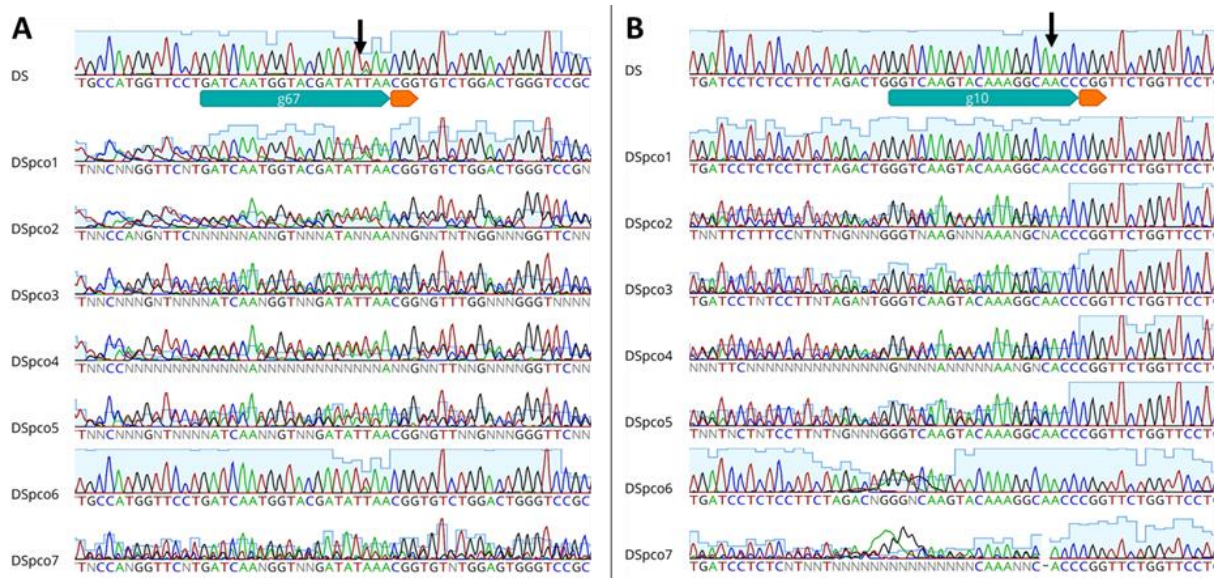



**Figure 4-12. Representative chromatograms from direct sequencing of PCR products after amplifying g67 and g10 target sites on the *vacuolar invertase* gene of the potato cultivar Desiree transformed with the *A. tumefaciens* harbouring the pFGC-ASVI vector.** (A): Sequence chromatogram of the *Vlnv* amplicon indicating g67 target site, (B): Sequence chromatogram of the *Vlnv* amplicon indicating g10 target site. The green arrows indicate gRNA target sites; the orange arrows indicate protospacer-adjacent motifs (PAMs). Black arrows point to the expected cleavage sites between the fourth and third nucleotide upstream of PAM. DS: sequence from wildtype Desiree. DSpco1 to DSpco7: sequences from Desiree transgenic events.

Ten shoots were recovered from transformation with *A. tumefaciens* harbouring the pFN117-ASVI vector. Two were from Atlantic (ALpFN1 and ALpFN2), and eight were from Desiree (DSpFN1 to DSpFN8). The presence of the ASVI construct in these ten putative transformants were confirmed by PCR screening for the construct (Figure 4-13). Overlapping signals were observed in sequencing chromatograms of PCR amplicons containing g67 and g10 target sites on the *Vlnv* gene. There were no overlapping peaks at the g7 target site on the *AS1* gene of ALpFN1 or ALpFN2, suggesting that

they could be homozygous mutations (Figure 4-14). The sequences containing the g4 target site on the *AS1* gene of all transgenic shoots were identical to the wildtype sequence. Overall, putative mutations in both genes were observed for 58% and 100% of recovered shoots from transformation using pFGC-ASVI and pFN117-ASVI vectors (Table 4-4)

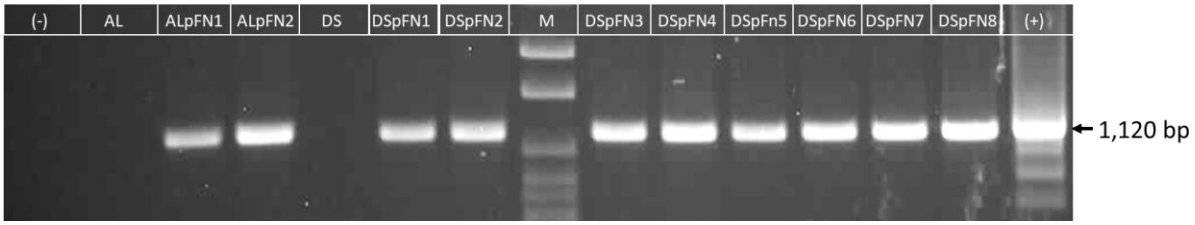



**Figure 4-13. Agarose gel electrophoresis image of products from PCR screening for integration of the ASVI construct in recovered shoots from leaf disc explants of potato cultivars Desiree and Atlantic transformed with *A.tumefaciens* harbouring pFN117-ASVI vector.** Lane (-): negative control. AL and DS: amplified PCR products from genomic DNA of wildtype Atlantic and Desiree, respectively. ALpFN1 and ALpFN2: amplified PCR products from genomic DNA of recovered Atlantic shoots. DSpFN1 to DSpFN8: amplified PCR products from genomic DNA of recovered Desiree shoots. Lane (+): amplified PCR products from plasmid DNA of pFN117-ASVI as a positive control. M: 100 bp to 3000 bp DNA ladder.

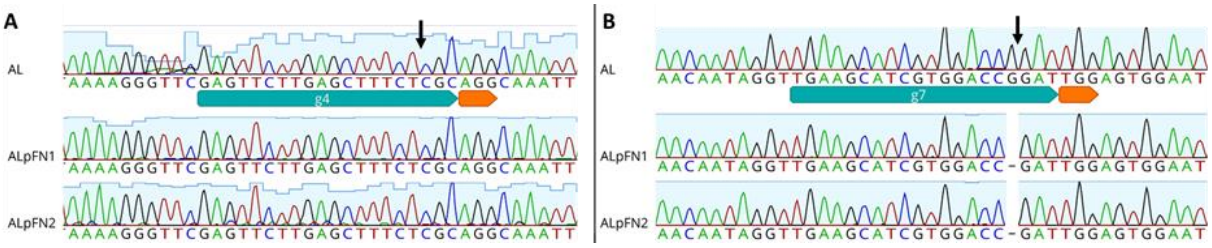



**Figure 4-14. Representative chromatograms from direct sequencing of products after PCR amplification of g4 and g7 target sites for the *asparagine synthetase 1* gene of the potato cultivar Atlantic transformed with the *A.tumefaciens* harbouring pFN117-ASVI vector** (A) and (B): g4 and g7 target sites on the *AS1* gene of potato cultivar Atlantic, respectively. The green arrows indicate target sites; the orange arrows indicate protospacer-adjacent motifs (PAMs). Black arrows point to the expected cleavage sites between the fourth and third nucleotide upstream of PAM. AL: sequence of wildtype Atlantic. AlpFN1 and ALpFN2: sequence of Atlantic transgenic events.

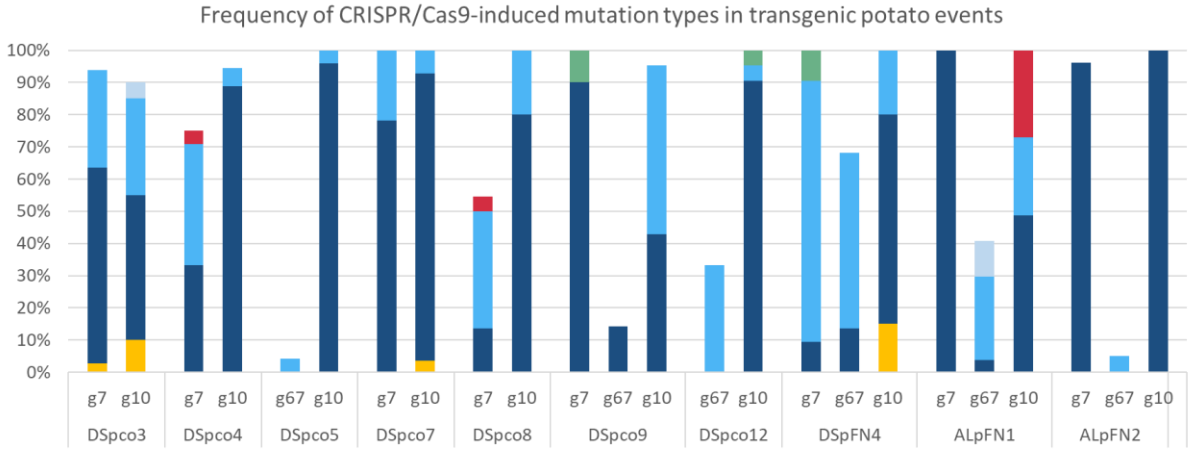
**Table 4-4. Mutation rates in *vacuolar invertase* and *asparagine synthetase 1* genes of Atlantic and Desiree transgenic edited events.** pFGC-g4g7 and pFGC-g671g0: vectors expressing Cas9 protein and dual-gRNA system. pFGC-ASVI and pFN117-ASVI: vectors expressing Cas9 protein and four gRNAs. *BlpR* gene: gene confers resistance to glufosinate-ammonium. ASVI construct: construct contains four gRNAs with the tRNA processing system. N/A: not applicable. PCR screening for the *BlpR* gene was done for regenerated shoots after transformations with *A.tumefaciens* harbouring pFGC-g4g7, pFGC-g67g10 and pFGC-ASVI vectors. PCR screening for the ASVI construct was done for regenerated shoots after transformation with *Agrobacterium tumefacines* harbouring pFN117-ASVI vector.

| Potato variety | Vector | Total leaf discs | No. of shoots | *BlpR* gene-positive shoots | No. of shoots containing ASVI construct | No. of shoots with target gene mutation | No. of shoots with mutation in both target genes |
|---|---|---|---|---|---|---|---|
| Atlantic | pFGC-g4g7 | 1,298 | 0 | 0 | N/A | 0 | 0 |
| | pFGC-g67g10 | 733 | 0 | 0 | N/A | 0 | 0 |
| | pFGC-ASVI | 156 | 0 | 0 | N/A | 0 | 0 |
| | pFN117-ASVI | 925 | 2 | N/A | 2 | 2/2 (100%) | 2/2 (100%) |
| Desiree | pFGC-g4g7 | 549 | 9 | 1/9 | N/A | 0/9 (0%) | 0/9 (0%) |
| | pFGC-g67g10 | 544 | 4 | 1/4 | N/A | 0/4 (0%) | 0/4 (0%) |
| | pFGC-ASVI | 294 | 12 | 11/12 | N/A | 10/12 (83%) | 7/12 (58%) |
| | pFN117-ASVI | 403 | 8 | N/A | 8/8 | 8/8 (100%) | 8/8 (100%) |

### 4.3.3 Assessing mutation types and frequencies

There was a total of 20 putative mutants generated from transformation with pFN117-ASVI and pFGC- ASVI vectors. As they were recovered from different transformation batches, there was variation in their developmental stages. Ten putative mutation events were ready to be transferred to the PC2 glasshouse for tuber formation during August to December 2020, and these were subjected to mutation analysis (see Chapter 6). PCR amplicons of the target sites of these ten events were cloned into pGEM T-easy vector and transformed into *E. coli*, and at least 18 clones were screened and sequenced (Figure 4-15). Among ten events, two had mutations on the *VInv* gene (Figure 4-15, events DSpco5 and DSpco12), while the rest had mutations on both *VInv* and *AS1*

genes. A total of 28 PCR amplicons containing target regions (g7, g67 or g10 target sites) of ten events were cloned and sequenced. Even though direct PCR amplicon sequencing chromatograms of the g67 target site of all events showed multiple base peaks, potentially indicating the presence of mutations in the sequences, no mutation was detected at this site for events DSpco3, DSpco4, DSpco7 and DSpco8 (Figure 4-15). Homozygous mutations were found at the g7 target site of AlpFN1 and g10 site of AlpFN2, which had the same 1 bp deletion in all examined clones (Figure 4-15). More than four allelic variations were detected at the target site g10 of DSpco3 (1 bp insertion, 1 bp deletion, 2 to 10 bp deletion and >10 bp deletion) and g67 site of ALpFN1 (1 bp deletion, 2 to 10 deletion, >10 bp deletion, combine mutation and wildtype allele), suggesting that these events consisted of chimeric cells.




| | Number of clones | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| target site | DSpco3 | DSpco4 | DSpco5 | DSpco7 | DSpco8 | DSpco9 | DSpco12 | DSpFN4 | ALpFN1 | ALpFN2 |
| g7 | 23 | 24 | n/a | 23 | 21 | 20 | n/a | 20 | 35 | 26 |
| g67 | 20 | 18 | 24 | 28 | 20 | 21 | 21 | 22 | 27 | 20 |
| g10 | 20 | 18 | 24 | 28 | 20 | 21 | 21 | 22 | 27 | 20 |

**Figure 4-15. Frequency of CRISPR/Cas9-induced mutation types in ten transgenic potato events.** g7: target site in the *asparagine synthetase 1* gene. g67, g10: target sites in the *vacuolar invertase* gene. DSpco3 to DSpco12: transgenic events generated from potato cultivar Desiree transformed with *A.tumefaciens* harbouring the pFGC-ASVI vector. DSpFN4: transgenic event generated from potato cultivar Desiree transformed with *A.tumefaciens* harbouring the pFN117-ASVI vector. ALpFN1 and ALpFN2: transgenic events generated from potato cultivar Atlantic transformed with *A.tumefaciens* harbouring the pFN117-ASVI vector.

The highest mutation frequency was recorded for the g10 target site on the *VInv* gene sequence (98.19%), followed by the g7 target site (90.63%) on the *AS1* gene and g67 target site (17.19%) on the *VInv* gene, no mutation was found at g4 on the *AS1* gene in any tested event (Figure 4-16 A). Deletions accounted for more than half of the induced mutations, at 65.8%. Insertions, substitutions and combination mutations were induced at a much lower frequency, 2.1%, 1.4% and 1.3%, respectively. Approximately one third (33%) of the 634 clones examined, no mutations were detected when compared to the wildtype sequence (Figure 4-16 B). Deletions ranged from 1 bp to 35 bp, with 1 bp deletions being the most common among mutated sequences (68.53%). Insertions and substitutions occurred at a much lower frequency (3.03% for insertion and 0.47% for substitution) and were limited to 1 bp (Figure 4-16 C). Regarding the SNP on the g67 target site of Desiree *VInv* gene, 85% (148) of 174 clones had only wildtype sequences; among those, 75% (111 clones) had the nucleotide T while 25% (37 clones) had the nucleotide A at the SNP site.

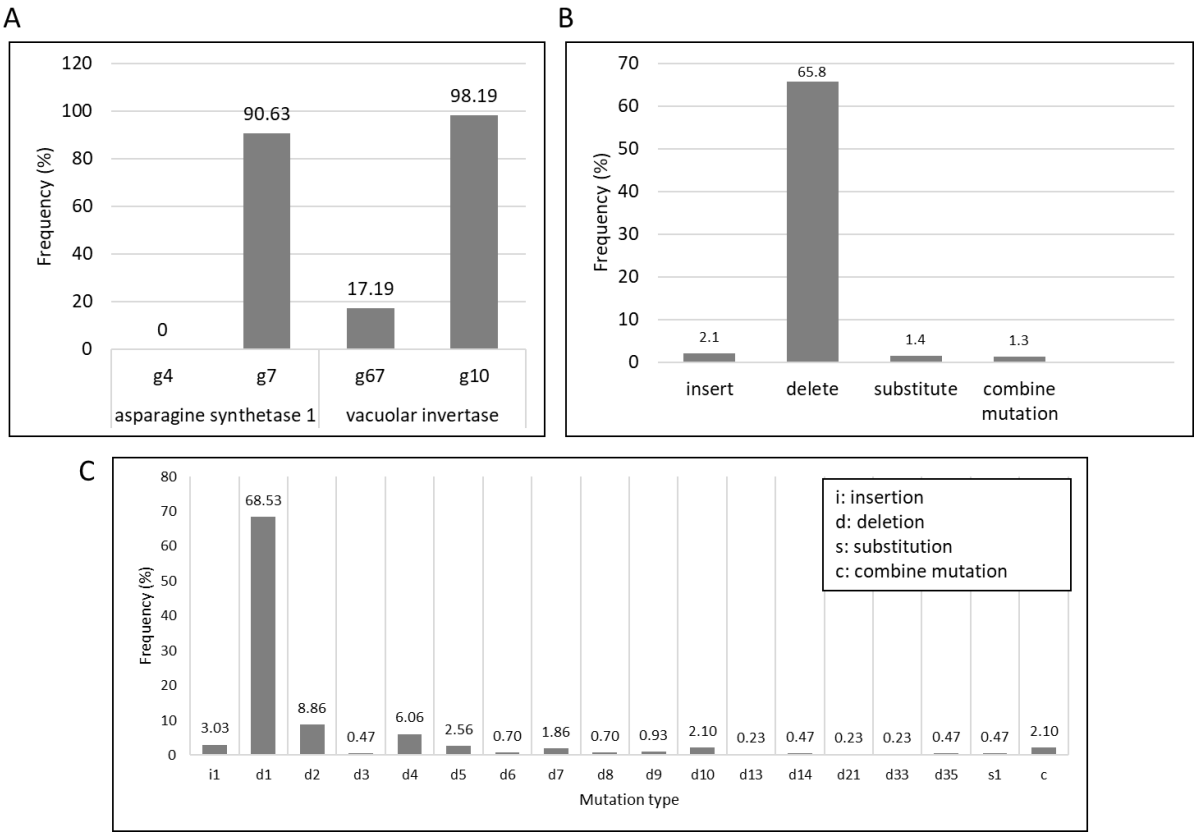



**Figure 4-16. CRISPR/Cas9-induced mutation type and frequency.** Graph A: Mutation frequency at each target site. Graph B: Frequency of total detected mutation types at the three target sites (g7 on *asparagine synthetase 1* gene, g67 and g10 on *vacuolar invertase* gene). Mutation frequency was calculated as the number of clones with mutations in the sequence divided by the total (634) clones sequenced. Graph C: Type and frequency of the mutations detected. The number after the alphabet at the x-axis indicates the number of edited nucleotides, e.g. i1 indicates one base insertion. The frequency of mutation type was calculated based on the number of clones carrying that type of mutation divided by the total clones with mutations.

### 4.3.4 *In silico* protein translation of edited gene sequences

#### 4.3.4.1 *Vacuolar invertase* gene

The translated protein sequence of the *VInv* gene (accession no. HQ110080.1) consisted of 639 amino acids. Deletions at the guide g67 target site of events ALpFN1, DSpco5, DSpco9, DSpco12 and DSpFN4 resulted in a frameshift, protein sequence changes, and premature stop codons. Stop codons were generated in all edited alleles of those events within the first 200 amino acids of their predicted protein sequence (Figure 4-17 B). The three-base deletion at g67 of ALpFN2 did not generate significant protein sequence changes, except for removing one amino acid (Figure 4-17 B).

The translation of edited sequences at the g10 target site revealed that premature stop codons were generated within the first 270 amino acids in all events (Figure 4-18 B). However, sequences without premature stop codons were detected in four events: DSpco3, DSpco7, Dspco12 and DSpFN4 (one sequence in each event); these proteins lost one to 11 amino acids instead (Figure 4-18 B).

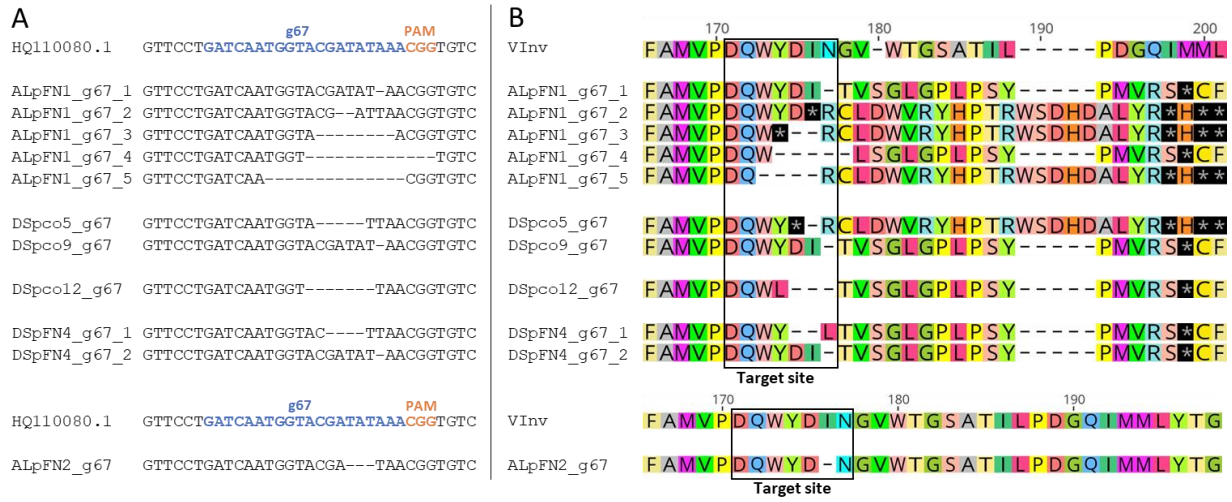



**Figure 4-17. Partial alignment of vacuolar invertase DNA and protein sequences of the reference sequence and edited events with mutations at g67.** (A): Alignment of DNA sequences; HQ110080.1: accession no of *vacuolar invertase* DNA sequence; g67: target site, blue bases; PAM: protospacer-adjacent motif, orange bases. (B): Alignment of predicted protein sequences; VInV: translation of *vacuolar invertase* reference sequence (accession no. HQ110080.1). The solid black boxes represent stop codons. Sequence name: first two letters indicate the cultivar (AL: Atlantic, DS: Desiree); the next three letters indicate vector construct (pFN: pFN117-ASVI, pco: pFGC-ASVI), the following number indicates an individual event, g67: target site and last number indicates the number of edited alleles.


**Figure 4-18. Partial alignment of vacuolar invertase DNA and protein sequences of the reference sequence and edited events with mutations at g10.** (A): Alignment of DNA sequences; HQ110080.1: accession no of *vacuolar invertase* reference sequence; g10: target site, in blue; PAM: protospacer-adjacent motif, in orange. (B): Alignment of predicted protein sequences; VInV: translation of *vacuolar invertase* reference sequence (accession no. HQ110080.1). The solid black boxes represent stop codons. Sequences with highlighted names do not contain premature stop codons. Sequence name: first two letters indicate the cultivar (AL: Atlantic, DS: Desiree); the next three letters indicate CRISPR/Cas9 construct (pFN: pFN117-ASVI, pco: pFGC-ASVI), the following number indicates an individual event, g10: target site, and last number indicates the number of edited alleles.

### 4.3.4.2 *Asparagine synthetase 1* gene

The predicted AS1 protein sequence translated from the reference DNA sequence (accession no. NW_006238977.1:c1565347-1560303) had 590 amino acids. Premature stop codons were generated within the first 55 amino acids in all events containing mutations at g7 (Figure 4-19), suggesting the interruption of the AS1 protein sequence and its activity in those events. Two alleles of DSpco3, one allele of DSpco8 and one allele of DSpFN4 did not have a premature stop codon in their translation sequence, but one or two amino acids were removed (Figure 4-19 B).

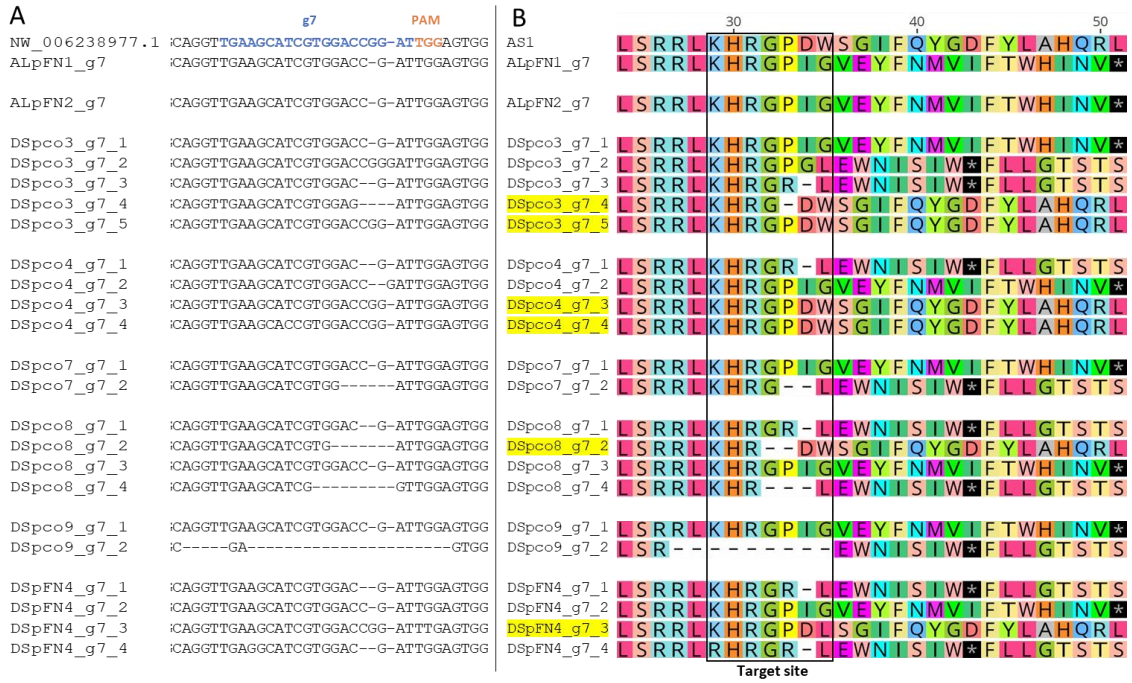



**Figure 4-19. Partial alignment of asparagine synthetase 1 DNA and protein sequences of the reference sequence and edited events with mutations at g7.** (A): Alignment of DNA sequences; NW_006238977.1: accession no of reference sequence; g7: target site, blue; PAM: protospacer-adjacent motif, in orange. (B): Alignment of predicted protein sequences; AS1: translation of *asparagine synthetase 1* reference sequence (accession no. NW_006238977.1). The solid black boxes represent stop codons. Sequences with highlighted names do not contain premature stop codons. Sequence name: first two letters indicate the cultivar (AL: Atlantic, DS: Desiree); the next three letters indicate CRISPR/Cas9 construct (pFN: pFN117-ASVI, pco: pFGC-ASVI), the following number indicates an individual event, g10: target site, and last number indicates the number of edited alleles.

## 4.4 Discussion

CRISPR/Cas9-induced mutations with up to 100% frequency were achieved in both the *VInv* and *AS1* genes in regenerated transgenic Desiree and Atlantic potato shoots. However, the mutation frequency varied significantly between the target sites, from no mutation at the g4 target site to 98.19% at the g10 target site. The variability of mutation efficiency has been studied to determine the controlling factors, although there is no clear explanation. For example, the presence of a thiamine (T) base within the four bases proximal to PAM, especially as the last base before PAM, is disfavoured by Cas9 (Xu et al., 2015). This finding seems to be true in the case of g67 (5'-GATCAATGGTACGATATTAA-3') and g4 (5'-GAGTTCTTGAGCTTTCTCGC-3') target sites which have a T base at the fourth position upstream of the PAM, but this was contradictory for the g7 (5'-

TGAAGCATCGTGGACCGGAT-3') target site, which has a T base at the 3'-end of the target sequence. On the other hand, cytosine (C) before PAM is also reported to be unfavourable for Cas9 binding (Doench et al., 2014). Again, a contradictory result was obtained as both g10 and g4 target sites have a C nucleotide at the end of the sequence; only g4 resulted in no mutation, while g10 resulted in 98.19% mutation efficiency. Most of the studies on the effectiveness of gRNA have been from studies on animal cells; thus, it may not be entirely applicable for plant cells. The g4 target site effectively guided Cas9 to cleave the *AS1* gene fragment *in vitro* (see section 3.3.3 of Chapter 3); thus, the presence of T as the fourth base and C as the last base before PAM may not be the reason for low efficiency as Cas9 still bound to this gRNA. A possible explanation for the low expression of g4RNA is due to the T-rich (eight Ts) sequence in the g4 target site, which could cause low expression of gRNA (Xu et al., 2015).

Unlike the g4 target site, the low mutation rate at the g67 target site was probably caused by the SNP in the target sequence. The SNP at the g67 target site of potato cultivar Desiree was not discovered during the initial sequencing when six clones were screened to assess the allelic variation present. It may be due to TA cloning bias, where a particular SNP will be picked up at a higher rate than another (Veillet et al., 2020). This SNP is located within the seed sequence of the target site, which is the 12$^{th}$ nt directly upstream of PAM. This SNP's location was also on the third base upstream of PAM, where the cleavage site of Cas9 is. Hence, the effectiveness of CRISPR/Cas9-induced DNA DSBs at the g67 target site might be affected by the mismatches at that SNP, as the seed sequence is known to be crucial for Cas9 recognition, binding and cleavage activity (Zheng et al., 2017).

Large deletions between two target sites on the same gene have been reported in the Arabidopsis *phytoene desaturase* gene (Li et al., 2013), chromosome 2 of *Japonica* rice (Zhou et al., 2014), tomato *argonaute 7* gene (Brooks et al., 2014) and recently in potato *granule bound starch synthase I* gene (Veillet et al., 2019a). The distance between two target sites can be as small as 24 bp (Li et al.,

2013) to larger than 100 kb (Zhou et al., 2014). The target sites on *VInv* and *AS1* genes were selected with 135 bp between the g67 and g10 target sites and 1,163 bp between g4 and g7 target sites (see chapter 3) to facilitate larger deletions. However, the 'drop-out' of a genetic fragment between the two target sites was not found in this study. The low or no mutation rate in g67 and g4 greatly reduced the possibility of large DNA fragment deletions that would be between the target sites g67 and g10 in the *VInv* gene and g4 and g7 target sites in the *AS1* gene.

The culture medium used is an important factor in plant transformation as it directly influences the possible number of shoots regenerated. Initially, the culture media of treatment T7 (see chapter 2) were used during the transformation process for the dual-gRNA vector constructs (pFGC-g67g10 and pFGC-g4g7). However, this treatment did not lead to a high shoot induction frequency in Atlantic (approximately 10%, see chapter 2,) and no transgenic shoots were obtained. Consequently, the regeneration of Atlantic was switched to using the optimised media (C52 medium for callus induction and shoot induction medium of treatment S3) with a 64.3% shoot regeneration frequency (see chapter 2), and then two transgenic events were obtained. More transgenic events (18) were obtained from Desiree cultured on regeneration media with a 100% shoot induction rate (treatment T5, chapter 2). From these, 58% to 100% of regenerated shoots were transgenic and contained edited genes. Therefore, improving the shoot induction frequency of Atlantic will undoubtedly increase the chances of obtaining gene-edited events.

Regarding promoters driving the expression of Cas9, two vectors were used to express Cas9 and gRNAs in plant cells: pFN117-Cas9 and pFGC-pcoCas9. Expression of the Cas9 coding sequence in the pFGC-pcoCas9 vector was driven by the 35SPPDK promoter (constitutive 35S enhancer fused to the maize C4 pyruvate orthophosphate dikinase basal promoter). The expression of Cas9 in the pFN117-Cas9 vector was driven by a 2X 35S promoter with the addition of tobacco mosaic virus (TMV) omega sequence enhancer, which was expected to increase translation efficiency of Cas9 *in vivo*. Plant transformation with the pFN117-ASVI vector derived from pFN117-Cas9 resulted in a

higher mutation frequency than in plants with the pFGC-ASVI vector (constructed from pFGC-pcoCas9 vector). Mutated alleles were detected in all regenerants transformed with pFN117-ASVI, while only 83% of transformants with pFGC-ASVI contained mutations. In addition, all edited events transformed with pFN117-ASVI had both *VInv* and *AS1* genes mutated; this rate was lower in those from pFGC-pcoCas9, at 58%. These results indicate that the Cas9 expressing cassette with 2X 35S promoter and TMV omega enhancer contributed to increasing the mutation frequency in edited events. Similar observation on the advantages of a translation enhancer was highlighted by (Kusano et al., 2018).

CRISPR/Cas9-induced mutations in *VInv* and *AS1* genes of potato were generated successfully in this chapter, with diverse mutation types and a mutation efficiency of up to 100%. Protein sequence predictions from *in silico* translations revealed potential knockouts for both *VInv* and *AS1* genes in edited events and a probable reduction in their protein levels and functionality. It would be possible to generate large gene fragment deletion by grouping the g10 target site in the *VInv* gene and the g7 target site in *AS1* with new target sites on the corresponding target gene sequence, possibly closer to each other. If sufficient funds were available, the gRNAs/constructs could be synthesised by a commercial provider, which would speed up the research progress. As demonstrated here, with the tRNA processing system, many gRNAs can be introduced into plant cells instead of just two or four to maximise the chances of inducing desired mutations.

# Chapter 5

# Gene editing with CRISPR/Cas9 via PEG-mediated protoplast transfection and RNP-particle bombardment

## 5.1 Introduction

CRISPR/Cas9 is a gene-editing method that employs site-directed nuclease (SDN) technology and is now widely used to edit a range of plant species. The gene edits or mutations resulting from this technology are classified as follows - SDN-1: the introduction of insertions or deletions (e.g. single point mutations) without introduced DNA, SDN-2: short insertion of a few base pair using an oligonucleotide DNA template, and SDN-3: longer insertion bases, up to a complete gene, using an introduced DNA template (Menz et al., 2020). Several countries have adopted this classification to regulate products derived from SDN technology. The edited plants obtained through SDN-1 mutations without external genetic material integrated into their genome are not classified as GMOs in America, Argentina, Australia, Brazil, Canada, Israel and Japan (Menz et al., 2020, Gupta et al., 2021), with other countries considering to adopt this approach. The generation of transgene-free gene-edited potato plants of existing cultivars requires the CRISPR/Cas9 system to be used as a ribonucleoprotein complex (RNP) instead of using transformation vectors. Unlike wheat or other crop plants, potato is highly heterozygous, making it impossible to backcross transgenic potato to obtain null segregant as this will remove the unique genetic combination of the given potato variety. The use of the RNP complex can avoid the transgene introduction into the potato genome. This complex can be delivered into plant cells either by PEG-mediated protoplast transfection or particle bombardment approach.

Direct delivery of RNPs has been achieved for some model and crop plant species, including Arabidopsis, tobacco, lettuce, rice, maize, wheat, tomato and potato (Woo et al., 2015, Svitashev et al., 2016, Liang et al., 2017, Andersson et al., 2018, Makhotenko et al., 2019, Veillet et al., 2019b, González et al., 2020, Zhao et al., 2021). Protoplast transfection of RNPs is the only delivery method reported for potato, and the highest mutation efficiency was 72% (Zhao et al., 2021). Biolistic bombardment with RNPs has been tested in other crop plants, and with variable editing efficiency:

up to 47% efficiency was achieved in maize (Svitashev et al., 2016) while only 0.56% to 4.2% was reported for wheat (Liang et al., 2017, Imai et al., 2020).

In this chapter, RNP complexes of Cas9 and four gRNAs were delivered into potato leaf disc-derived calli or leaf tissue via particle bombardment to generate transgene-free gene-edited plants. The protoplast transfection method from an established protocol by Nicolia et al. (2015) was tested with plasmid DNA to lay out a foundation for delivering RNPs into potato protoplasts.

## 5.2 Methodology

### 5.2.1 Protoplast transfection assay

#### 5.2.1.1 Preparation of media and solutions

Media and solutions for protoplast isolation and transfection were prepared according to Nicolia et al. (2015) with modifications. The 10X base solution contained 1.9 g/L $KNO_3$, 0.44 g/L $CaCl_2$, 0.37 g/L mg $MgSO_4.7H_2O$ and 0.17 g/L $KH_2PO_4$. The iron stock solution contained 1.9 g/L $FeSO_4.7H_2O$ and 1.4 g/L $Na_2EDTA$ in Milli-Q water. The 10X macro stock solution contained 74 g/L $KNO_3$, 49.2 g/L $MgSO_4.7H_2O$ and 3.4 g/L $KH_2PO_4$. The 1000X micro stock contained 1.5 g/L $H_3BO_3$, 5 g/L $MnSO_4.H_2O$, 1 g/L $ZnSO_4.7H_2O$, 0.12 g/L $Na_2MoO_4.2H_2O$, 0.012 g/L $CuSO_4.5H_2O$, 0.012 g/L $CoCl_2.6H_2O$ and 0.38 g/L KI. The 200X vitamins stock was made with 0.5 g/L pantothenic acid, 0.5 g/L choline chloride, 1 g/L ascorbic acid, 0.01 g/L p-aminobenzoic acid, 0.5 mg/L nicotinic acid, 0.5 g/L pyridoxine-HCl, 5 g/L thiamine-HCl, 0.2 g/L folic acid, 0.005 g/L biotin, 0.01 g/L cyanocobalamin, 0.005 g/L cholecalciferol. The 50X organic acid stock contained 1 g/L pyruvic acid, 2 g/L fumaric acid, 2 g/L citric acid monohydrate and 2 g/L DL-malic acid. Sugar stock contained 6.25 g/L sorbitol, 6.25 g/L sucrose, 6.25 g/L fructose, 6.25 g/L ribose, 6.25 g/L xylose, 6.25 g/L mannose, 6.25 g/L rhamnose monohydrate, 6.25 g/L cellobiose and 2.5 g/L myo-inositol. All solutions were prepared in Milli-Q water, filter-sterilised and stored at 4°C, except for the vitamin stock stored at -20°C.

Medium E was prepared by mixing 1X macro stock, 0.147 g/L $CaCl_2.2H_2O$, 10 mL/L iron stock, 1X micro stock, 1X vitamins stock, 20 mL/L sugar stock, 1X organic acids stock, 0.5 g/L casein hydrolysate, 33.7 g/L glucose, 30.92 g/L mannitol, 1 g/L BSA (bovine serum albumin), 1 mg/mL thiamine HCl, 2 mg/mL zeatin riboside and 2 mg/mL 2,4-D in Milli-Q water. pH was adjusted to 5.6 with 1 M KOH and the medium was filter sterilised and then stored at 4°C.

Alginate solution was made by dissolving 28 g/L alginic acid-Na salt and 72.88 g/L sorbitol in Milli-Q water followed by autoclaving. The floating solution was prepared by dissolving 72.88 g/L sorbitol and 7.351 g/L $CaCl_2.2H_2O$ in Milli-Q water; the solution was then filter-sterilised. Both alginate and the floating solution were kept at 4°C after sterilisation. Setting agar was prepared in two steps: 72.88 g/L sorbitol and 8 g/L agar were dissolved in Milli-Q water and autoclaved; 7.351 g/L $CaCl_2.2H_2O$ was dissolved in Milli-Q water, filter-sterilised and then combined with the autoclaved sorbitol + agar solution before pouring a 0.5 cm layer into a 50-mL sterile plastic container.

PEG (polyethylene glycol) solution was freshly prepared on transfection day at 25% concentration by dissolving 250 g/L PEG 4000, 72.8 g/L mannitol, and 328.16 g/L $Ca(NO_3)_2$ in Milli-Q water. Transformation buffer 1 (TB1) was made by dissolving 34.6 g/L mannitol, 14.68 g/L $CaCl_2.2H_2O$ and 5 g/L MES (morpholinoethane sulfonic acid) in Milli-Q water; pH was adjusted to 5.6. Transformation buffer 2 (TB2) was made by dissolving 91 g/L mannitol, 3.04 g/L $MgCl_2.6H_2O$ and 1 g/L MES in Milli-Q water, pH 5.6. All solutions were filter-sterilised, TB1 and TB2 were stored at 4°C.

#### 5.2.1.2 Protoplast isolation from leaves

Protoplasts were isolated from *in vitro* leaves, according to the protocol of Nicolia et al. (2015) with modifications. About 20-25 fully expanded leaves (1 g) of Desiree or Atlantic were detached from 4- to 6-week-old *in vitro* plantlets, cut into thin strips using a sharp blade in sterile MS solution supplemented with 20 g/L sucrose, pH 5.6. The strips were incubated in freshly prepared 60 mL of plasmolysis solution (0.5 M sorbitol, filter-sterilised) for 30 minutes in the dark. Leaf strips were then transferred into freshly prepared 15 mL digestion solution (1X base solution, 37.35 g/L

mannitol, 0.87 g/L $CaCl_2$, 10 g/L cellulase RS (Yakult Pharmaceutical Industry Co.), 2 g/L macerozymes (Yakult Pharmaceutical Industry Co.), filter-sterilised into a 50-mL centrifuge tube). The tube was wrapped in aluminium foil and kept on a rocker at the lowest speed for three to four hours at room temperature.

After digestion, a sieve with 0.38 µM aperture was pre-wetted with wash solution (0.45 M mannitol, autoclaved), the solution containing isolated protoplasts was filtered through the sieve (solution 1). The sieve was then washed with 5 mL wash solution (solution 2). The two filtered solutions (solutions 1 and 2) were combined and divided into four 5-mL portions into four 10-mL conical centrifuge tubes. The tubes were centrifuged at 70 *x g* for 5 min (minimum acceleration and deceleration) at room temperature. The liquid supernatant was discarded, protoplast pellets were gently resuspended in 2.5 mL of 20% sucrose by gentle flicking, and protoplast suspensions from two tubes were combined into one. One millilitre of autoclaved 0.3 M KCl was carefully layered on top of the sucrose-protoplast suspension, and care was taken not to mix the two solutions. The tubes were centrifuged at 50 *x g* for 15 min (minimum acceleration and deceleration) at room temperature. A thick band of protoplasts formed at the KCl and sucrose solution interface which was carefully collected by pipetting and transferred into a new 10-mL conical centrifuge tube containing 3 mL of wash solution. The mixture was topped up to 7 mL with wash solution and centrifuged at 70 *x g* for 5 min (minimum acceleration and deceleration). The liquid supernatant was discarded, protoplasts were gently resuspended in 1 mL of medium E (for regeneration), or 3 mL of TB1 (for transfection) and solutions from two tubes were combined into one. A 15-µL of the protoplast-containing solution was used for quantifying protoplast density using a haemocytometer, and the remaining protoplasts were kept at 4°C in the dark during counting.

After counting, protoplasts were either used for transfection or cultured for regeneration. For regeneration, protoplast density was adjusted to 1.6 x $10^5$/mL with either medium E or medium E mixed with alginate solution at a 1:1 ratio to create alginate lenses. Protoplasts in medium E were

transferred to a 5-cm diameter glass petri dish and kept at 22°C in the dark. The protoplasts resuspended in medium E with alginate solution were gently dropped on setting agar using a 1000-mL pipette tip and allowed to set at room temperature. Two millilitres of the floating solution were then added to release the alginate lens containing protoplasts from setting agar, and the lenses were transferred to a sterile 5-cm diameter glass petri dish containing medium E. The protoplasts were kept in the dark at 22°C and medium E was replaced bi-weekly.

#### 5.2.1.3 PEG-mediated protoplast transfection with plasmid DNA

The tube containing protoplasts in TB1 was centrifuged at 50 *x g* for 10 min (minimum acceleration and deceleration). TB1 solution was discarded, and the concentration of protoplasts was adjusted to $1.6 \times 10^6$ by adding TB2. For every transfection, 100 µL of protoplasts (approximately 160,000 protoplasts) was added to a 10-mL conical centrifuge tube, followed by 10 µg of plasmid DNA (pFN117-ASVI vector), and one volume (110 µL to 140 µL, depending on the volume of added plasmid DNA) of 25 % PEG 4000 solution was added and gently mixed by flicking. After incubation for three minutes at room temperature, transfection was stopped by adding 5 mL of wash solution. The protoplasts were collected by centrifugation at 50 *x g* for 5 min (minimum acceleration and deceleration), resuspended in 100 µL of medium E and transferred to a sterile 5-cm diameter glass petri dish. Protoplasts were kept in the dark at 22°C for two days before DNA extraction. The control for PEG-mediated protoplast transfection was also done simultaneously with the same protoplast quantity, plasmid DNA and transfection conditions but without PEG.

#### 5.2.1.4 DNA extraction and molecular analysis of transfected protoplasts

After two days, transfected protoplasts in medium E were transferred to a 1.5-mL tube and centrifuged at maximum speed for 5 min. Medium E was discarded, and protoplasts were washed by adding 500 µL of wash solution followed by centrifugation at maximum speed for five min. Wash solution was discarded, and protoplasts were frozen in liquid nitrogen before homogenising using a tissue lyser at 22/s frequency for 1.5 min (TissueLyser II, QIAGEN). DNA was extracted from

protoplasts following the method described in section 3.2.2 of Chapter 3. The volumes of CTAB, chloroform: isoamyl alcohol (24:1) and ice-cold isopropanol used were adjusted to 300 µL due to the small sample size. PCR and sequencing of extracted protoplast DNA were done according to section 4.2.5 (Chapter 4).

### 5.2.2 Particle bombardment with RNPs

#### 5.2.2.1 Preparation of gold particles for bombardment

Gold particles (approximately 0.6 µm in diameter, Bio-Rad) were freshly prepared before bombardment according to the manual of Biolistic® PDS-1000/He Particle Delivery System (Bio-Rad) with minor adjustments. Briefly, for every ten shots with RNPs, 4.5 mg of gold particles were sterilised in 1 mL of 70% ethanol by vigorous vortexing for 5 min. The particles were soaked in 70% ethanol for 15 min before centrifugation at 5,000 rpm for 5 min. The ethanol was discarded, and particles were washed with 1 mL of sterile, nuclease-free water by vortexing for 1 min. The particles were incubated at room temperature for 5 min, centrifuged at 5,000 rpm for 5 min, and the water was removed by pipetting. This washing step was repeated two more times before adding 5 µL of NEB 3.1 buffer (New England Biolabs) and 45 µL of nuclease-free water to make 50 µL solution of the gold particles at 90 mg/mL concentration

#### 5.2.2.2 RNP assembly of Cas9 protein and gRNA

Lyophilised Cas9 protein (Proteowa Pty Ltd) was dissolved in nuclease-free water to make a 1 µg/µL protein solution. All gRNAs were prepared according to section 3.2.7 in chapter 3. The RNP complex was assembled separately for each gRNA (g4 RNA, g7 RNA, g67 RNA and g10 RNA) by mixing 10 µg of Cas9 protein with 10 µg of gRNA, 1X of NEB 3.1 buffer and sterile, nuclease-free water in a 50-µL reaction. The reaction was incubated at 37°C for 15 min in a thermocycler. Then 25 µL from each RNP reaction was mixed with 50 µL of gold particles (90 mg/mL), making the final volume 150 µL and particle density of 30 mg/mL. For each bombardment, 15 µL of RNP particle solution was spread on the microcarrier and air-dried in a laminar flow hood.

#### 5.2.2.3 Bombardment of RNPs into leaf-derived calli of potato

Leaf discs (0.7 cm in diameter) were prepared according to section 4.2.4.1 (Chapter 4). The discs were cultured on callus induction medium (MSGV + 5 mg/L NAA + 2 mg/L BAP + 16 g/L glucose + 2.8 g/L Gelrite, pH 5.6) for five days before bombardment. A group of three callus discs were arranged at the centre of a 90 mm petri dish (without overlapping) containing MS medium supplemented with 20 g/L sucrose, 0.2 M mannitol and solidified with 2.8 g/L gelrite, pH 5.6. The explants were bombarded twice with RNP-coated particles at a 9-cm target distance with the BioRad biolistic device using 1,100 psi rupture discs. After bombardment, explants were transferred to callus induction medium for another two days before being transferred to shoot induction medium. Atlantic explants were transferred to MSGV supplemented with 6 mg/L BAP, 5 mg/L $GA_3$, 16 g/L glucose, pH 5.6 while Desiree explants were transferred to MSGV supplemented with 0.02 mg/L NAA, 0.15 mg/L $GA_3$, 2.2 mg/L zeatin riboside, 16 g/L glucose, pH 5.6. Regenerated shoots were carefully isolated from calli to avoid selecting clones, and these were cultured on MS with 20 g/L sucrose, pH 5.6 for rooting and development into plantlets.

#### 5.2.2.4 Molecular analysis of regenerated shoots from RNP-treated calli

Leaves of regenerated shoots from RNP-treated explants were used for DNA extraction following the CTAB method described in section 3.2.2 of Chapter 3. PCR and sequencing were performed with primers flanking four target sites as mentioned in section 4.2.5 of Chapter 4. PCR products showing multiple peaks in the sequencing chromatogram were cloned according to section 3.2.4 (Chapter 3). Sequencing results were analysed on Geneious Prime software (Biomatters).

The only gene-edited event generated from RNP-particle bombardment was subjected to *in silico* protein translation to access putative changes in its protein sequences. The mutations detected at the g10 target site on the *VInv* gene were included in the reference sequence of the corresponding gene (accession no. HQ110080.1), replacing the wildtype target sites. The untranslated regions and

introns were removed from both the reference and mutant sequences before translating *in silico* using Geneious Prime software (Biomatters).

## 5.3 Results

### 5.3.1 Protoplast isolation and transfection with plasmid DNA

Protoplasts were isolated from leaves of potato cultivars Atlantic and Desiree at an average of 2.35 x $10^5$ protoplasts per gram leaf fresh weight. In liquid medium E, protoplasts exhibited no noticeable changes in the first two days after isolation. On the third day, protoplast expansion was observed, and small protoplast groups were formed (Figure 5-1). However, the colour of protoplasts gradually turned from green to a darker colour, and no further change in protoplast shape was observed after ten days (Figure 5-1). In the fourth week, protoplasts from both varieties were deformed and lost their round shape accompanied by the dark colour, and free chloroplasts were seen in the medium as they were released from burst protoplasts (Figure 5-1). The discolouration of protoplasts was also observed in the alginate lenses two weeks post isolation.

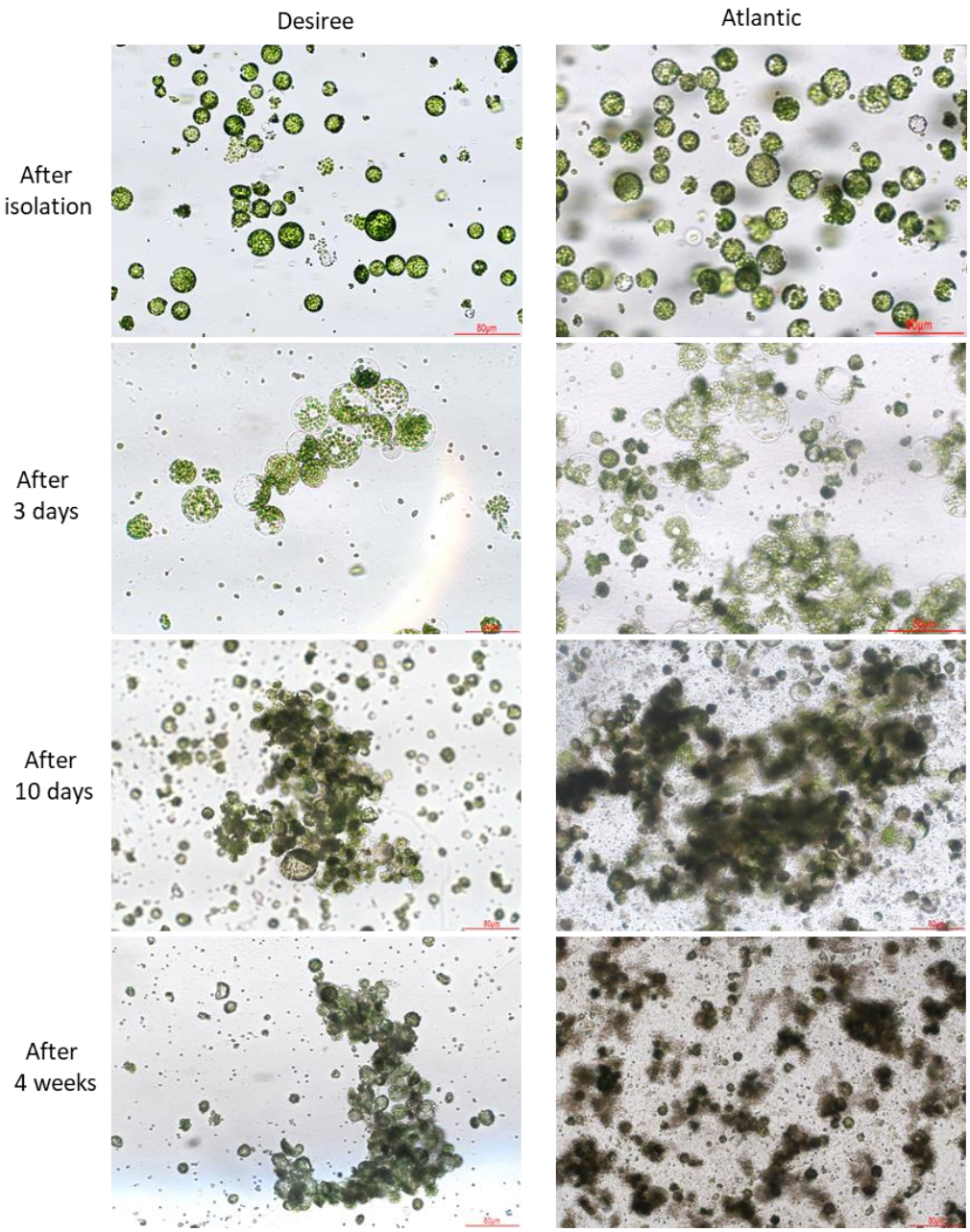



**Figure 5-1. Isolated protoplasts from *in vitro* leaves of potato cultivars Desiree and Atlantic.** Protoplasts were resuspended in liquid medium, and pictures were taken immediately after isolation, three days, ten days and four weeks later.

Protoplasts that were transfected with plasmid DNA were subjected to DNA extraction after two days. The limited amount of DNA extracted from transfected protoplasts was only sufficient for PCR amplification of target regions. There was a band in the negative control of PCR amplifying the g4 target site (lane 1, Figure 5-2), but then it was absent when the negative control was re-run on a different gel, suggesting that the observed contamination was carried over from the adjacent well in

the first run. No PCR product was observed for g7, g67 and g10 target site flanking primers from PEG-DNA transfected protoplast (lanes 6, 10, 14, Figure 5-2). The PCR products amplified from the control protoplasts appeared to have similar sizes with positive control in g4, g67 and g10, except for a smaller product for the g7 target site (lanes 7, Figure 5-2). The difference in the size of PCR products at the g7 target site was analysed by sequencing clones, and it was due to a 36-bp deletion in one of the wildtype alleles (P2 and DS1, Figure 5-3). On the other hand, no mutation was detected from direct PCR amplicon sequencing of the g4 target site in transfected protoplasts.

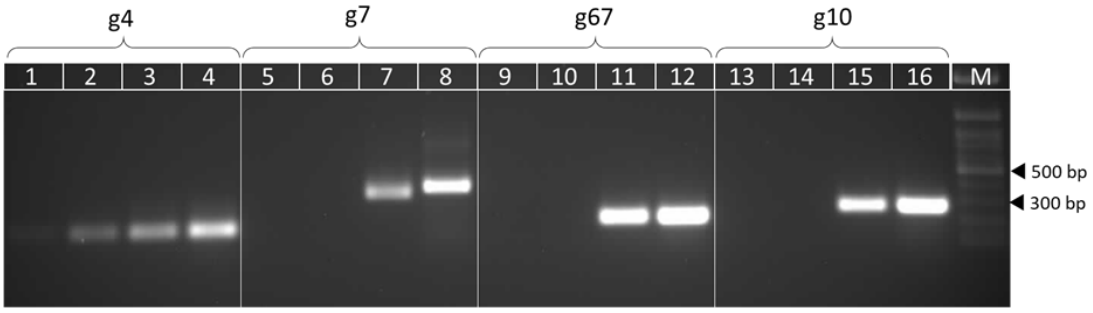



**Figure 5-2. Gel electrophoresis of products from PCR amplified target regions in potato cultivar Desiree after protoplast transfection.** g4, g7: Target regions of the *asparagine synthetase 1* gene. g67, g10: Target regions of the *vacuolar invertase* gene. Lanes no. 1, 5, 9 and 13: Negative controls. Lanes no. 2, 6, 10 and 14: PCR amplification of DNA extracted from protoplasts transfected with 25% PEG. Lanes no. 3, 7, 11 and 15: PCR amplification of DNA extracted from protoplasts that were transfected without PEG. Lanes no. 4, 8, 12 and 16: Positive controls using DNA from Desiree wildtype. M: 100 – 3000 bp DNA marker.

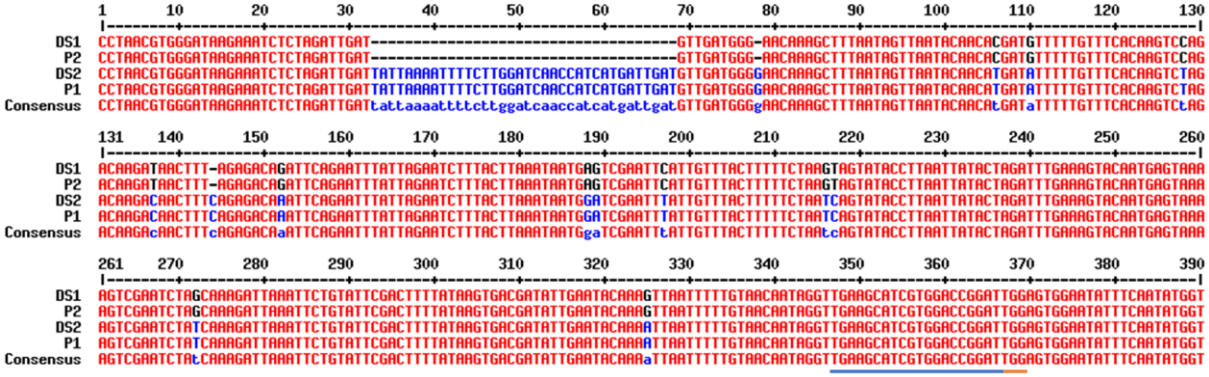



**Figure 5-3. Multiple sequence alignment of g7 target site amplified from DNA of Desiree wildtype and control protoplasts.** DS1 and DS2: Sequences from Desiree wildtype. P1 and P2: Sequences from transfected Desiree protoplasts. The g7 target site is underlined in blue (from position 347 to 366), and the protospacer adjacent motif (PAM) is underlined in orange (from position 367 to 369).

### 5.3.2 Particle bombardment with RNP-coated gold particles

A total of 294 pre-cultured leaf disc derived calli were used for particle bombardment with RNPs (206 explants from Atlantic and 88 from Desiree). Shoots emerged from explants within two weeks after bombardment, and individual shoots were transferred to fresh media without any selection agent for rooting. Sequencing results from 343 shoots (166 Atlantic shoots and 227 Desiree shoots) were analysed and one shoot from Desiree contained a putative mutation, detected from the mixed signals in its sequencing chromatograms (DSRNP217) (Figure 5-4 A), resulting in an editing efficiency at 0.4%. Clones of the g10 target site in this event were sequenced, and there was one base deletion at the fourth position upstream of PAM in g10, which was present in 20% of 35 tested clones (Figure 5-4 B). The translated VINV protein sequence from wildtype is 639 amino acids in length. A pre-mature stop codon was generated in the first half of the protein sequence translated from the edited *VInv* allele in the DSRNP217 event (Figure 5-4 C). No mutation was detected on the other three target sites after direct PCR amplicon sequencing.

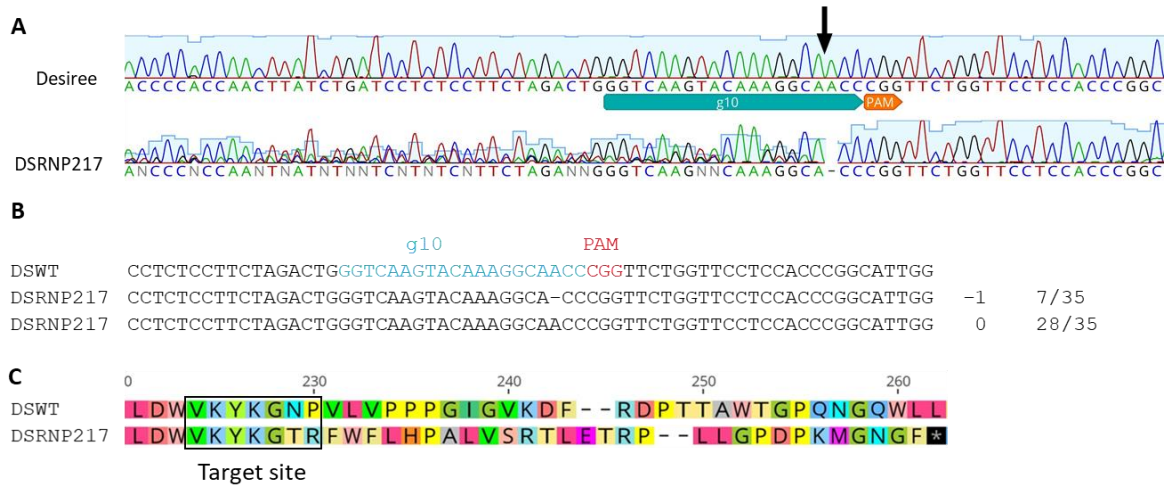



**Figure 5-4. PCR amplicon sequencing and *in* silico protein translation of Desiree gene-edited event DSRNP217.** DSRNP217 was generated from potato cultivar Desiree leaf calli bombarded with a ribonucleoprotein (RNP) complex of Cas9 protein and four gRNAs (g4RNA, g7RNA, g67RNA and g10RNA). (A): Alignment of PCR amplicon sequences of Desiree wildtype and edited event DSRNP217. g10: Target site in the *vacuolar invertase* gene. The black arrow indicates the expected cleavage site. PAM: protospacer-adjacent motif. (B): Mutation analysis of clones sequenced from the DSRNP217 event. DSWT: Sequence of the *VInv* gene from Desiree wildtype. (C): *In silico* translated protein sequences from Desiree wildtype and edited event DSRNP217.

## 5.4 Discussion

Direct delivery of RNPs into tobacco, wheat, tomato, and potato cells has been achieved successfully using PEG-mediated protoplast transfection (Andersson et al., 2018, Lin et al., 2018, González et al., 2020, Zhao et al., 2021, Nicolia et al., 2021). This method can result in up to a 72% editing efficiency in potato protoplasts (Andersson et al., 2018, González et al., 2020, Zhao et al., 2021). However, regenerated plants from protoplasts often suffer from somaclonal variation, with the number of regenerants carrying unwanted chromosomal variation accounted for up to 95% of total generated shoots from potato protoplasts (Jones et al., 1983, Fossi et al., 2019). Besides, protoplast transfection is a challenging method that requires specific technical skills in handling, and technical errors can reduce protoplast yield and quality (Craig et al., 2005). As seen in this chapter, the established protocol did not entirely guarantee the success of protoplast transfection.

Isolated protoplasts can be cultured in a liquid medium, and this was often used in early work on potato protoplasts (Kikuta et al., 1986, Jones et al., 1989, Foulger and Jones, 1986), but immobilising protoplasts in alginate can be beneficial. Alginate is supposed to prevent protoplast from agglutinating, reduce polyphenol production, and promote cell wall regrowth and cell division (Moon et al., 2021). Thus, protoplasts were expected to survive better and form more calli in alginate lenses. However, in experiments reported here, the quality of the isolated protoplasts was disappointing, and protoplast viability quickly declined. The protoplasts did not grow and divide either in liquid medium or alginate lens during the culture process. The protoplast transfection experiments were carried out to evaluate the potential of this method for RNP delivery. However, more work and time were required to optimise the culture and regeneration process to improve the efficiency of protoplast transfection. For example, alginate lenses that contain protoplasts can be cultured on a solid medium instead of a liquid medium, as it can promote better callus formation from protoplasts (Moon et al., 2021). In general, protoplast isolation and culture from potato can

also be an 'art'; that is, it takes time to optimise such systems, and despite the donor plants being grown in controlled conditions, responses to potato in tissue culture can vary with the season.

The particle bombardment approach is a more novel approach for RNP delivery than protoplast transfection, and no specific editing efficiencies for potato are available at present (Makhotenko et al., 2019). Nevertheless, particle bombardment does offer advantages over the protoplast transfection approach. Firstly, explants used for bombardment are typically leaf discs or stem sections that regenerate shoots readily, with a lower frequency of somaclonal variation and genomic instability than shoots regenerated from protoplasts (Fossi et al., 2019). Secondly, the regeneration process for bombarded explants is relatively straightforward, and makes use of established protocols in *Agrobacterium*-mediated transformation. Thirdly, shoot induction can be achieved in a shorter time from bombarded explants (leaf discs or stem sections) than from cultured protoplasts. Shoots emerged from leaf discs within three weeks post bombardment in this study, while protoplasts may take 3-6 months of culture to regenerate shoots (Jones et al., 1989).

In this work, the RNP-particle bombardment approach yielded a 0.4% editing efficiency, and this was similar to what was achieved for wheat (Liang et al., 2017), but lower than the reported frequency of 1.9% after particle bombardment of potato with plasmid DNA (Romano et al., 2001). There are several reasons why RNP bombardment is relatively inefficient compared to using expression vectors. The first is that there is less editing machinery (Cas 9 and gRNA) available in RNP-bombarded cells: plasmid DNA integrates into the plant genome, and expression can continue, while RNPs gradually degrade in bombarded cells. The second is that a selectable marker is usually used to select cells with integrated DNA of Cas9/gRNAs and these cells are likely to have edited sequences in target genes.

Increasing the number of shots bombarded per explant can improve the editing efficiency as RNPs will be delivered into more cells. The 0.4% efficiency reported in this chapter resulted from double bombardments per explant. In comparison, in wheat embryos that were bombarded four times, up

to 4.2% of regenerants carried mutant alleles (Imai et al., 2020), 7.5 times higher than the 0.56% from wheat embryos bombarded once (Liang et al., 2017). The pre-mature stop codon generated in the predicted protein sequence of the mutated *VInv* allele in the DSRNP217 event indicates that the mutated protein is truncated after translation, so its function is likely to be reduced.

The effectiveness of direct delivery of RNPs via particle bombardment was demonstrated in this chapter. Although the editing efficiency was low, it could be improved by optimising the process and increasing the number of bombarded explants. For potato, this method holds tremendous potential as an RNP delivery method for generating non-transgenic edited events. Alternatively, improving the efficiency of culture and regeneration of shoots from potato protoplasts would enable many more edited events to be generated, with the disadvantage of increased somaclonal variation in the resultant plants.

# Chapter 6

# Assessing the levels of sugars in cold-stored tubers and acrylamide content in crisps from gene-edited potato plants

## 6.1 Introduction

In this project, the *VInv* and *AS1* genes were targeted using the CRISPR/Cas9 system to reduce the accumulation of glucose and fructose (hexose sugars) in tubers during cold storage and acrylamide formation in high temperature-processed potato products. The edited gene sequences in the generated events were transcribed *in silico* which indicated that premature stop codons had been introduced into exon 3 of the *VInv* gene and exon 2 of the *AS1* gene, suggesting possible knockout of these genes (see Chapter 5). In previous studies, in which RNAi and TALENs were used to silence *VInv* and *AS1* genes, effective suppression of the accumulation of hexose sugars was achieved, together with a reduced acrylamide content in modified potatoes (Rommens et al., 2008, Bhaskar et al., 2010, Ye et al., 2010, Wu et al., 2011, Chawla et al., 2012, Zhu et al., 2014, Zhu et al., 2016). Therefore, it was reasonable to expect that the edited events generated in this study with edited *VInv* and *AS1* genes would have significantly reduced hexose sugars and acrylamide levels.

In this chapter, the sugar composition in cold-stored tubers and acrylamide in fried potato products were evaluated and compared between the wildtype and gene-edited events. Tubers were harvested from two-month-old plants growing in a glasshouse and were cold-stored at 4°C for four months. The total tuber weight per plant and tuber size were assessed to obtain preliminary data on the performance of gene-edited plants under glasshouse conditions. Soluble sugars were extracted from cold-stored tubers, and these were subjected to an enzymatic assay to determine the content of glucose, fructose, and sucrose. Crisps were made from cold-stored tubers by deep frying the potato tuber slices in vegetable oil, and acrylamide was extracted from those crisps for quantification using an ELISA method. The colour intensity of the fried crisps was assessed to compare the crisps made from cold-stored tubers of gene-edited potato plants with those from wildtype potatoes.

## 6.2 Methodology

### 6.2.1 Growing potato plants for tubers

Three plants each of the gene-edited event and wildtype plants were grown in Physical Containment Level 2 (PC2) glasshouse. Due to the different growth stages, events were divided into two batches growing from August to October 2020 and from September to November 2020. Each plant was grown in a 5 L pot filled with pasteurised Murdoch soil mix (soil mixed with 20 g/L dolomite, 15 g/L $CaCO_3$, 40 g/L Grower's Blue NPK fertiliser, 40 g/L Osmocote® all-purpose fertiliser plus trace elements). Plants were watered daily, and tubers were harvested after two months. Harvested tubers were counted, washed, air-dried, and weighed before storage in paper bags at 4°C, in the dark for four months. Tuber length and width were measured using the Fiji image processing software with photos taken after harvesting (Schindelin et al., 2012). Due to a waterlogging event in the second week of the ALpFN1 growth period and leaf miner infestation starting from the fourth week of DSpco7 growth, only two replicates were analysed for each of these events.

### 6.2.2 Sugar assay

#### 6.2.2.1 Extraction of soluble sugars

After four months, 4°C cold-stored tubers were brought to room temperature. These were peeled, cut into small pieces, and homogenised in liquid nitrogen using a mortar and pestle. A 300 mg portion of this homogenised sample was mixed with 1 mL of 80% ethanol in a 2-mL tube. The tube was vortexed before incubation at 70°C for an hour with continuous agitation on a heat block. The tube was then centrifuged at 10,000 *x g* for 2 min, and the supernatant was collected in a new 2-mL tube. The extraction was done separately for each of the three tubers per event, and the extracts were stored at -20°C.

#### 6.2.2.2 Sugar quantification assay

The hexose sugars and sucrose in tuber extracts were quantified using the enzymatic method (Jones et al., 1977, McKibbin et al., 2006). The assay was performed in a microplate; each 200-µL reaction contained 100 mM imidazole, 10 mM $MgCl_2$, 1.1 mM ATP, 0.5 mM $NADP^+$, 0.14 U glucose-6-phosphate dehydrogenase (Cat. No. G6378, Sigma-Alrich) and 20 µL of the tuber extract at room temperature. Initial absorbance at 340 nm was recorded as the absorbance baseline for each reaction. For glucose, 0.12 U hexokinase (Cat. No. H4502, Sigma-Alrich) was added to the reaction, followed by a 10-min incubation at room temperature, and absorbance was recorded at 340 nm. For fructose quantification, 1 U phosphoglucose isomerase (Cat. No. P5381, Sigma-Alrich) was added, the reaction was incubated for 10 min at room temperature, and the absorbance at 340 nm was recorded. Finally, 4 U invertase (Cat. No. I4504, Sigma-Alrich) was added for sucrose quantification, followed by the same incubation time and absorbance reading. Absorbance was measured using a Beckman Coulter DTX 880 Multimode Detector microplate reader with an A340 nm absorbance filter and 10 nm bandwidth. Three readings were recorded for each sample after each enzyme-addition step. Standard curves were generated for each sugar type using concentrations of 0 to 100 nmol. The sugar concentrations in each sample were determined by plotting the absorbance values against the standard curves.

### 6.2.3 Cold-stored tuber processing and assessment of potato crisp colour intensity

Cold-stored tubers were peeled, cut into 2-mm thick slices using a mandoline slicer and deep-fried in vegetable oil at 191°C for 60 s (Bhaskar et al., 2010). The oil temperature was monitored with a digital food thermometer. Fried crisps were cooled on paper towels and photographed to record their colour. The colour intensity of potato crisps was measured using the FIJI image processing software, with a value of 255 being the brightest (white) and the value 0 being the darkest (black) (Schindelin et al., 2012).

### 6.2.4 Acrylamide ELISA assay

#### 6.2.4.1 Acrylamide extraction

Acrylamide extraction was performed according to the manufacturer's instructions for the Acrylamide-ES ELISA kit (Eurofins). Three 15-mL tubes were prepared with labels for each sample: waste, #1 and #2. The SPE column was removed from the packaging, put in the waste tube, and conditioned with 2 mL of 100% methanol. The tube was centrifuged at 500 rpm, for at least 4 min, to ensure all liquid passed through, and the flow-through was discarded. The column was rinsed twice with 2 mL of Milli-Q water, each with the same centrifugation step. Flow-through was discarded after each rinse, and the conditioned SPE column was moved to tube #1. The ENV + SPE column was conditioned using the same method as the SPE column and was left inserted in the waste tube after discarding the flow-through from the final rinse.

Fried crisps from each sample were homogenised in liquid nitrogen using a mortar and pestle, and 2 g of the powdered sample was put in a 50 mL extraction tube (provided with the kit) with 40 mL of Milli-Q water. The tube was vortexed and then kept on an orbital shaker at 250 rpm for 30 min at room temperature. After that, the tube was vortexed briefly, and the sample was then allowed to sediment for 5 min. The 0.45 µm filter provided with the extraction kit was pushed into the extraction tube using the filter plunger. Each filtered sample was transferred to four separate 2 mL tubes (1.5 mL filtrate in each), and these were centrifuged at 14,000 *x g* for 5 min at room temperature. A 250 µL aliquot of liquid supernatant from each tube was collected avoiding the top fat layer and the solids at the bottom. A total of 2 mL of liquid supernatant was transferred to the conditioned SPE column in tube #1. The tube was then centrifuged at 500 rpm for 4 min or until all liquid passed through the column. The SPE column was rinsed with 1 mL of Milli-Q water followed by centrifugation at the same speed, and the eluate was collected in the same tube #1, making the total eluate volume 3 mL. One mL of this eluted sample was passed through the conditioned ENV + SPE column in the waste tube by centrifugation at 500 rpm for 4 min. The remaining 2 mL of eluted

sample was passed through the conditioned ENV + SPE column with the same centrifugation, and the content in the waste tube was discarded. The column was rinsed twice with 1 mL of Milli-Q water each time, and the liquid in the waste tube was discarded. The column was dried by centrifugation at 2,400 rpm for 10 min, and then it was inserted into tube #2. The acrylamide extract bound to the column was eluted with 2 mL of 60% methanol with two elutions (1 mL each) and centrifugation at 500 rpm for 4 min. The eluate (2 mL in total) was transferred to a clean 4-mL brown glass vial (provided with the kit) and stored at 4°C for further analysis.

#### 6.2.4.2 Derivatisation of acrylamide

The acrylamide extract, acrylamide standards and the control were derivatised according to the manufacturer's protocol (Eurofins Scientific). Briefly, 250 µL of sample/standard/control was transferred to a clean 4-mL brown glass vial (provided with the kit), and 50 µL of derivatisation reagent was added. The vial was vortexed vigorously for 15 s and incubated at 50°C for an hour. The sample was cooled to room temperature before adding 2 mL of acrylamide assay buffer.

#### 6.2.4.3 ELISA assay

An aliquot of 50 µL of the derivatised sample/standard/control was added to a secondary goat anti-rabbit antibody-coated well of the microtiter plate (Figure 6-1). A 50 µL of acrylamide-HRP enzyme conjugate were added to each well, followed by 50 µL of primary rabbit anti-acrylamide antibody solution. The content in each well was mixed individually by pipetting, and the plate was sealed and incubated for an hour at 4°C. After incubation, the solution was decanted, and the plate was inverted on a paper towel to remove all liquid. Each well was washed three times with 250 µL of cold wash buffer each, and the plate was inverted on a paper towel after each wash to ensure the removal of all liquid. To each well, 150 µL of substrate solution was added and mixed well by pipetting, and then the plate was incubated in the dark for 30 min. After 30 mins, 100 µL of stop solution was added to each well, and the solution was mixed thoroughly. Absorbance at 450 nm was

read within 15 minutes of adding the stop solution using a Beckman Coulter DTX 880 Multimode Detector microplate reader.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Std 0 | Std 4 | Atlantic | ALpFN2 | DSpco4 | DSpco8 | DSpco12 | | | | | |
| B | Std 0 | Std 4 | Atlantic | Desiree | DSpco4 | DSpco8 | DSpFN4 | | | | | |
| C | Std 1 | Std 5 | Atlantic | Desiree | DSpco5 | DSpco8 | DSpFN4 | | | | | |
| D | Std 1 | Std 5 | ALpFN1 | Desiree | DSpco5 | DSpco9 | DSpFN4 | | | | | |
| E | Std 2 | Std 6 | ALpFN1 | DSpco3 | DSpco5 | DSpco9 | | | | | | |
| F | Std 2 | Std 6 | ALpFN1 | DSpco3 | DSpco7 | DSpco9 | | | | | | |
| G | Std 3 | Control | ALpFN2 | DSpco3 | DSpco7 | DSpco12 | | | | | | |
| H | Std 3 | Control | ALpFN2 | DSpco4 | DSpco7 | DSpco12 | | | | | | |

**Figure 6-1. Layout of the microtiter plate for the ELISA assay.** Std 0 to Std6: standard 0 to standard 6. Control: acrylamide control. Extracted acrylamide samples were analysed in triplicate.

### 6.2.5 Statistical analysis

Raw data from sugar assay was processed using Microsoft Excel software: the initial (baseline) absorbance value was subtracted from the measured absorbance value for each sugar type (glucose, fructose, or sucrose), a standard curve for each sugar type was generated and used to calculate the corresponding sugar concentration in the extracted sample. The acrylamide ELISA assay results were analysed using the four-parameter logistic (4PL) method on MyAssays (https://www.myassays.com/four-parameter-logistic-curve.assay) with the dilution factor of the sample as 20. Welch Two Sample t-test (at 95% confidence interval) was used to determine differences in tuber size, crisp colour intensity, sugar content and acrylamide content between wildtype and gene-edited events. Statistical analysis was performed using R ver.3.6.3 (R Core Team, 2020), and statistical significance was assessed at $p \leq 0.05$ level. Column charts were generated using the R package "ggplot2" (Wickham, 2009), with error bars indicating standard deviation.

## 6.3 Results

### 6.3.1 Total weight and size of harvested tubers from wildtype and gene-edited plants

Tuber formation was evident one month after transferring the plants to the glasshouse. Atlantic tuber sizes ranged from 1.5 cm to 5 cm, and Desiree tuber sizes were from 0.7 cm to 6 cm in length (Figure 6-2 A and C). Among two events derived from Atlantic, the size of tubers from the ALpFN2 event was comparable with those of wildtype ($p \leq 0.05$), while tubers from the ALpFN1 event appeared to be significantly ($p \leq 0.05$) smaller (Figure 6-2 A). A reduction in total tuber weight per plant was also observed for ALpFN1, with an average tuber weight of 14.6 g per plant, lower than the 79.1 g per plant for wildtype (Figure 6-2 B). The tuber sizes of all Desiree-derived gene-edited events were similar to Desiree wildtype with no significant differences ($p \leq 0.05$) in either tuber length or width (Figure 6-2 C). Three Desiree-derived events: DSpco3, DSpco8, and DSpco12 produced approximately twice the total tuber weight per plant of wildtype while a low total tuber weight per plant (8.2 g) was recorded for DSpco7 (Figure 6-2 D).

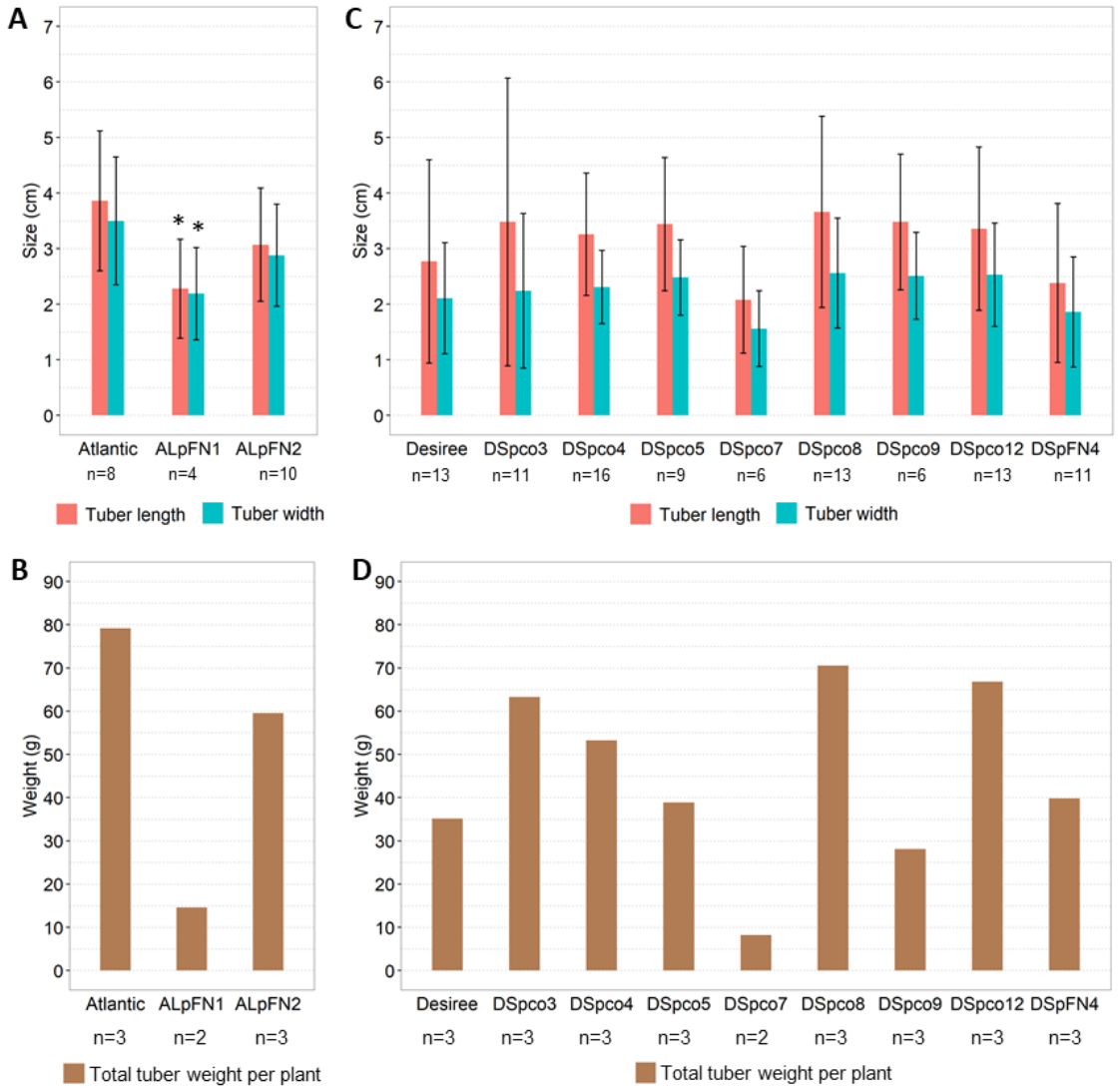



**Figure 6-2. Potato tuber size and total tuber weight per plant.** (A) and (B): Tuber size and total tuber weight per plant of Atlantic wildtype and gene-edited events. (C) and (D): Tuber size and total tuber weight per plant of Desiree wildtype and gene-edited events. Error bars indicate standard derivation, asterisk (*) indicates statistical significance at $p \leq 0.05$ relative to the corresponding wildtype, n represents the number of replicates.

### 6.3.2 Sugar content in cold-stored tubers

A significant reduction ($p \leq 0.05$) in glucose and fructose concentration was measured in two gene-edited events for Atlantic: ALpFN1 and ALpFN2 (Figure 6-3 A and B). The glucose content measured for tubers of AlpFN1 and ALpFN2 was significantly ($p \leq 0.05$) lower at 0.02 mg/gfw and 0.87 mg/gfw compared to 2.9 mg/gfw in wildtype tubers (Figure 6-3 A). Fructose was present at a higher concentration than glucose in ALpFN1 and ALpFN2, but its concentration was still significantly ($p \leq$

*0.05*) lower than that in wildtype tubers, with 0.13 mg/gfw in ALpFN1 and 1.66 mg/gfw in ALpFN2 compared to 2.79 mg/gfw in wildtype (Figure 6-3 B). The sucrose concentration in ALpFN2 was comparable with wildtype, while the sucrose concentration detected in ALpFN1 was significantly ($p \leq 0.05$) lower than wildtype, with an approximately 32.4% reduction (2.81 mg/gfw in ALpFN1 compared to 4.5 mg/gfw in Atlantic wildtype, Figure 6-3 C).

In contrast to Atlantic gene-edited events, the glucose concentration in six out of eight Desiree gene-edited events (DSpco3, DSpco4, DSpco5, DSpco7, DSpco8 and DSpco9) was not different from wildtype at $p \leq 0.05$ (Figure 6-3 D). A significant reduction ($p \leq 0.05$) in glucose concentration was only observed for DSpco12 and DSpFN4 events, for which the glucose content was 55.4% and 35.6% of that detected for the wildtype (1.6 mg/gfw in DSpo12 and 1.03 mg/gfw in DSpFN4 compared to 2.89 mg/gfw glucose for Desiree wildtype, Figure 6-3 D). Five out of eight Desiree gene-edited events had significantly ($p \leq 0.05$) lower fructose concentration than wildtype (DSpco4, DSpco8, DSpco9, DSpco12 and DSpFN4, Figure 6-3 E). The sucrose concentration in all Desiree gene-edited events was comparable to the wildtype at $p \leq 0.05$ (Figure 6-3 F).

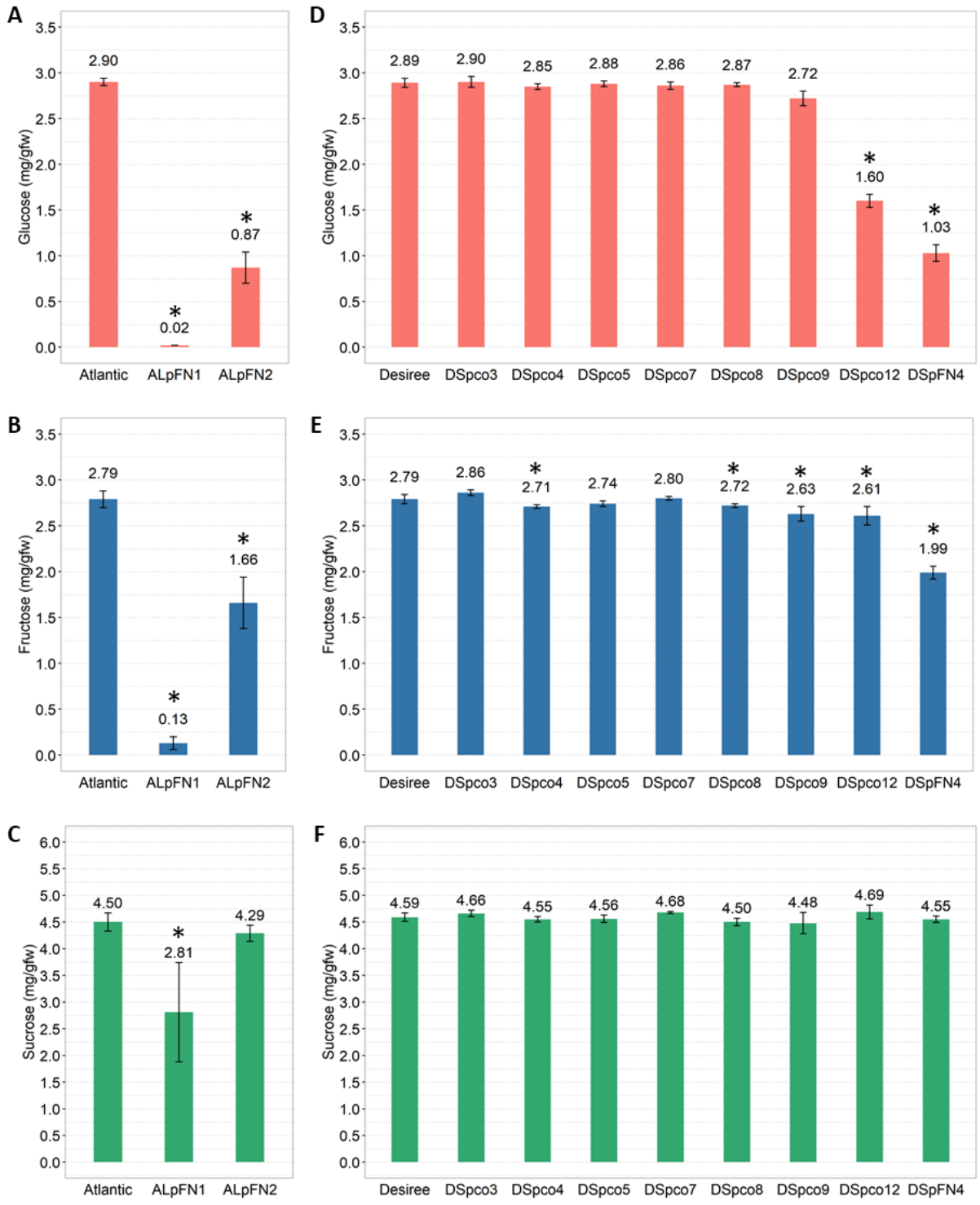



**Figure 6-3. Concentration of glucose, fructose, and sucrose in cold-stored potato tubers of wildtype and gene-edited events.** (A), (B) and (C): Concentration of glucose, fructose, and sucrose in cold-stored tubers of Atlantic wildtype and gene-edited events, respectively. (D), (E) and (F): Concentration of glucose, fructose, and sucrose in cold-stored tubers of Desiree wildtype and gene-edited events, respectively. Concentrations are expressed as milligram per gram fresh weight of tuber. Bars represent the average concentration for three replicate tubers, with three technical replicates measured from each tuber. Error bars represent standard deviation. Asterisks (*) indicate values that were significantly ($p \leq 0.05$) different from those of wildtype.

### 6.3.3 Colour intensity of fried potato crisps

The potato tuber slices were fried in two separate batches, and their colour intensity after frying was compared to the corresponding wildtype slices of the same batch. Browning of tuber slices started approximately after frying for 30 s. Tuber slices from the events ALpFN1 and ALpFN2 did not turn brown even after 60 s of frying, and the crisps had a distinctively lighter colour (Figure 6-4 A and B) with significantly ($p \leq 0.05$) higher (lighter) colour intensity than Atlantic wildtype (Figure 6-5 A and B). Crisps made from tubers of five out of eight Desiree gene-edited events were lighter in colour with significantly ($p \leq 0.05$) higher colour intensities than wildtype (Figure 6-4 C and D, Figure 6-5 C and D). Crisps made from tubers of the other three Desiree gene-edited events, DSpco3, DSpco5 and DSpco7, developed similar browning as the wildtype crisps ($p \leq 0.05$) (Figure 6-5 C and D).

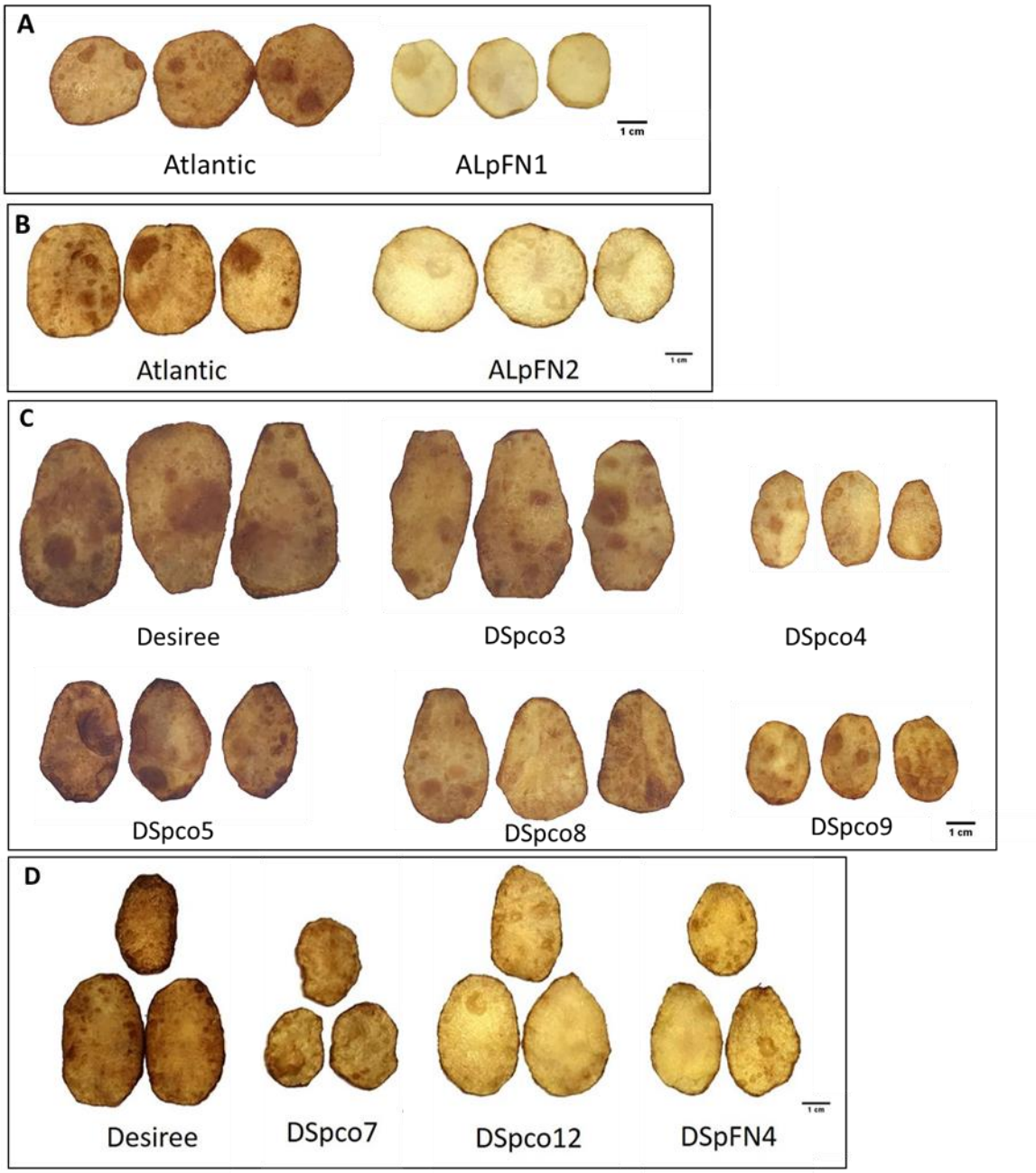



**Figure 6-4. Crisps made from potato tubers of wildtype and gene-edited events** (A) and (B): Crisps made from tubers of Atlantic wildtype and gene-edited events. (C) and (D): Crisps made from tubers of Desiree wildtype and gene-edited events. Tubers were stored at 4°C for four months, then peeled and sliced with a thickness of 2 mm before deep-frying at 191°C in vegetable oil for 60 seconds. Scale = 1 cm.

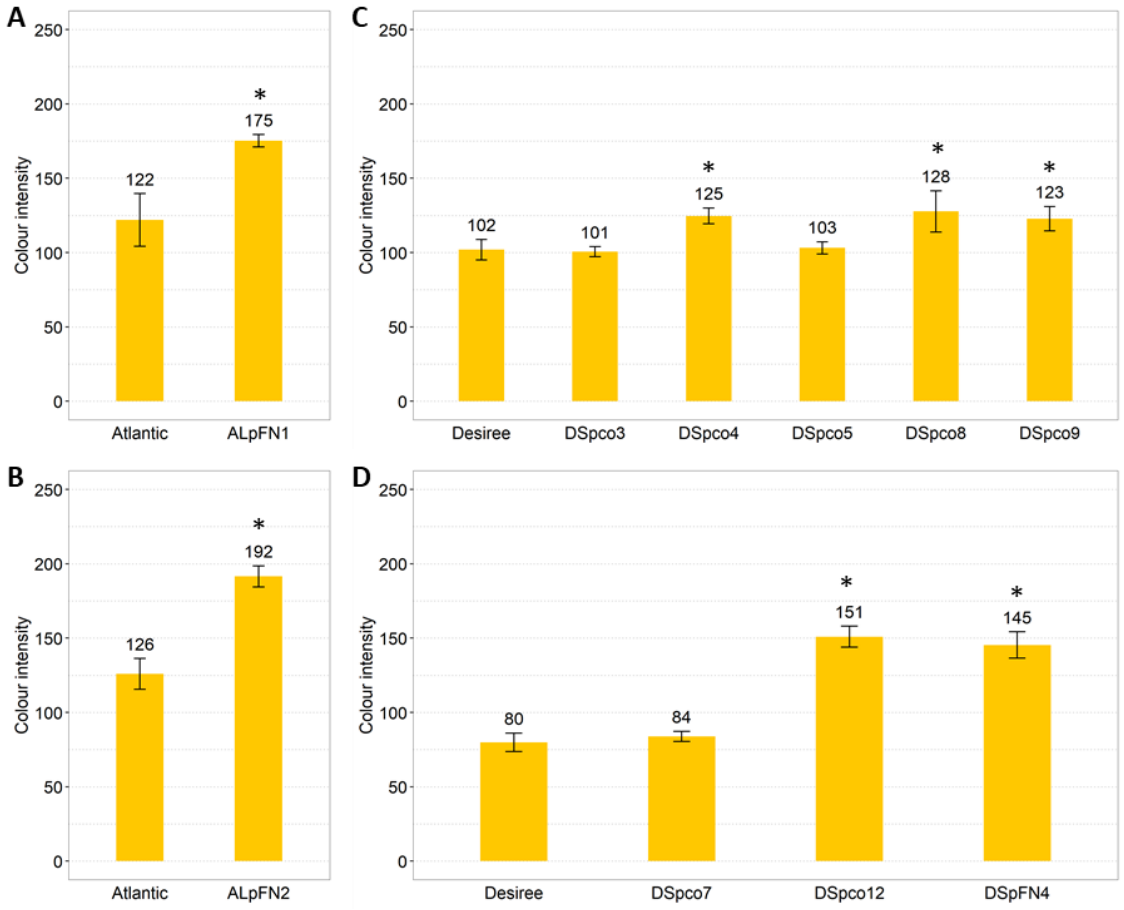



**Figure 6-5. Colour intensity of potato crisps.** Graphs (A) and (B): Colour intensity of crisps made from cold-stored tubers of Atlantic wildtype and gene-edited events. Graphs (C) and (D): Colour intensity of crisps made from cold-stored tubers of Desiree wildtype and gene-edited events. Tubers were stored at 4°C for four months before being deep-fried at 191°C for 60 s. Bars represent the mean value of colour intensity from six crisps. Error bars represent standard deviation. Asterisks (*) indicate significance at $p \leq 0.05$ relative to wildtype. Value of colour intensity ranges from 0 being the darkest (black) to 250 being the brightest (white).

### 6.3.4 Acrylamide content in fried crisps

The acrylamide in fried potato crisps was extracted and analysed using an ELISA kit (Eurofins Scientific). The acrylamide content in Atlantic crisps was approximately one-third of Desiree: 1,222 ng/g and 3,614 ng/g, respectively (Figure 6-6). The acrylamide content in crisps made from tubers of ALpFN1 and ALpFN2 was significantly ($p \leq 0.05$) lower than in crisps from wildtype potatoes, with a reduction of 72.8% for ALpFN1 and 70.1% for ALpFN2 (Figure 6-6 A). A significant ($p \leq 0.05$) reduction in acrylamide content was measured in crisps made from cold-stored tubers of six Desiree gene-edited events (DSpco3, DSpco7, DSpco8, DSpco9, DSpco12 and DSpFN4, Figure 6-6 B). Crisps

made from cold-stored tubers of DSpco7 and DSpco8 had 1,604 ng/g and 1,647 ng/g acrylamide, respectively, which was less than 50% of the acrylamide content detected in crisps made from cold-stored tubers of Desiree wildtype (Figure 6-6 B). The acrylamide content in crisps made from DSpco3, DSpco9 and DSpFN4 was 733.7 ng/g, 1,084 ng/g and 1,041 ng/g, respectively, and these values were less than 30% of the acrylamide measured for the wildtype crisps (Figure 6-6 B). Crisps made from cold-stored tubers of the event DSpco12 contained the least acrylamide among crisps from tubers of Desiree gene-edited events, with a reduction of 85.3% acrylamide compared to wildtype crisps (Figure 6-6 B). Interestingly, crisps from tubers of DSpco5 contained 21% more acrylamide than wildtype crisps (significant at $p \leq 0.05$) (Figure 6-6 B).

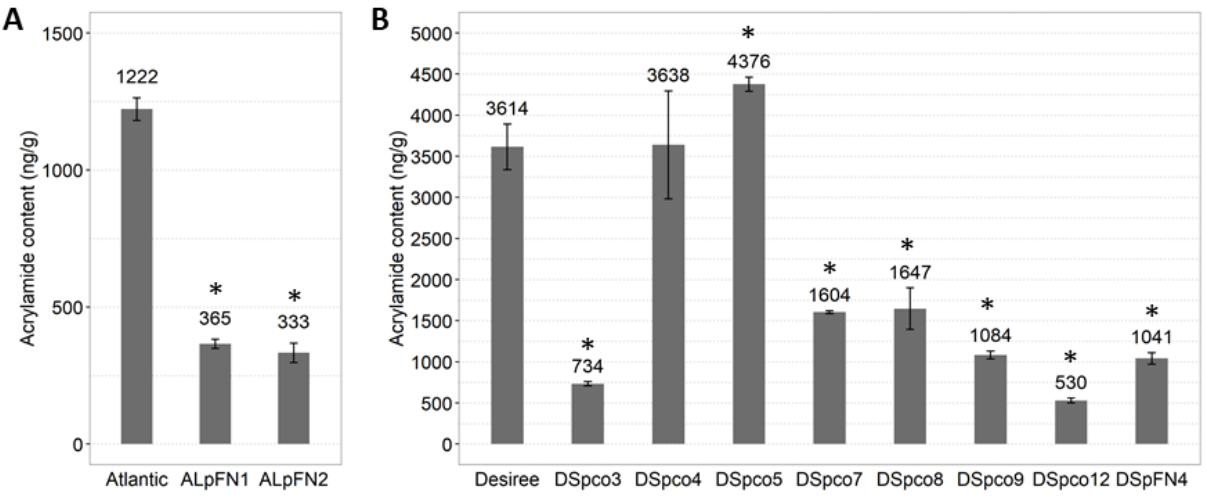



**Figure 6-6. Acrylamide content in potato crisps.** Graph (A): Acrylamide content in crisps made from cold-stored tubers of Atlantic wildtype and gene-edited events. Graph (B): Acrylamide content in crisps made from cold-stored tubers of Desiree wildtype and gene-edited events. Acrylamide content is expressed as ng per gram of crisps. Crisps were made from cold-stored tubers by deep-frying 2-mm thick slices in vegetable oil for 60 s. Bars represent average acrylamide content from three samples, with three technical replicates per sample. Error bars represent standard deviation. Asterisk (*) indicate significance at $p \leq 0.05$ relative to wildtype

## 6.4 Discussion

Potato plants of the wildtype and gene-edited events were grown in a PC2 glasshouse from *in vitro* culture to collect the tubers. Among all tested gene-edited events, DSpco7 and ALpFN1 plants had the lowest total tuber weight per plant, and the tubers from ALpFN1 were smaller than those of the wildtype. Editing of the *VInv* and *AS1* genes was not expected to affect either tuber weight or size

negatively since there was no yield reduction or abnormal tuber phenotype when *VInv* and *AS1* genes were knocked out in potato varieties Ranger Russet, Russet Burbank and Katahdin using RNAi (Rommens et al., 2008, Bhaskar et al., 2010, Chawla et al., 2012, Zhu et al., 2016). The low total tuber weight per plant and smaller tuber size in ALpFN1 and DSpco7 probably resulted from waterlogging and leaf miner infestation during their growth in the glasshouse. In addition, provenance may also affect the growth of *in vitro* plants when they are transferred to soil. Because of the limited number of gene-edited plants available for a field trial and time restrictions resulting from Covid-19 lockdowns, plants were grown in a glasshouse to harvest tubers. The size and yield of tubers of edited potato plants reported here cannot be taken as a reflection of any differences that might be present under standard growth conditions.

In relation to yield, it was reported that *AS1*-silenced RNAi potato lines exhibited a lower yield than wildtype when they were grown in the field from seed tubers (Chawla et al., 2012). The expression level of *AS1* in those RNAi lines was not presented, but the low yield could be caused by extreme silencing of the *AS1* gene, which interrupted the flow of nitrogen within the plant (Lam et al., 2003). This could be the case of the ALpFN1 and DSpco7 events which had high mutation frequencies for the *AS1* gene. Such results indicate that the expression of the *AS1* and asparagine levels in edited events needs to be assessed further to investigate any effects of different degrees of editing on tuber yields. Field trials with larger plant populations, undertaken over at least two years, would be needed to remove provenance effects and provide a proper assessment of the phenotype and yield of the gene-edited events developed here.

In most of the gene-edited events that were generated, the concentrations of glucose and fructose were significantly lower than that in the wildtype ($p \leq 0.05$). However, for three Desiree gene-edited events (Dspco3, DSpco5 and DSpco7), there was no significant difference in fructose concentration. Also, for six Desiree gene-edited events (DSpco3, Dspco4, DSpco5, Dspco7, DSpco8 and DSpco9), glucose concentrations were similar to that of the wildtype ($p \leq 0.05$), even though they all had mutated *VInv* genes. It is possible that not all target alleles had been edited in some cases, so

wildtype allele(s) were still present (DSpco3, DSpco4, DSpco9), and these genes could be transcribed and translated to generate functional VINV protein. The variation in mutation types and frequency is expected to result in a range of VINV activity, which would be reflected in differing levels of hexose sugars present in tubers. Similar observations were mentioned in studies by Clasen et al. (2016) and Tuncel et al. (2019), in which the correlation between mutation frequency and protein activity was studied. Additionally, mutated alleles that did not have a frameshift mutation or premature stop codon introduced in the transcript (in DSpco3 and DSpco7) are unlikely to have greatly modified VINV protein production and activity. Hence, the vacuolar invertase translated from edited *VInv* genes containing wildtype allele(s), or mutated alleles without a premature stop codon, would still have partially or fully functional protein, with enough activity to convert sucrose to hexose sugars.

Although glucose and fructose concentration in gene-edited events were reduced, sucrose concentration remained the same as that of wildtype ($p \leq 0.05$). It was expected that a reduction in glucose and fructose would be balanced by an increased sucrose accumulation, as less sucrose was hydrolysed (Ye et al., 2010, Bhaskar et al., 2010, Wu et al., 2011, Clasen et al., 2016). However, contradictory results were obtained in this study, in which the sucrose content in the gene-edited events did not increase. The reduction in VINV activity may not be sufficient to change the sucrose level substantially in some events, or sucrose may be redirected to other pathways. Sucrose in plant cells is not only degraded by VINV in the vacuole but also by cytosolic invertase in the cytosol to hexose sugars or by sucrose synthase (SuSy) to fructose and uridine diphosphate glucose (UDP-glucose) (Ferreira and Sonnewald, 2012, Stein and Granot, 2019). SuSy is the predominant pathway of breaking down sucrose in sink tissues, but when tubers mature, are detached from plant and stored, SuSy activity declines, and VINV activity becomes predominant (Ross and Davies, 1992, Ferreira and Sonnewald, 2012). It is plausible that in edited events with reduced activity of VINV, sucrose is not accumulated but is hydrolysed by other enzymes instead, resulting in a similar sucrose level as in the wildtype tubers.

As anticipated, the acrylamide content measured in crisps made from cold-stored tubers of gene-edited events was lower than those of wildtype tubers. The VINV and AS1 proteins are two key components for acrylamide formation in the Maillard reaction (Zhu et al., 2016). Among ten tested events, eight carried mutations in both *VInv* and *AS1* genes; two events, DSpco5 and DSpco12, had only the edited *VInv* gene. The cooked crisps from DSpco12 (with only the edited *VInv* gene) contained the least acrylamide compared to other Desiree gene-edited events with mutations in the *VInv* and *AS1* genes. This low acrylamide content could result from the lower hexose sugar production in this event, as acrylamide formation is thought to be controlled mainly by hexose sugar levels (Muttucumaru et al., 2014). Nevertheless, the importance of AS1 in acrylamide formation was noted in other events with no significant ($p \leq 0.05$) change in hexose sugar content, as there was a significantly ($p \leq 0.05$) lower acrylamide in these than in crisps from wildtype. For example, tubers of DSpco7 accumulated similar hexose sugar levels as the wildtype tubers, but crisps made from these tubers contained 56% less acrylamide. It appears that the edited *AS1* gene/product in this event was sufficient to reduce the subsequent acrylamide formation in potato crisps, despite the presence of normal level of hexose sugars in the tubers.

Overall, some of the CRISPR/Cas9-induced mutations generated in this study led to a reduction of hexose sugar accumulation in cold-stored tubers and reduced acrylamide content in fried potato crisps. Silencing *VInv* alone can reduce acrylamide production but editing both the *VInv* and *AS1* genes reduced the acrylamide content in fried potato products more effectively.

# Chapter 7

# General discussion

## 7.1 Overview

Potato is the most important tuber crop and plays a crucial role in world food security. A major use of potatoes is their processing into crisps and French fries. Potatoes that accumulate hexose sugars and exhibit browning after frying are rejected by processors, causing economic losses and waste. There is also a health risk for consumers from acrylamide formation when potatoes are deep-fried because acrylamide is classified as probably carcinogenic to humans. Therefore, generating potato lines that accumulate less hexose sugar during cold storage and have low acrylamide forming potential can reduce food waste and are healthier for human consumption.

Conventional methods of potato breeding are time-consuming and require the screening of large populations of germplasm to find desired combinations of alleles. In addition, potato is highly heterozygous and a tetraploid, which further complicates the breeding process, which may take up to 20 years. By making use of new breeding technologies such as CRISPR/Cas9, the long breeding cycles can essentially be eliminated, and an existing well-performing potato variety can be improved for single or multiple traits without changing other qualities.

The aim of this project was to develop potato lines which are healthier for human consumption, with low acrylamide-forming potential and reduced cold-induced sweetening. The strategy was to apply the CRISPR/Cas9 gene-editing system to alter the *vacuolar invertase* (*VInv*) and *asparagine synthetase 1* (*AS1*) genes in potato. The aim was accomplished through experimental work, and the following objectives were achieved:

1. Optimisation of micropropagation and regeneration systems for potato (*Solanum tuberosum L.*) cultivars Atlantic and Desiree
2. Identification and characterisation of the target genes *VInv* and *AS1*
3. Identification of gRNAs targeting *VInv* and *AS1* genes and *in vitro* evaluation of gRNA efficiency
4. Delivery of the CRISPR/Cas9 system into plant cells via *Agrobacterium*-mediated transformation and regeneration of plants from those cells to obtain edited events

5. Direct delivery of the CRISPR/Cas9 into plants via protoplast transfection or particle bombardment for generating transgene-free gene-edited plants
6. Identification of edited plants and sequencing to determine the form of gene editing
7. Biochemical analysis of potato tubers to assess their acrylamide-forming potential and accumulation of hexose sugars after cold storage

In summary, *in vitro* regeneration systems to form shoots from leaf explants were optimised for potato cultivars Desiree and Atlantic; these resulted in shoot induction rates of 100% and 64.29%, respectively. Two target genes, *VInv* and *AS1,* were cloned and sequenced from both varieties to design target-specific gRNAs with no potential off-targets in the potato genome. Two gRNAs were designed to target two sites (g67 and g10) of the *VInv* genes, spaced 135 bp apart in the exon 3. Likewise, the *AS1* gene was also targeted simultaneously by two gRNAs at two sites on exon 1 (g4) and exon 2 (g7), spaced 1,162 bp apart. All gRNAs corresponding to the target sites successfully guided Cas9 to cleave target DNA at the expected sites in the *in vitro* Cas9 cleavage assays. Four transformation vectors expressing Cas9 protein with either dual-gRNAs or four gRNAs were constructed for *Agrobacterium*-mediated plant transformation. Twenty transgenic gene-edited events were obtained, and 17 of these had mutations in both target genes. The Cas9 protein and gRNAs were also assembled as ribonucleoprotein (RNP) complexes and were delivered directly into leaf tissue-derived calli via particle bombardment. After regenerating shoots from calli and screening for mutation, one non-transgenic edited event was obtained from a total of 343 shoots, yielding a 0.44% mutation frequency for the RNP-particle bombardment method. This event was edited at the g10 target site of the *VInv* gene.

The accumulated glucose and fructose levels in cold-stored tubers of the ten edited events analysed were as low as 0.02 mg/gfw and 0.13 mg/gfw in Atlantic-derived ALpFN1 event, respectively; compared to 2.9 mg/gfw glucose and 2.79 mg/gfw fructose in the Atlantic wildtype tubers. The

acrylamide content in crisps from tuber slices of Desiree-derived DSpco12 events was 85% lower than that in crisps of Desiree wildtype tubers.

## 7.2 The two-step regeneration system yielded a high shoot induction rate in potato cultivars Atlantic and Desiree

As developed in this study, 100% of Desiree leaf disc explants generated shoots when cultured on a two-step regeneration system consisting of a callus forming and shooting medium. Desiree has been used often as the variety of choice for optimising various protocols and experimental methods for potato transformation because it responds well in tissue culture (Wheeler et al., 1985, Butler et al., 2016, Butler et al., 2015, Zhou et al., 2017, Veillet et al., 2019b, Zhan et al., 2019). The regeneration efficiency of Atlantic explants (leaf discs) was relatively lower, with 64.3% producing shoots from the two-stage regeneration system. However, this rate was still better than other established regeneration systems for Atlantic. Three out of seven systems tested were developed explicitly for Atlantic, yet one was not reproducible; one resulted in 10.4% shoot regeneration, and the other resulted in a 32.5% shoot induction rate (T3, T6 and T7 treatments, respectively, see Chapter 2) (Peng et al., 2008, Han and Lee, 2015, Nadakuduti et al., 2019b). Except for the 10.4% shoot induction rate from the T6 treatment, which was similar to that reported, outcomes from other replicated treatments differed from reported results. When comparing the published regeneration systems for Atlantic, which used multiple media types, the regeneration system developed for Atlantic in this study was relatively simpler, with two media, for callus and shoot formation, and BAP and NAA as the only plant growth regulators (PGR) supplemented in the culture media (Han and Lee, 2015, Nadakuduti et al., 2019b).

Potato is a tuber crop that must be vegetatively propagated to maintain its genetic composition. The generation of gene-edited potato plants also depends on having an efficient *in vitro* regeneration system. The success of Cas-9 mediated gene editing depends on various factors such as the Cas9 activity, the efficiency of gRNAs *in vivo* and the plant's efficiency in repairing the DNA double-strand

breaks correctly (Peng et al., 2016, Campenhout et al., 2019). Therefore, an effective regeneration system with a high shoot induction rate increases the chances of obtaining gene-edited potato plants.

Microtubers, which can be induced *in vitro*, are known to have similar morphology to field-grown tubers, making them ideal for tuber analysis in the laboratory without glasshouse or field growth experiments (Barker, 1953, Estrada et al., 1986). Although microtubers were not used in the current study, they could be used in other studies which do not require large quantity of tubers, such as in protein production or gene expression studies. Furthermore, microtubers are also useful for germplasm conservation or seed tuber production in breeding programs as they can be induced in a shorter time than field-grown tubers (Donnelly et al., 2003).

## 7.3 High frequency of CRISPR/Cas9-induced mutations with the use of multiple gRNAs

Up to 100% of the regenerated potato plants obtained after *Agrobacterium*-mediated transformation in this study had CRISPR/Cas9-induced mutations. Transformations with *A. tumefaciens* harbouring vectors expressing Cas9 and four gRNAs resulted in 58% to 100% of regenerated plants carrying two edited target genes, while no mutation was obtained from transformation with vectors expressing Cas9 and dual-gRNA targeting one gene. These results indicate that increasing the number of gRNAs can improve gene-editing efficiency as the total number of gRNAs in the transformation vector affects mutation frequency (Xie et al., 2015, Kusano et al., 2018). With the incorporation of multiple gRNAs in a single vector, enhancing Cas9 expression with translation enhancers such as the TMV omega used in this study, can avoid the competition for Cas9 among gRNAs, which potentially reduces the mutation frequency (Xie et al., 2015, Kusano et al., 2018). In addition, integrating a translation enhancer in the Cas9 expressing sequence also increases multiplex gene-editing efficiency. A higher mutation frequency (100%) was obtained from

transformation with the pFN117-ASVI vector carrying TMV omega than transformation with pFGC-ASVI vector (58%). The improvement in gene-editing efficiency obtained with the TMV omega translation enhancer was similar to the use of dMac3 translation enhancer in potato (Kusano et al., 2018).

Although the efficiency of all four gRNAs was confirmed via *in vitro* Cas9 cleavage assays, this does not guarantee the performance of these gRNAs *in vivo*. The mutation frequency observed at four target sites varied greatly. For the *AS1* gene, no mutation was detected at g4, while g7 yielded a 90.6% of mutation frequency. For the *VInv* gene, the mutation frequency at g10 was 98.2%, which was much higher than the 17.2% mutation frequency obtained at g67. Guide RNAs that yielded low CRISPR-induced mutation frequency despite having criteria-satisfied sequences and high *in vitro* performance have been reported previously in Arabidopsis, rice and Chinese cabbage (*Brassica rapa* spp. *pekinensis*) (Feng et al., 2013, Jeong et al., 2019, Zhang et al., 2016b). The *in vitro* gRNA efficiency is thought to be affected by certain sequence features of the gRNA itself and epigenetic factors (Doench et al., 2014, Xu et al., 2015, Liang et al., 2016, Zhang et al., 2016b). The results that the nucleotide composition of gRNAs can affect gene-editing efficiency are inconsistent, as discussed in Chapter 4, while epigenetic factors are not fully understood at present. Thus, to ensure efficient editing of a given gene, it is recommended that multiple gRNAs are selected for the CRISPR system, since using this strategy results in increasing the chances of generating mutations.

## 7.4 Mutations in target genes affected the activity of translated proteins

The *VInv* and *AS1* genes were targeted to reduce the accumulation of hexose sugars during cold storage and acrylamide in tuber products after processing at high heat. VINV hydrolyses sucrose into glucose and fructose in the vacuoles of tuber cells, and its activity is increased by low temperatures such as 4°C during cold storage (Malone et al., 2006). The AS1 enzyme converts aspartate into asparagine, a free amino acid that reacts with hexose sugars in the Maillard reaction

at high temperature (120°C or higher) to form acrylamide (Mottram et al., 2002, Rommens et al., 2008).

Detected mutation types in gene-edited events included deletions from 1 bp to 35 bp, 1-bp insertion, 1-bp substitutions, and a combination of different mutation types in one allele. All CRISPR/Cas9 induced mutations caused frameshift resulting in changes in amino acid sequences, and most of them generated premature stop codons in their predicted protein sequences. Because the gRNAs were designed to target exons near the 5'-end of the selected genes, changes in protein sequence at the start meant that the following protein sequences were also altered. The wildtype VINV protein sequence encodes 639 amino acids, and premature stop codons were introduced in edited events as early as the 174$^{th}$ position of the predicted protein sequence of the mutated *VInv* gene. Likewise, the wildtype AS1 protein encodes 590 amino acids, and mutated *AS1* alleles were predicted to have premature stop codons within positions 34 to 51 of the translated amino acid sequence. Those premature stop codons suggest that proteins translated from mutated genes are likely to be truncated and may have partially or completely lost their function (Tuncel et al., 2019, Nishitani et al., 2016). In particular, VINV and AS1 activities were expected to be reduced, leading to a reduction in sucrose being processed into hexose sugars, and less asparagine available for acrylamide formation from the Maillard reaction.

As expected in edited events with premature stop codons in VINV and AS1 protein sequences, reductions in the content of hexose sugars and acrylamide were found in tubers and fried crisps. For example, VINV and AS1 protein translation in ALpFN1 events was expected to stop at amino acid positions 174 and 51, respectively. In cold-stored tubers of this event, hexose sugars were 0.02 mg/gfw glucose and 0.13 mg/gfw fructose, a remarkable 145 times lower than the 2.9 mg/gfw glucose and 21.5 times lower than 2.79 mg/gfw fructose present in cold-stored tubers of wildtype event. The acrylamide content in crisps from cold-stored tubers of the same edited events was one-third of the 1222 ng/g acrylamide present in crisps from wildtype cold-stored tubers. The reduction

in acrylamide content indicates that the function of translated proteins from mutated *VInv* and *AS1* genes was either partially or completely lost.

However, not all edited events exhibited changes in sugar levels. For example, the gene-edited event DSpco5 contained mutations in all its sequenced clones of the *VInv* gene with interrupted protein translation at the amino acid position 175. Tubers from this event accumulated a comparable amount of hexose sugars as did wildtype tubers after cold storage. Thus, the VINV protein in this edited event must have remained functional, although a premature stop codon was introduced in its mRNA transcript. In principle, mRNA transcripts that contain premature stop codons are eliminated by the endogenous nonsense-mediated mRNA decay (NMD) mechanism (Popp and Maquat, 2016). However, the position of the premature stop codon determines the susceptibility of an mRNA transcript to NMD. The premature stop codon in the predicted VINV protein sequence of the DSpco5 event did not fall within 50 nt from the exon-exon junction, making them susceptible to the NMD (Popp and Maquat, 2016). On the other hand, translation can still be initiated from an in-frame ATG start codon (Makino et al., 2016, Harries et al., 2005). This translation re-initiation mechanism is active in plants as a regulator of gene expression, as has been observed in human cells, in which proteins were produced from genes containing CRISPR-induced mutations (Makino et al., 2016, Merchante et al., 2017). It is plausible that the unexpected protein production from an edited gene in events such as DSpco5 was caused by translation re-initiation or from a wildtype allele that was not picked up in clone sequencing.

## 7.5 RNP-particle bombardment is a promising approach to generate transgene-free gene-edited plants

Introducing the CRISPR/Cas9 system into plant cells as an RNP complex avoids the integration of foreign DNA in the plant genome. Two commonly used delivery methods, protoplast transfection and particle bombardment, were tested in chapter 5 of this thesis. Although PEG-mediated protoplast transfection with RNP in potatoes has been reported to produce up to 68% mutation

frequency, optimisation is required for efficient protoplast transfection and regeneration (Andersson et al., 2018, González et al., 2020). The quality of protoplasts isolated in this work was not ideal, and this contributed to poor results for transfection. Even with high-quality protoplasts, shoot regeneration from potato protoplasts and calli may take up to three months or more, and regenerated shoots often exhibit high levels of tissue culture-induced somaclonal variation, which is manifested by undesirable chromosomal changes (Jones et al., 1983, Fossi et al., 2019).

In this study, it was found that delivery of RNPs *via* particle bombardment was more achievable than by protoplast transfection. RNP-coated gold particles were bombarded into leaf derived calli, and shoots were induced from the third week post bombardment. The mutation frequency obtained from the RNP-particle bombardment of potatoes in this study was 0.44%, comparable with 0.56% reported in wheat (Liang et al., 2017). RNP-particle bombardment is a relatively new strategy for gene-editing of potatoes. This approach was only applied in a functional study on the role of the nuclear protein coilin in potato in virus resistance in which CRISPR/Cas9 was used to knock down the related gene and no mutation frequency was mentioned (Makhotenko et al., 2019). It is noteworthy that during the regeneration and shoot induction stages of bombarded explants, the progress of this project was affected by two months of lockdown and exclusion from the laboratories due to the Covid-19 pandemic. Approximately a quarter of the regenerated shoots forming in culture were lost from contamination and overgrowth before it was possible to subject them to mutation screening. Hence, the number of edited events would probably have been greater, and more than the one edited event obtained from screening the remaining 343 available shoots after lockdown. The edited event generated from the RNP bombardment approach contained a 1-bp deletion at the g10 target site of the *VInv* gene, which introduced a premature stop codon in its predicted protein sequence, suggesting an interruption in protein function.

The effectiveness of CRISPR/Cas9 with four gRNAs was clearly demonstrated in the edited plants regenerated after *Agrobacterium*-mediated transformation. RNP-particle bombardment with those

gRNAs can be followed up to generate more non-transgenic edited events. As found in edited events generated from *Agrobacterium*-mediated plant transformation after selection, the properties targeted were significantly modified. Hexose sugar accumulation in tubers and acrylamide formation potential in crisps were significantly reduced ($p \leq 0.05$) after cold storage. Therefore, apart from the need to screen more shoots regenerated after RNP-particle bombardment, it can be expected that SDN-1 non-GM plants can be generated, which will exhibit similar characteristics to those found in GM edited plants. This is highly significant, since this approach holds great potential for commercial development of edited plants, as they are not regulated as GMOs in increasingly more countries.

## 7.6 Conclusion and future recommendations

This study successfully demonstrated that CRISPR/Cas9 gene editing could be used to improve important traits in widely grown potato cultivars such as Atlantic and Desiree, without loss of the variety. There is increasing interest by consumers in food, and they are becoming more health-conscious. Tubers of the generated gene-edited events accumulated less hexose sugars during cold storage, and fried potato crisps produced lower acrylamide with less browning ($p \leq 0.05$). The first trait is important to potato processors since it can reduce losses, whereas a reduction in acrylamide levels is an excellent health trait. Further experiments on expression of edited gene and protein production/ protein concentration are recommended as they will provide better insight on the effect of editing on plant phenotype.

The *Agrobacterium*-mediated plant transformation method yielded more CRISPR/Cas9-induced mutations, and these demonstrated the further potential of gene-editing in potato. The RNP-particle bombardment approach to generate transgene-free edited events demonstrates the potential for commercialisation of potatoes developed using this strategy. RNP complexes can be assembled using designed gRNAs to obtain similar improved properties reported for the transgenic

edited events. Although GM modified potato plants are being grown commercially in the USA, transgene-free edited potatoes are likely to be more acceptable to a broader range of consumers.

Other properties that can be modified by gene-editing include tubers with higher levels of vitamins and amino acids to improve their nutritional value. For example, targeting *methionine gamma-lyase* (*MGL*), which catalyses methionine degradation, can alter the methionine catabolic pathway and increase free methionine content in potato tubers (Kumar and Jander, 2017). Improved vitamin A levels in potato tuber can also be achieved using CRISPR/Cas9 to target *lycopene ε-cyclase* (*LCY-e*) to redirect lycopene to β-carotene (provitamin A) biosynthesis (Diretto et al., 2006). Designing new gRNAs targeting *MGL* and *LCY-e* genes in events developed from this project would generate even more elite potato lines. Such potato lines can be cold stored for an extended period without high acrylamide formation in processed products while providing extra vitamin A and methionine for human health. For ease of development, *Agrobacterium*-mediated plant transformation can be used for functional studies because of the higher mutation frequency. Results from such studies can be applied to RNP-particle bombardment to develop better potatoes for commercial growth.

An important aspect of the wider use of gene-edited potatoes and other crops is the need for harmonisation of international regulations, so that they do not constitute non-tariff barriers to international trade. With the exclusion of EU countries and New Zealand, SDN-1 edited crops are no longer regulated as GMOs in an increasing number of countries. These include the USA, Canada, most countries in South America, South Africa, Japan and Australia, with many other countries considering their policies and regulations to de-regulate SDN-1 gene-edited products (Menz et al., 2020, Gupta et al., 2021). As this trend continues, there is optimism that at least SDN-1 gene-edited produce will be used more fully to increase both the quantity and quality of food for future generations.

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