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Agrobacterium-mediated transformation protocol for diploid potato and targeting the *StPDS* gene using CRISPR-Cas9

MSc THESIS

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and the thesis is accepted for the defense process.

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Head of Department Supervisor

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bp	base pair
Cas9	CRISPR-associated protein 9
cDNA	Complementary DNA
CRISPR	Clustered Regularly Interspaced Short Palindromic Region
DMT	Double Monoploid Transformation
E. coli	*Escherichia coli*
ELF1a	Elongation Factor 1-alpha
GA3	Gibberellic acid
gRNA	guide RNA
HDR	Homology-Directed Repair
ID	Identification
MSB5	Murashige and Skoog Basal Medium with 5% Sucrose
NHEJ	Non-Homologous End Joining
ORF	Open Reading Frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGSC	Potato Genome Sequencing Consortium
pJET 1.2	Cloning vector
qPCR	Quantitative Polymerase Chain Reaction
*Sa*Cas9	*Staphylococcus aureus* Cas 9
sgRNA	Single-guide RNA
SNP	Single Nucleotide Polymorphism
SOC	Super Optimal broth with Catabolite repression
StPDS	*Solanum tuberosum Phytoene Desaturase* gene
Timentin	Ticarcillin and Clavulanic Acid
UTR	Untranslated Region

1. Introduction

Potato (*Solanum tuberosum* L.), a member of the Solanaceae family, is an essential tuber crop that plays a significant role in ensuring food security worldwide (Amir et al., 2018; Gonzales et al., 2020; Reyniers et al., 2020; Das et al., 2021). It is primarily grown for its source of energy, minerals, protein, fats, and vitamins (Naumann et al., 2020). Despite its importance, potato remains vulnerable to biotic and abiotic stresses, and potato breeders have been attempting to increase its resistance, accounting the era of climate change.

Cultivated potato crops suffer from a variety of pests and diseases caused by air-borne and soil-borne pathogens, including fungi, viruses, insects, and bacteria. These diseases range from less destructive to the most destructive, with respect to yield loss, quality decrease, and lethality to crops. The most destructive, intensively studied, and economically significant diseases affecting potato include bacterial wilt (*Ralstonia solanacearum*), late blight (*Phytophthora infestans*), early blight (*Alternaria solani*), and potato blackleg (*Pectobacterium atrosepticum*) (https://cipotato.org/crops/potato/potato-pests-diseases/).

The intensity and impact of diseases have directed efforts into identifying and understanding the genes responsible for susceptibility in potato to increase its resistance. In recent years, researchers have explored various plausible genetic targets using CRISPR-Cas9, particularly the polygenic traits to confer resistance against pathogens, improve economically significant traits, and to optimize regeneration protocols for challenging plant species.

Tetra-allelic knockouts of *StDND1*, *StDMR6*, *StCHL1* in *Solanum tuberosum* 'Désirée' are reported to have increased resistance to late blight (Kieu et al., 2021). Similarly, targeted mutants of *StALS1* resulted in herbicide tolerance in 'Désirée' and the diploid variety MSX914-10 (Butler et al., 2015). The knockout of the transcription factor *StMYB44* resulted in increased phosphate transport in potato paving a way to evade phosphate starvation and deficiency stress (Zhou et al., 2017). CRISPR-Cas indeed accelerates potato breeding to achieve set objectives as elaborated by Tiwari et al. (2022).

The advent of genetic engineering technologies and the availability of nuclear and organelle genome sequences of homozygous doubled-monoploid potato breeding lines have made it possible to manipulate specific genes, resulting in improved crop traits, increased yield, and minimal off-target effects. However, the ploidy level of potato continues to present a more challenging task due to the presence of multiple alleles, leading to reduced gene editing efficiency and a laborious process.

Domesticated potatoes are tetraploids (2n = 4x = 48). They are highly heterozygous, making it difficult to breed due to inbreeding depression and reduced fertility (Nadolska-Orczyk

et al., 2007; Leisner et al., 2018). On the other hand, genome editing for diploid potato (2n = 2x = 24) varieties is more convenient due to the presence of only two sets of chromosomes, as demonstrated in *Solanum chacoense* M6 (Huang et al., 2020) and *Solanum tuberosum* group Phureja (Butler et al., 2020). Hence, diploid potatoes provide a promising platform for genome editing studies, particularly for overcoming the challenges associated with tetraploid potatoes. They provide a simpler way to induce mutations precisely, maintain and trace introduced traits, and select desirable traits (Bethke et al., 2022*)*. They additionally offer the advantage to detect gene functions which can be interpreted for applications in tetraploid varieties.

Furthermore, studies focused on protocol optimization to improve the efficiency of CRISPR-Cas system in relation to potato breeding have increased, with the *StPDS* gene being commonly used as a model for genome editing in potato and other crops like tobacco, tomato (Pan et al., 2016) among others. This is due to its easy phenotype observation of depigmentation and dwarfism caused by PDS deficiency, which is responsible for catalysing phytoene into ζ-carotene and involved in carotenoid biosynthesis (Pan et al., 2016; Zhou et al., 2018; Bánfalvi et al., 2020).

Therefore, by using a diploid potato cultivar DG 82-330 targeting the *StPDS* gene as a model gene, this study aims to test the CRISPR-Cas9 editing and *Agrobacterium* transformation protocols with focus on parameters affecting transformation and regeneration.

Additionally, the study aims to investigate the potential effects of targeting the 5' untranslated region (UTR) of exon 1 of the *StPDS* gene, which contains the start codon and initiates the synthesis of the first few amino acids of the *St*PDS protein (Lakshmi et al., 2021). Disrupting this region can influence the translation of the entire *St*PDS protein. Thus, comprehending the impact of CRISPR-Cas9 on these regions is crucial to understand the possible consequences of targeting not only the translated regions but also the 5' UTR.

Overall, this study aimed to test CRISPR-Cas9 editing for the *StPDS* gene in diploid potato and investigate the potential impacts of targeting a specific region of the gene. The results of this study provide valuable insights for the development of precise and effective genome editing protocols for *S. tuberosum*. Notably, our study highlights the cruciality of considering the relationship between CRISPR-Cas9 and *Agrobacterium*-mediated transformation parameters, including inoculation time, gRNA design, plant growth regulator ratios, and explant type. The insights gained from this study will inform further research in the field and contribute to the advancement of tailored editing protocols for potato genetics

1.1. Objectives of the study

- ✓ To test *Agrobacterium*-mediated transformation protocols for the diploid DG 82-330 potato cultivar, targeting the *Solanum tuberosum PDS* gene.
- ✓ To investigate the potential effects of targeting the 5'UTR of exon 1 of the *StPDS* gene in potato with CRISPR-Cas9.

2. Literature review

2.1. Diploid cultivars

As there is a growing interest in the use of the CRISPR-Cas9 system in potato breeding, most studies have so far focused on tetraploid cultivars (Bethke et al., 2022). However, there is now increasing attention being given to the use of this system in diploid potato cultivars. A recent study by Yasmeen et al. (2023), has highlighted the differences in regenerative ability between diploid and tetraploid potatoes.

Diploid breeding has several advantages, including the ease of removing homozygous plants with deleterious recessive alleles from the breeding population (Jansky et al., 2016; Zhang et al., 2021). Additionally, the ability to produce uniform offspring is the basis of inbred hybrid breeding, and the entire breeding process is more efficient, leading to a more rapid rate of genetic gain (Bethke et al., 2022). Diploid breeding also makes it easier to select for desirable traits that are only expressed when two recessive alleles are present (Sanford et al., 1996; Ronning et al., 1999; Yencho et al., 2000). It additionally is advantageous that the ploidy level can be manipulated upon requirement (Carputo and Barone, 2005). However, the diploid potato presents a significant challenge due to its self-incompatibility. In order to address this issue, researchers such as Hosaka and Hanneman (1998a, b), Kao and McCubbin (1996) and Dzidzienyo et al. (2016) have conducted studies to gain a better understanding of the underlying mechanism and genes associated with self-incompatibility in potato. Several studies have been conducted to investigate the mechanism and genes related to self-compatibility in potato. Hosaka and Hanneman's study (1998a) on the *S*/ gene associated with self-compatibility is particularly noteworthy as it highlights the importance of incorporating genes that promote self-compatibility in developing parents for diploid potato breeding.

Early studies on potato transformation assays favoured cultivars such as ‘Désirée’ and ‘Bintje’ (De Block, 1988; Nahirñak et al., 2022). However, it has been demonstrated that transformation efficiency is dependent on genotypes, and that the effectiveness of obtaining transgenic plants can vary significantly among different potato genotypes (Conner et al., 1992; Dale and Hampson, 1995; Kumar et al., 1995; Heeres et al., 2002; Han et al., 2015; Bakhsh, 2020). Therefore, to achieve efficient and targeted gene editing in all potato genotypes, it is important to optimize transformation protocols according to the specific genotype being studied, as determined by Heeres et al. (2002). To achieve this several approaches including various delivery methods and transformation protocols have been analyzed.

2.2. Gene Transfer Methods in Potato

Identifying an efficient gene editing system functional in diploid varieties necessitates the identification of suitable delivery method. Several methods are available for delivering CRISPR-Cas9 constructs in potato. These include *Agrobacterium*-mediated transformation, direct transfer of DNA, and Polyethylene glycol (PEG)–mediated protoplast transformation (Halterman et al., 2016; Qi et al., 2019). *Agrobacterium*-mediated transformation is widely used for genetically modifying dicotyledonous plants such as tobacco (Slavov et al., 2005), torenia (Li et al., 2007), tomato (Sui et al., 2007) and potato (Sandhya et al., 2020). It was first reported by Chilton and her colleagues in the early 1980s (Chilton et al., 1983) and subsequently by Ooms et al. (1986).

When used as a vector with CRISPR-Cas system in potato breeding, *Agrobacterium*-mediated transformation is deployed with dicot-origin promoters such as Arabidopsis (*At*U6p) or potato (*St*U6p/U3p) and non-plant promoters such as CaMV 35S (Belhaj et al., 2013; Sandhya et al., 2020). The ‘Désirée’ and ‘Bintje’ potato cultivars are the most used cultivars for optimization of transformation protocols (Chakravarty and Wang, 2010). Other cultivars with economic significance in most parts of the world, such as 'Shepody', 'Kennebec', 'Russet Burbank', and 'B101', have been studied and evaluated for their ability to regenerate. This has helped to expand the knowledge base for the regeneration of both diploid and tetraploid cultivars (Vinterhalter, 2008; Han et al., 2015; Halterman et al., 2016; Nadakuduti et al., 2018; Bruce and Shoup Rupp, 2019; Wang et al, 2020; Kaur et al., 2020; Nahirñak et al., 2022).

However, the efficacy of transformation is highly dependent on several factors, including bacterial strain, media composition, pre-culture time, co-cultivation time, inoculation time, bacterial concentration, wound type, insert size, selective agent, vector, genotype, type of explant, and the number of sgRNAs (Yong et al., 2006; Kaur and Devi, 2019; Bakhsh, 2020).

Although great efforts have been made to optimize these parameters, potato transformation protocols remain highly genotype dependent (De Block, 1988; Conner et al., 1992; Dale and Hampson, 1995; Kumar et al., 1995; Heeres et al., 2002; Han et al., 2015; Bakhsh, 2020). Therefore, it is crucial to tailor the transformation protocol to the specific genotype being targeted to achieve maximum efficiency. *Agrobacterium*-mediated transformation is a widely used and there are effective methods for delivering CRISPR-Cas9 constructs in potato breeding, however, its success depends on a variety of factors that must be optimized for specific genotype.

2.3. Parameters Influencing Agrobacterium Transformation of Potato

Agrobacterium tumefaciens-mediated transformation in potato, first reported by An et al. (1986) using leaf and stem segments of ‘Russet Burbank’ (tetraploid) and ADX262-9 (diploid),

has increasingly been reported as the most efficient method to deliver a gene of interest in potato (Fatahillah et al., 2016; Farhanah et al., 2017; González et al., 2020).

As confirmed by several reports, transformation and shoot organogenesis is influenced by numerous factors. This includes explant type and age, basal media components, plant growth regulators, infection time, degree of injury, type of strain and the genotype among others (Melchers et al., 1989; Yong et al., 2006; Aggarwal et al., 2011; Kaur and Devi, 2019; Bakhsh, 2020).

The genotype in potato transformation is crucial (De Block, 1988; Heeres et al., 2002). Bakhsh (2020), too, affirmed the genotype dependency phenomena by using five cultivars of potato infected with *Agrobacterium tumefaciens* strain LBA4404. Potato genotypes are particularly known to have varying regeneration competence (Visser et al., 1989).

The degree of injury, with leaf explants, has been reported to be vital (Trujillo et al., 2001; Banerjee et al., 2006; Kaur et al., 2020). Intense cuts, especially with small sized leaves can reduce transformation frequency and severe damage to the leaf (Newell et al., 1991; De Block, 1988).

Another critical parameter is the type of strain used. Several studies have used various strains in diploid or tetraploid potato genotypes. The LBA4404 remains the most used strain in potato transformation protocols, but alternative strains like GV2260, GV3101, C58C1 and others better than LBA4404 such as EHA105, have also been successfully applied in transformation of potato. One of the reported drawbacks with the strain EHA105 is its difficulty to be eliminated from cultures (Hood et al., 1986; Terakami et al., 2007; Aggarwal et al., 2011; Kaur et al., 2020). It requires high levels of cefotaxime of approximately about 200 mg/l to 400 mg/l (Maheswaran et al., 1992; Terakami et al., 2007; Kaur et al., 2020).

Other than leaf explants, stem segments and tuber discs are commonly used (Beaujean et al, 1998). The transformation efficiencies varied among explants, where some reports reported leaves to have higher organogenesis and efficiency, while others have reported stem segments or tuber discs to be better for transformation. (Banerjee et al., 2006; Vinterhalter et al., 2008; Wang et al., 2020).

Additional principal factors affecting transformation efficiency in potato include the optical density of the bacterial suspension, centrifugation step, the length of the co-cultivation and selectable marker (Bruce et al., 2019). In potato, the commonly used marker gene is *neo* or *nptII* which codes for a neomycin phosphotransferase. It inactivates aminoglycoside type antibiotics, including kanamycin and neomycin (Yenofsky et al., 1990) by catalyzing the transfer of the terminal phosphate of ATP (Numata et al., 2016).

The optimal optical density values are between 0.5 and 0.8, depending on the construct and the growth medium. Co-cultivation periods should be between 24 and 96 hours, with 48 hours being the most reported (Wang et al., 2020).

Although several factors contribute to successful plant transformations, the genotype remains the highly cited factor to influence transformation (Makarova et al., 2011). There exists several protocols for potato transformation and regeneration but unfortunately, there does not exist a universal protocol that can be followed across all potato genotypes (De Block, 1988; Trujillo et al., 2001; Chakravarty et al., 2007; Han et al., 2015; Heeres et al., 2002) requiring customization of the protocol for the genotype before proceeding with genome editing projects.

2.4. CRISPR-Cas9 System

The CRISPR-Cas9 system provides acquired immunity against invading foreign DNA via RNA-guided DNA cleavage (Wiedenheft et al., 2012), and was first identified in the *Escherichia coli* genome by Ishino et al. (1987). This system is distinguished into two distinct classes based on the sequence, structure, and functions of the Cas proteins, and six types based on the conservation of genes and organization of the locus (Makarova et al., 2011). The widely used type II system from *Streptococcus pyogenes* Cas9 (*Sp*Cas9) targets DNA and belongs to class 2, using a single effector component effector protein, with RuvC and HNH nuclease domains (Perez et al., 2018; Moon et al., 2019).

The CRISPR-Cas9 system (**Figure 1**) is composed of two main components: the Cas9 endonuclease and a guide RNA (gRNA) (Wiedenheft et al., 2012). The gRNA, which is 17-20 base pairs long, is designed to target a specific sequence of DNA and directs the Cas9 endonuclease to generate double strand DNA break (DSBs), 3 to 4 bp upstream of the PAM (5′-NGG-3’) protospacer adjacent motif (Cao et al., 2020). The generated DNA breaks can be repaired using either the non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathway, and result to either insertions or deletions, or gene knockout through frameshift mutations, depending on the cell type and the experimental design (Johnson and Jasin, 2001; Holger, 2005; Symington and Gautier, 2011).

CRISPR-Cas9-mediated gene editing has revolutionized the field of molecular biology, emerging as a popular tool for genome modification (Doudna and Charpentier, 2014). Unlike traditional transgenic approaches, one major advantage of genome editing is that its product may not involve foreign DNA, making it a unique and powerful breeding tool with promising applications in agriculture (Townsend et al., 2009; Sprink et al., 2015; Kanchiswamy et al., 2015; Kim and Kim, 2016; Langner et al., 2018; Mohanta et al., 2017; Zhang et al., 2018a, b).

Compared to its predecessors, such as Zinc Finger Nucleases (ZFNs) (Gao et al., 2010; Osakabe et al., 2010; Zhang et al., 2010) and Transcription Activator-like Effector Nucleases (TALENs) (Cermak et al., 2011; Li et al., 2012) that rely on protein-guided recognition mechanisms (Amancio et al, 2017; Lino et al, 2018), CRISPR-Cas9 technology relies on a simplified RNA-guided system. It offers greater specificity, efficiency, and versatility in

targeting genomic sequences, allowing researchers to modify genes more precisely and efficiently (Cong et al., 2013; Mali et al., 2013).

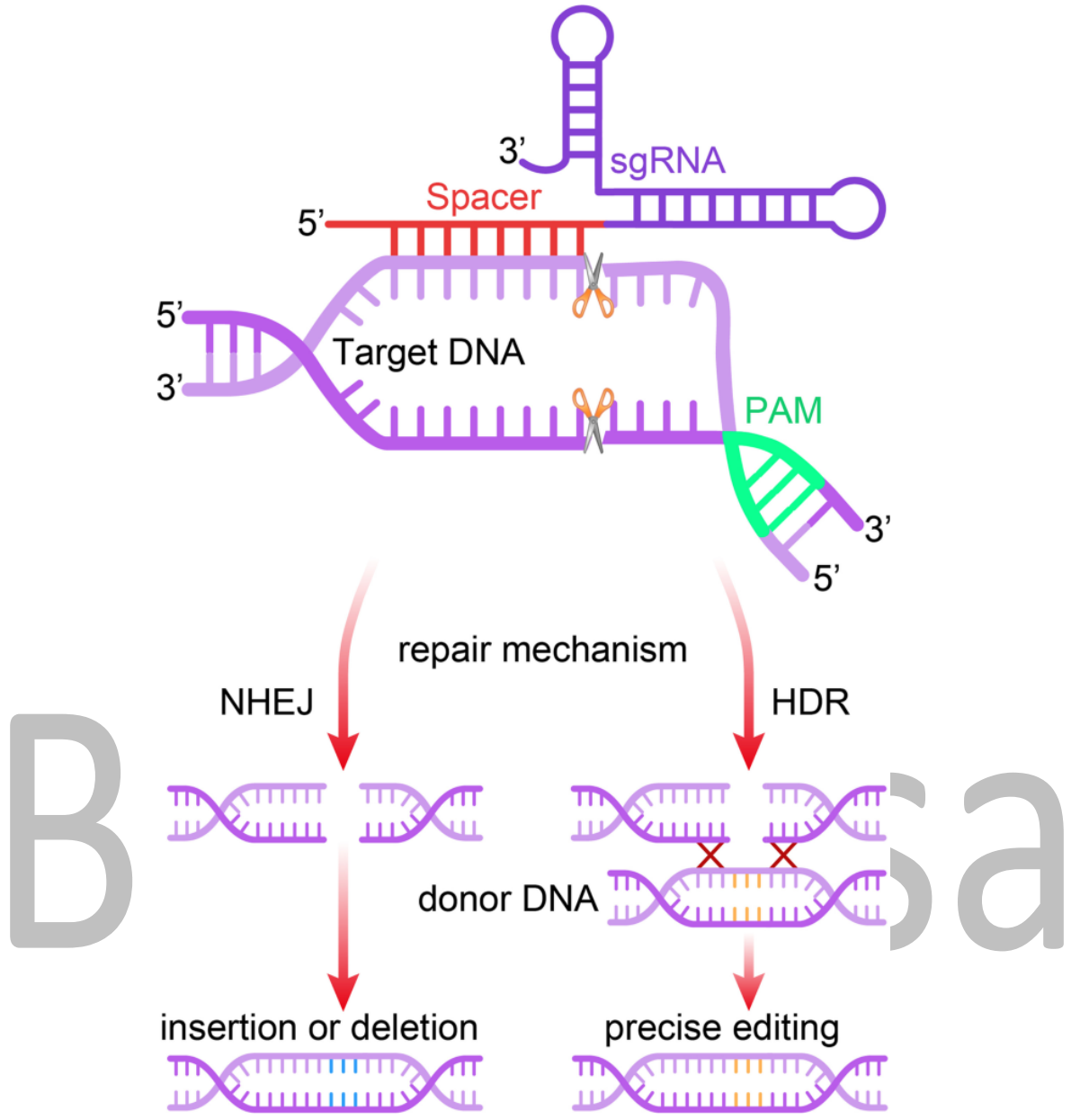


Figure 1: Illustration of CRISPR-Cas9 gene-editing mechanism using a single guide RNA (sgRNA) that directs Cas9 endonuclease to generate a double-strand DNA break (DSB) at a precise location 3 to 4 base pairs upstream of the 5'-NGG-3' PAM sequence, which can be repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR).

2.5. Application of CRISPR-Cas system in Potato

CRISPR-Cas9 has revolutionized gene editing in a variety of organisms, including plants. In 2013, Shan et al. utilized CRISPR-Cas9 to generate the first transgenic rice with albino and dwarf phenotypes by knocking out the *OsPDS-SP1* and *OsBADH2* genes. Since then, the technology has been applied to potato plants as well. Wang et al. (2015) tested the efficiency of CRISPR-Cas9 to generate knockouts targeting the *StIAA2* gene in a double haploid potato cultivar. They also demonstrated the native U6 promoter's ability to promote expression of the sgRNA and drive the Cas9 enzyme targeting the *N. benthamiana phytoene desaturase* (*NbPDS*) gene.

Moreover, CRISPR-Cas9 has been utilized for economically significant traits in potato plants, including improved tuber starch quality (Andersson et al., 2017, 2018; Kusano et al., 2018; Johansen et al., 2019; Tuncel et al., 2019; Veillet et al., 2019a; Sevestre et al., 2020; Zhao et al., 2021), tuber quality, and disease resistance to potato virus Y (PVY) and late blight caused by *Phytophthora infestans* (Dangol et al., 2019; Hameed et al., 2020; Hofvander et al., 2021). The technology has also been used to alter the carotenoid biosynthesis pathway in potato plants (Khromov et al., 2018; Bánfalvi et al., 2020; Butler et al., 2020) and to confer herbicide tolerance (Butler et al., 2015, 2016; Veillet et al., 2019b, 2020a,b,c).

Although, CRISPR-Cas9 technology has shown great potential for potato breeding, it still faces certain limitations and challenges. However, significant progress has been made, which include the development of new variants such as *Fn*Cas9, derived from the Gram-negative bacterium *Francisella novicida*, which was reported by Price et al. (2015), and Cas12a and Cas13 proteins (Hou et al., 2023), like the variant *Ls*Cas13a from *Leptotrichia shahii*, which was studied by Aman et al. (2018).

Additionally, base editors reported by Veillet et al. (2019), and *Staphylococcus aureus* (*Sa*Cas9), which recognizes a PAM of (5'-NNGRRT-3'), reported by Ran et al. (2015) and Veillet et al. (2020) are other examples of advancements made in CRISPR-Cas technology. These developments hold promise for overcoming some of the limitations of this technology and improving its application in potato breeding.

Furthermore, the development of efficient transformation techniques suitable for a wide range of cultivars, genotypes, and ploidy levels, notably in potato breeding, is still ongoing as a means of increasing CRISPR-Cas effectiveness and efficiency.

Table 1: Published reports on the application of CRISPR-Cas9 genome editing for *StPDS* in diploid or tetraploid cultivars (Tiwari et al., 2022**)**

Target gene	**Trait**	**CRISPR system**	**Delivery/transformation system**	**Genotype**	**Key findings**	**Reference**
StIAA2	Phenotype	Cas9	*Agrobacterium*	*S. tuberosum* Gp Phureja	Mono- and bi-allelic homozygous mutants (83%)	Wang et al. (2015)
PDS and *coilin*	Carotenoid biosynthesis	Cas9	*In vitro* study without delivery	'Chicago'	Stimulated activity *in vitro*	Khromov et al. (2018)
Phytoene desaturase (*StPDS*)	Carotenoid biosynthesis	Cas9	*Agrobacterium*	'Désirée'	Mutants (2–10%)	Bánfalvi et al. (2020)
StPDS	Carotenoid biosynthesis	Cas9	*Agrobacterium rhizogenes*	Diploid, self-compatible F_1 hybrid DMF1 (DM1-3 × M6)	Transgenic hairy roots mutants (64–98%)	Butler et al. (2020)
StDMR6-1 and *StGBSSI*	Phenotype	Cas9	*Agrobacterium*	'Désirée'	*Sp*Cas9-NG application in genome editing	Veillet et al. (2020a)
StPDS	Anthocyanin pigmentation in tubers	Cas9	Particle bombardment	'Russet Burbank'	Reduced anthocyanin pigmentation	Butler et al. (2020)
StPDS	Carotenoid biosynthesis	Cas9	*Agrobacterium*	XuanShu 2-Tetraploid	Knockout mutants with mutation frequency of 46.67% and clear albino phenotypes	Ma (2019)

PDS gene structure

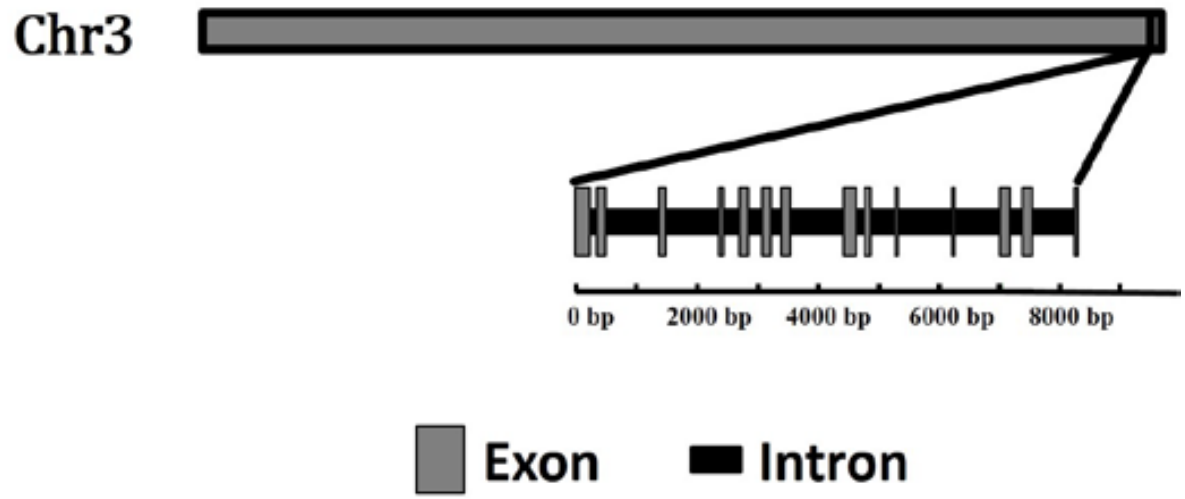


Figure 2: Chromosomal location and genomic structure of *StPDS* (Ping et al., 2018).

The CRISPR-Cas9 system has proven effective in targeting various genes in potato, including the *Phytoene Desaturase (PDS)* gene (**Table 1**). PDS is a crucial enzyme in carotenoid biosynthesis, and its deficiency leads to depigmentation and dwarfism in plants, making it a useful model system for evaluating genome editing methods in potato. PDS has been studied in different plant species, such as arabidopsis, tomato, citrus, strawberry, and potato, with the *StPDS* gene located on chromosome 3 (**Figure 2**), spanning from position 61,769,961 to 61,781,673. The gene consists of 14 exons, 13 introns, and an ORF region of 1,752 bp, encoding a 583-amino acid protein (Ping et al., 2018).

Exon 1 of the *StPDS* gene annotated as gene ID PGSC0003DMT400023665 (Potato Genome Sequencing Consortium, 2012) spans the 5' upstream sequence position 61,771,634 to 61,772,022 bp. The coding region of the *StPDS* gene is composed of eight exons with intronic regions in between. Exon 1 only consists of the 5'UTR, and Exons 9 to 13, 3' downstream of the *StPDS* gene sequence, also consist of an untranslated coding region. The structure of exon 1 and the entire gene provides valuable information for understanding the mechanisms of PDS gene regulation and developing efficient gene editing strategies in potato and other plant species (Fioretto et al., 2022).

3. Material and Methods

3.1. Plant material

The potato varieties 'Désirée' and DG 82-330 were grown *in vitro* at 23°C under long-day conditions (16 h light and 8 h dark) in controlled environment in solid Murashige and Skoog (MS) medium supplemented with vitamins (Duchefa), containing sucrose (20 g/L) at a pH of 5.6-5.8. Leaves and stems of four-week-old plants were used for tissue culture experiments.

3.2. Tissue culture of DG 82-330 potato

Two protocols (P2 and P3) were tested and compared. Both the stem segments and the leaves were pre-cultured for three days in the media with components as listed in **Table 2** with a pH of 5.6-5.8 at 23°C.

Table 2: Primary media for Protocol 2 and Protocol 3

Components	**Protocol 2**	**Protocol 3**
MS with vitamins	4.4 g/L	4.4 g/L
Sucrose	30 g/L	30 g/L
Gelrite	2 g/L	-
Agar	-	7 g/L
Hormones		
Indole acetic acid (IAA)	4 mg/L	-
Benzyl Amino Purine (BAP)	0.04 mg/L	-
2,4-dichlorophenoxyacetic acid (2,4-D)	-	4 mg/L
Trans-zeatin riboside (TZR)	-	0.8 mg/L

After preculture, the explants were transferred to the media from **Table 2** for P2 and media from **Table 3** for P3. After two weeks both P2 and P3 were put into media comprising components as listed in **Table 3** with a final pH of 5.6-5.8 along with appropriate selection and antibiotics. P2 was sub-cultured weekly and P3 in every 14 days. Observations were taken in intervals of 7 days.

Table 3: Media for induction of shoots for Protocol 2 and Protocol 3

Components	**Protocol 2**	**Protocol 3**
MS with vitamins	4.4 g/L	4.4 g/L
Sucrose	30 g/L	30 g/L
Gelrite	2 g/L	-
Agar	-	7 g/L
Hormones		
TZR	2.5 mg/L	4 mg/L
Gibberellic acid	0.2 mg/L	2 mg/L

Shoots were regenerated on the media as listed in **Table 4**.

Table 4: Media for rooting/maintaining of plant lines

Components	Amount
MS with vitamins	4.4 g/L
Sucrose	20 g/L
Agar	7 g/L
pH	5.6-5.8

3.3. *Agrobacterium*-mediated Transformation of DG 82-330 Potato

The *Agrobacterium tumefaciens* strain C58C1 harbouring pKSE-PDS construct was used for the transformation of *Solanum tuberosum* DG 82-330 potato using P2 and P3 for two experimental repeats. Stem segments and leaves from in vitro propagate plants were inoculated with the C58C1 *Agrobacterium tumefaciens* at an OD_{600}: 0.6-1.0.

In P2, explants were dipped in the *Agrobacterium* suspension for about 10 s per explant, while in P3, explants were inoculated for 3-10 min in groups. The infected explants were co-cultivated in refreshed media as listed in **Table 2**, for 3 days and 4 days for P2 and P3, respectively, in the dark at 23°C. After co-cultivation, the explants were transferred to media as described above (**Table 2**) but supplemented with 150 mg/L kanamycin. Kanamycin-resistant shoots were regenerated into plants on selective media (**Table 4**).

3.4. Detection of transformants

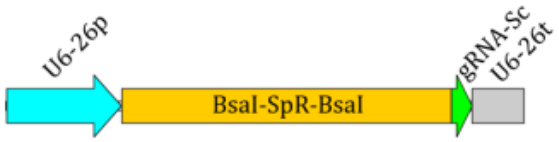
Genomic DNA of kanamycin-resistant regenerates and wild-type diploid potato plants were extracted using GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific) and used as a template for PCR amplification of the integrated transgene by using the primers U6-26p-F and the CRISPR-target lower strand (**Table 5**). The transformation efficiency was calculated according to the results of electrophoresis of the PCR products.

3.5. CRISPR-Cas9 editing of StPDS Gene

In this study, we aimed to test the protocol for manipulating the *StPDS* gene's 5' UTR region in exon 1 using the CRISPR-Cas9 system. This region possibly contains regulatory elements crucial for gene expression.

Vector Construction for Genome Editing

Sequence of U6-26p-driven gRNA expression cassette without target



(U6-26p)-(BsaI-SpR-BsaI)-(gRNA-Sc)-(U6-26t)

CGACTTGCCTTCCGCACAATACATCATTTCTTCTTAGCTTTTTTTCTTCTTCTTCGTTCATACAGTTTTTTTTTGTTTATCAGCTTAC
ATTTTCTTGAACCGTAGCTTTCGTTTTCTTCTTTTTAACTTTCCATTCGGAGTTTTTGTATCTTGTTTCATAGTTTGTCCCAGGATTA
GAATGATTAGGCATCGAACCTTCAAGAATTTGATTGAATAAAACATCTTCATTCTTAAGATATGAAGATAATCTTCAAAAGGCCCC
TGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATATGGGAAAGAACAATAGTATTTCTTATATAGGCCCATTTAAGTTGAAAAC
AATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGTTTATATACAGCTAGAGTCGAAGTAGTGATTGGGA
GACCAACCCAGTGGACATAAGCCTGTTCGGTTCGTAAGCTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAG
AACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGGGGTACAGTCTAT
GCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGATGTTACGCAGCAGG
GCAGTCGCCCTAAAACAAAGTTAAACATCATGGGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGC
GTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAG
TGATATTGATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTGGAAACTTC
GGCTTCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCC
AGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGA
TCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTC
CTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGCGAAAT
GTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAAT
GGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCT
CGCGCGCAGATCAGTTGGAAGAATTTGTCCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTAGCTAGA
AATTCGTTCAAGCCGACGCCGCTTCGCGGCGCGGCTTAACTCAAGCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAAC
TATCAGGTCAAGTCTGCTTTTATTATTTTTAAGCGTGCATAATAAGCCGGTCTCGGTTTTAGAGCTAGAAATAGCAAGTT
AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGCAAAATTTTCCAGATCGATTTCTTC
TTCCTCTGTTCTTCGGCGTTCAATTTCTGGGGTTTTCTCTTCGTTTTCTGTAACTGAAACCTAAAATTTGACCTAAAAAAAATCTCA
AATAATATGATTCAGTGGTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCCGATGAGATAAACCAATA

Notes:

1. SpR, spectinomycin-resistance gene; gRNA-Sc, gRNA scaffold.
2. Enlarged and boxed letters indicate *Bsa*I sites.

Figure 3: The gRNA expression cassette sequence for dicots without target. The U6-26 promoter is indicated in blue. The sgRNA scaffold is depicted in green, and the U6-26 terminator in grey.

The genomic DNA was extracted from 4-week-old 'Désirée' (a reference control) and DG 82-330 potato varieties using GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific). *StPDS* primers Forward and Reverse (**Table 4**) were used to amplify the gene using the genomic DNA of the two varieties as template at different temperatures using the Phire Plant Direct PCR Master Mix (Thermo Scientific). The obtained PCR products were run on gel to confirm the size of products and efficiency of primers.

PCR-products were then purified and vector-cloned using GeneJET PCR Cloning Kit (Thermo Scientific) and sequenced. The obtained sequences were evaluated to create sequence specific target for CRISPR-Cas editing.

The CRISPR target sgRNA sequence (5'-GTGGCCAAACCACCGAAAGCAGG-3'), was designed via the CRISPOR.org web tool. The upper and lower strands (**Table 5**) without PAM and comprising overhangs to generate sticky ends as described by Xing et al. (2014) were designed and ordered. Both the strands (final conc. of 100 µM) were then annealed at 65°C for 5 min followed by slow cooling to room temperature (25°C) in the PCR machine. It was then built into the pKSE401 binary vector (100 ng/µL) behind the AtU6-26 promoter before the gRNA scaffold using *Bsa*I enzyme as described by Xing et al. (2014) shown in **Figure 3**, in a total volume of 10 µL. The T-DNA also comprises the Cas9 gene driven by the CaMV 35S promoter. The construct also contained *neo*mycin *phosphotransferase* gene as a selectable marker and was transformed into competent *E. coli*, using the following steps.

Tube comprising competent *E. coli* cells of strain Topp10 was taken out from -80°C storage and placed on ice for 30 min. Ten µL of the constructed vector was pipetted into the melted *E. coli* and mixed. A heat shock of 42°C for 45 s was given, followed by immediate addition of 500 µL of SOC solution. The tubes were gently shaken for 1 h at 37°C and then spread onto solid LB medium in Petri dishes containing kanamycin.

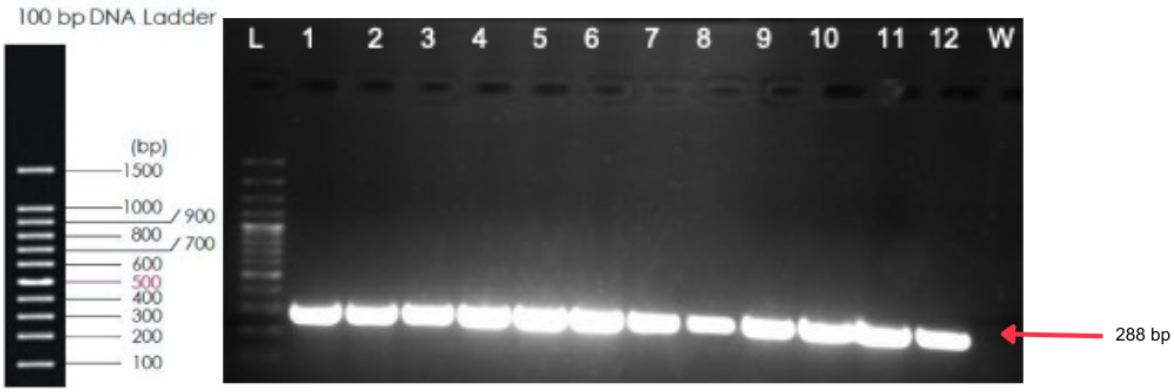


Figure 4: Colony PCR performed using U6-26p-F, showing approximately 288 bp.

The plates were grown overnight at 37°C. The obtained colonies were tested for the presence of the vector by PCR using primers U6-26p-F and the CRISPR-target lower strand (**Table 5**) as published by Xing et al. (2014) at 60°C.

The positive colonies were grown for plasmid extraction. The extracted plasmid was mobilized into C58C1 *Agrobacterium* strain using the method of Weigel and Glazebrook (2006). The presence of the target was checked via colony PCR as shown in **Figure 4**. The constructs were also sequenced using U6-26p-F prior to plant transformation as seen. The C58C1 *Agrobacterium* harbouring the pKSE-*StPDS* construct offering kanamycin resistance was used to transform DG 82-330 potato plants.

Table 5: Sequences of primers and targets used

Name	**Sequence**	**Annealing temperature (°C)**
U6-26p-F	5'-TGTCCCAGGATTAGAATGATTAGGC-3'	60
U6-26t-R	5'-CCCCAGAAATTGAACGCCGAAGAAC-3'	60
StPDS-F	5'-GTGGGACGTGTTAAATTTTGGTT-3'	61
StPDS-R	5'-TTTGTGGTGGTTTGGCAGTT-3'	61
pJET1.2-F	5'-CGACTCACTATAGGGAGAGCGGC-3'	?
StPDS upper	5'-ATTGGTGGCCAAACCACCGAAAGC-3'	?
StPDS lower	5'-AAACGCTTTCGGTGGTTTGGCCAC-3'	?
Ef1qPCR-F	AAACGGATATGCTCCAGTGC	60
Ef1qPCRR	GAACGCCTGTCAATCTTGGT	60

3.6. Mutation Detection and Transformation

To evaluate the efficacy of the CRISPR-Cas9 system in targeted gene editing, we amplified the target region of *StPDS* gene using *StPDS* primers Forward and Reverse from the extracted genomic DNA of the regenerates obtained through transformation under suitable kanamycin selection. The PCR products were then purified and subjected to enzymatic digestion set up as listed in **Table 6** at 55°C for 15 min and then run-on gel. In the wild type, a 95 bp product was expected to be digested off whereas accounting to CRISPR edition in the recognition site of the used enzyme the regenerants were expected to remain undigested as seen in **Figure 11C**.

Table 6: Digestion reaction

Components	**Amount**
PDS PCR product purified	500 ng
*Bsl*I restriction enzyme	0.5 µL
10x 3.2 Cutsmart Buffer	2 µL
Water	added up to
Total	20 µL

The amplified *StPDS* PCR products comprising the target region for edition from the candidate mutant plants and the wild-type control were subsequently cloned into the cloning vector pJET1.2 and transformed into *E. coli* Topp10 strain to obtain colonies with recombinant plasmids. The plasmids were extracted using the GeneJET Plasmid Miniprep Kit from the positive colonies after a confirmation of the presence of the plasmid with PCR product using primers U6-26p-F and U6-26t-R (**Table 5**).

The plasmids were sent for sequencing for analysis of CRISPR edition with pJET1.2 Forward primer (**Table 5**). To compare the sequencing results, the wild type (DG 82-330) sequence was used as control and the target sequence were aligned. The mutation was observed based on nucleotide differences, particularly 4 bp upstream of the PAM sequence (5′-AGG-3′).

3.7. RNA extraction and qPCR analysis

To assess the expression levels of the *StPDS* gene in nine edited DG 82-330 plants, we conducted quantitative polymerase chain reaction (qPCR) analysis. To ensure accurate normalization of the *StPDS* gene expression, we used the *Elongation Factor* (ELF1a) housekeeping gene as a reference. Total RNA was extracted and purified using the Direct-zol RNA Miniprep kit (Zymo Research) from 700 mg of frozen leaves and shoots tissue from 3-4-week-old plants, which were stored at -80°C. Regenerated plants were selected with P2 and P3 and whether they were stem segments or leaf regenerates.

Tissue homogenization was performed using a tissue homogenizer. The RNA concentration and purity were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm and 280 nm. The RNA was then transcribed into cDNA using the Revert-Aid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions.

The reactions were performed in triplicates in a 10 µL sample/well, in an Applied Biosystems Fast 7500 instrument. The synthesized cDNA was diluted 3x and used as a template for qPCR amplification. The *StPDS* Forward and Reverse primer was standardized for qPCR and the control (non-edited DG 82-330) leaf cDNA sample served as template at 60°C. The following temperature profile as listed in **Table 7** was set up. Reaction efficiencies were calculated based on the standard curve method. Efficiency of EF1α PCR, a reference gene (Nicot et al., 2005; accession No. AB061263.1), was 88.3%, whereas efficiency of *StPDS* PCR was 93.1%.

Table 7. qPCR conditions

Step	**Duration**
Initial denaturation 95°C	20 s
Denaturation at 95°C	5 s *40 cycles
Annealing/Elongation at 60°C	30 s

To quantify *StPDS* expression, *StPDS* primers (**Table 4**) were run on all the generated CDNA as templates at standardized conditions. The expression levels were calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001) with an efficiency correction step according to Pfaffl (2004).

4. Results

The main objective of this study was to test *Agrobacterium*-mediated transformation protocols for a diploid *Solanum tuberosum* (DG 82-330) and targeting the *StPDS* gene by CRISPR-Cas9 editing.

To the best of our knowledge, the 5'UTR of exon 1 of the *StPDS* gene has not been studied in previous research. In this results section, we present our findings on the effects of targeting the 5'UTR of exon 1 of the *StPDS* gene on gene expression and morphology of the potato plants.

4.1. Agrobacterium-mediated transformation

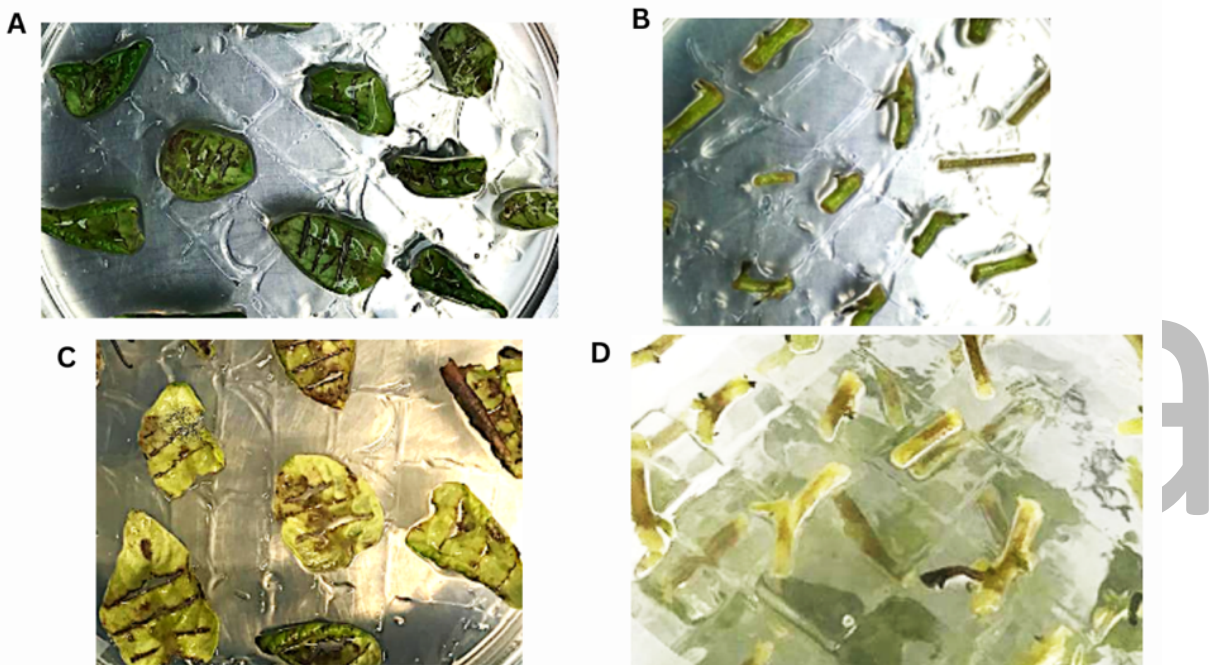


Figure 5: The explants of Protocol 2 (A-B) and Protocol 3 (C-D), two weeks after inoculation. In both protocols, leaves (A and C) and shoots (B and D) were used as explants.

The aim of this study was to test regeneration and transformation efficiency of the diploid potato cultivar DG 82-330 using leaf explants and stem segments (**Figure 5**) under two different protocols (P2 and P3) as tabulated in **Table 2**. More precisely, focusing on explant type and state, the inoculation time and plant growth regulators as key parameters. Protocol 2 was derived from Nadolska-Orczyk et al. (2007) and protocol 3 from Yadav and Sticklen (1995) combined with transformation protocol as reported by Douches et al. (1998).

To evaluate and compare DG 82-330 explant performance, we used the studies of Visser et al. (1991) on 'Désirée', 'King Edward', and B101 cultivars reporting that leaf explants were very sensitive to injury and that their state as starting material influenced regeneration and

transformation rates. Critical factors in this regard included leaf explant size, cut neatness, and the source of carbon (Han et al., 2015). In our study, browning and necrosis were observed in some of the small and badly injured DG 82-330 leaf explants, while others survived and developed further, particularly on P2.

P2 leaf explants successfully formed callus after 4 weeks and further developed into buds, eventually differentiating into healthy-looking shoots (**Figure 6**). Each leaf explant produced multiple shoots, and were more structurally stable, changing colour more consistently (from green to crispy-whitish), and grew in size (into an imbibition-like state). On the other hand, P3 leaf explants showed no callus formation (**Figure 5**), nor further organogenesis. Only aberrant phenotypic and morphological changes were noted, such as changes in leaf colour and structure, from green to pale white and dark brown, respectively. Notably, the structure of the leaves did not change, including the cut width, which was observed in P2 leaf explants.

Figure 6: Callus induction and shoot formation for P2 leaf explants.

Stem segments organogenesis on the other hand was more vigorous (5-9 cm) in P2 as compared to P3 (3-7 cm). P2 stem segments developed into bushy, purple shoots with small to medium-sized young leaves, and adventitious roots (**Figure 7A**, left image). In contrast, P3 shoots were whitish, pale, with very few small-sized leaves, and not bushy, with only one shoot regenerating from each stem segment explant (**Figure 7A**, right image). The first stem segments shoots transferred to selection media with kanamycin formed good root system compared to P3 (**Figure 7D** and **7E**).

Combined, the observed response of the used explants was attributed to the ratio of plant growth regulators (PGRs) TZR/GA3 and inoculation time, particularly with regard to the leaf explant to constantly and healthy development while in good rigid structural state.

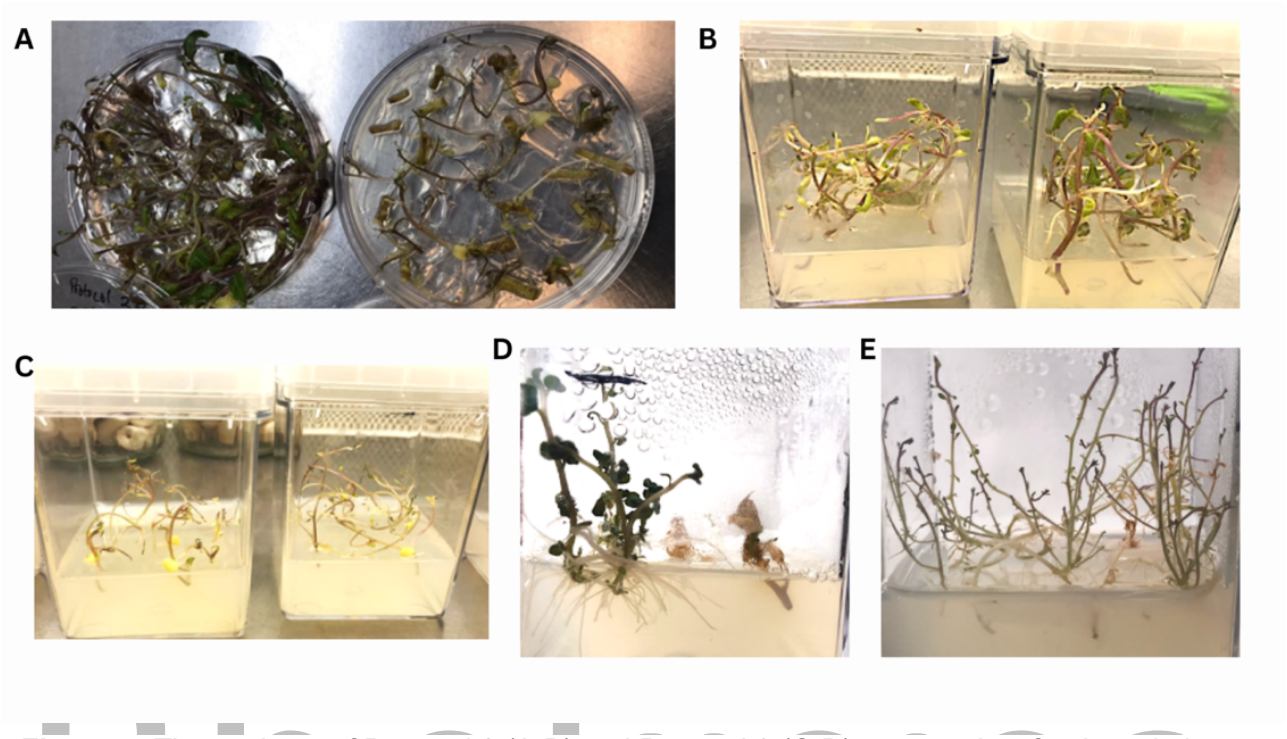


Figure 7: The explants of Protocol 2 (A-B) and Protocol 3 (C-D), two weeks after inoculation. In both protocols, leaves (A and C) and shoots (B and D) were used as explants.

To further investigate whether the regeneration rate was linked to explant type, we conducted a statistical analysis using the MONOVA overall test and follow-up analysis test, including Wilk's Lambda test. The results indicated a statistically significant relationship between the regeneration rate and explant type, with a *p*-value of 0.0003115, marked in red(as shown in the table below).

```
> summary(mod, test="Wilks")
          Df   Wilks approx F num Df den Df    Pr(>F)
Explant    1 0.67445   9.8951      2     41 0.0003115 ***
Residuals 42
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
>
```

Moreover, we evaluated callus induction and shoot regeneration, which is known to vary among different cultivars of various ploidy levels, particularly those with *andigena* genetic background. Previous studies with 'Désirée' have reported such variations (An et al., 1986;

Rocha Sosa et al., 1989; Banerjee et al., 2006; Han et al., 2015). In our study, we observed changes in phenotype and morphology during organogenesis of explants, with more variations observed in shoots transferred to kanamycin selection media (2-3 weeks old). Notably, leaf regenerates formed good root systems at quicker rates than stem segment regenerates for both P2 and P3.

Overall, with regards to regeneration rate, our results demonstrate that the of regeneration of explants for a diploid potato can be achieved.

Protocol 2 is the more suitable protocol for DG 82-330, most distinctively because callus and shoot induction (**Figures 8 and 9**) were more successful with good regenerates suitable for further applications. The overall state and organogenesis of leaf and stem explants by P2 confirms the compatibility of DG 82-330 to the components in P2. P2 components like the PGRs ratio can be further optimized to increase the regeneration rate.

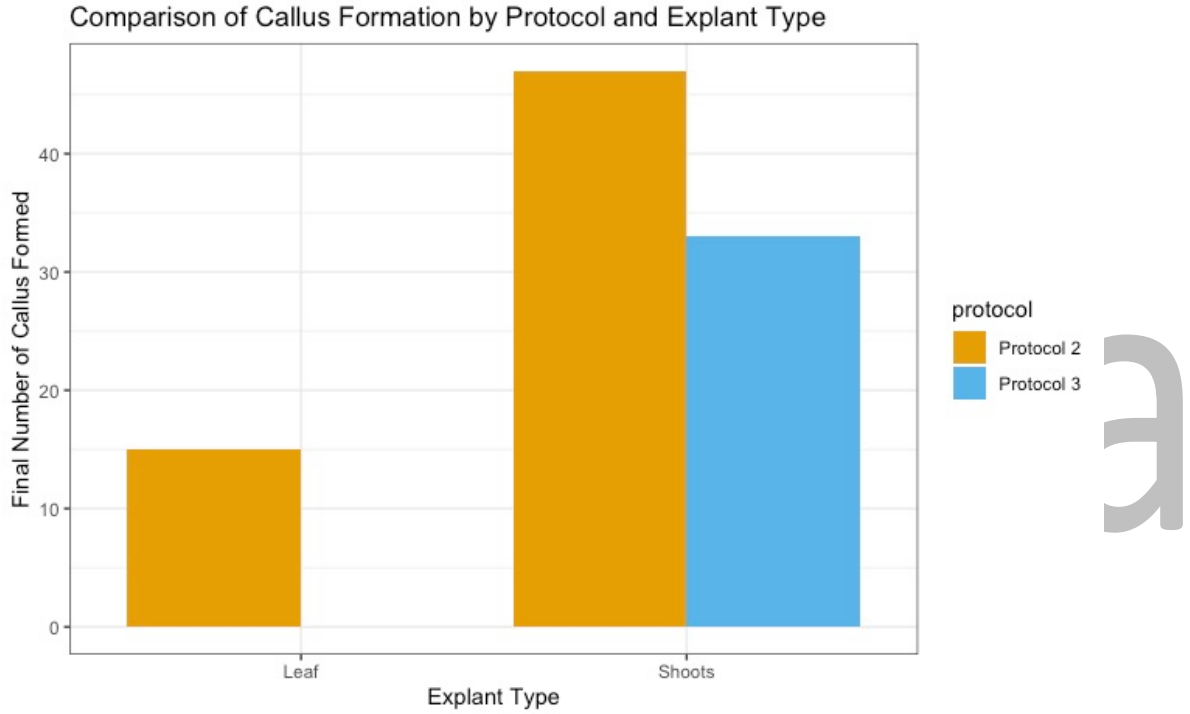


Figure 8: Comparison of callus formation by protocols and explants (shoots-stem segments).

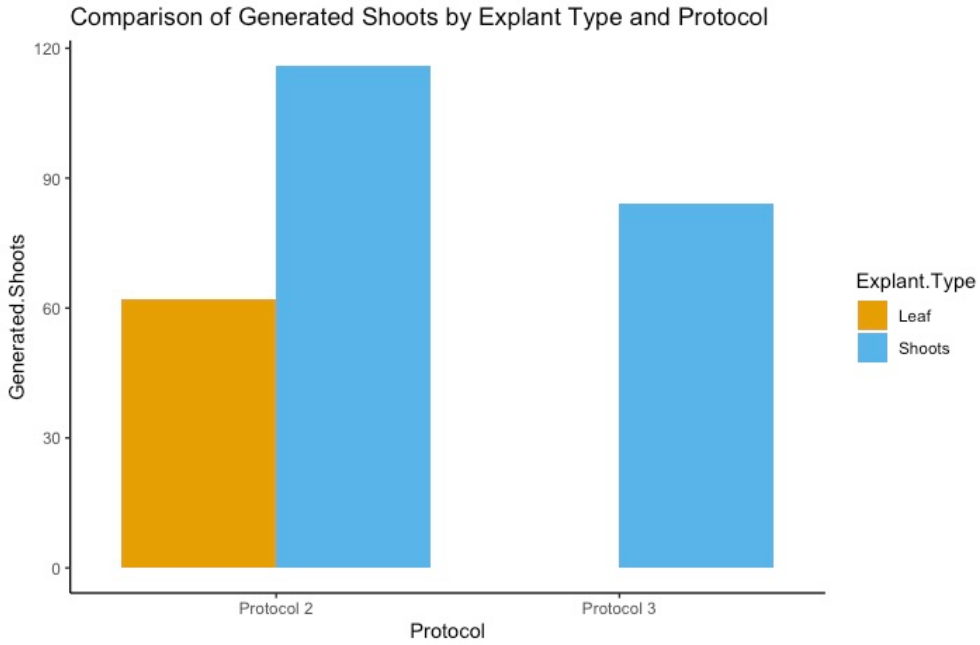


Figure 9: Comparison of generated shoots by protocol and explant ((shoots-stem segments).

Lastly, contaminations as illustrated below (**Figure 10**) were observed in most of the replicates, especially in P3 which was frequently infected by either bacteria or fungi. The causes of the contamination were not investigated but were not associated with the culture protocol. Rather, more sterile tissue culture practises should be strictly followed and observed.

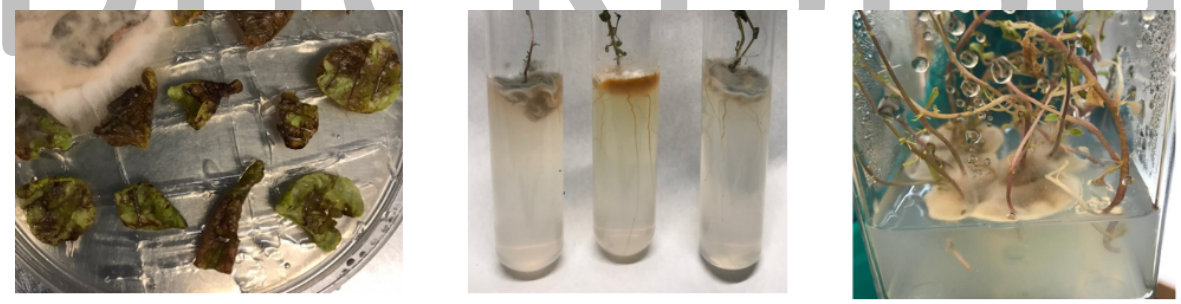


Figure 10: Contaminations from 2nd week to the stage of propagating shoot tips in tubes, after kanamycin selection.

4.2. Molecular Analysis of CRISPR-Cas9 Induced Mutations in *StPDS* Gene

Molecular analysis of CRISPR-Cas9 induced mutations in the *StPDS* gene was carried out on the generated plants. DNA and total RNA extraction were performed, and the extracted DNA was utilized as a template to amplify the 345 bp *StPDS* sequence through polymerase chain reaction (PCR).

To test for the presence of *StPDS* exon 1 insert, PCR was performed for kanamycin resistant rooted DG 82-330 plants 4,5,6,7,8,15,17,18, and control plant (**Figure 11**). The PKSE401 vector with a 6500-bp backbone size was used and a 345-bp insert of the exon 1 *StPDS*. The PCR products were run on a 2% agarose gel along with a 1-kb DNA ladder (L) to assess the size of the amplified fragments. The presence of a strong band in the gel indicates the amplification of the target DNA fragment in the respective plant sample.

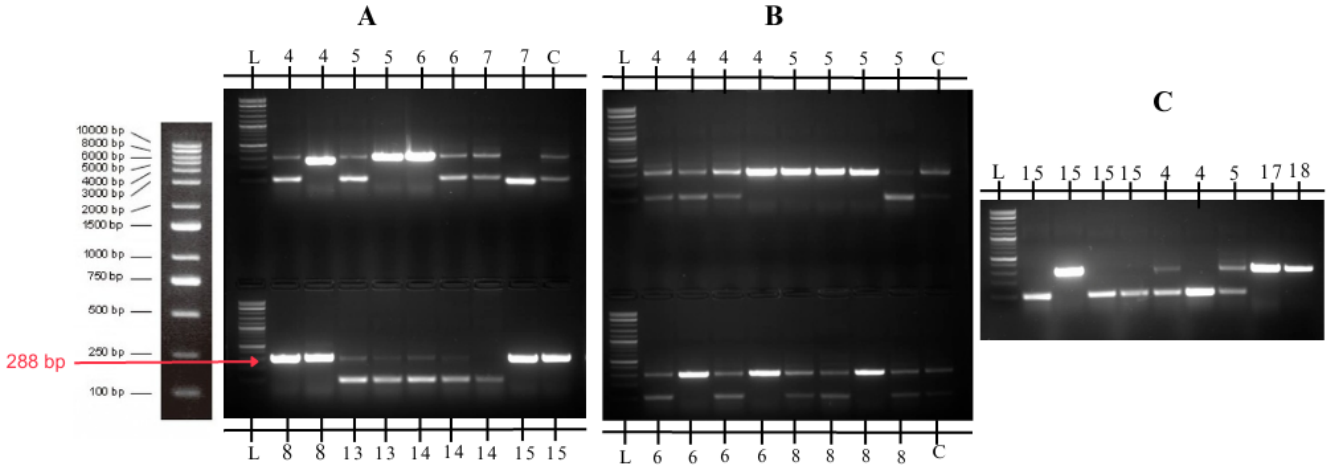


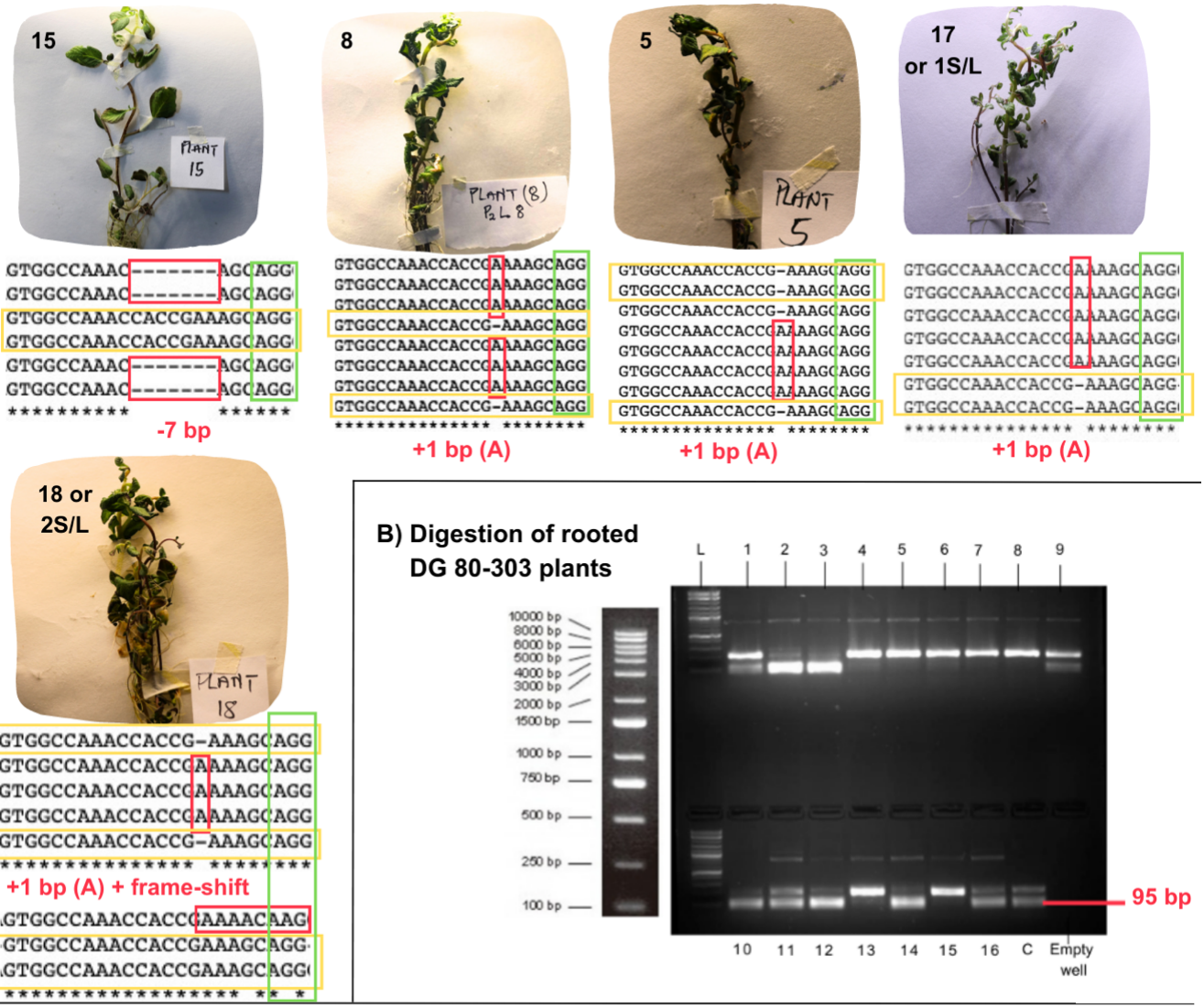
Figure 11: The gel images (A, B, C) show the outcomes of colony PCR performed on leaf and shoot explant regenerates from both P2 and P3. L, size marker; C, control plant.

Based on the expected product size, a strong band of approximately 288 bp on the gel confirmed the presence of the insert in the plant sample. As a result, the plants that tested positive (**Figure 11**) were selected for further analysis (4, 5, 6, 8,15,17 and 18). However, some plants showed both negative and positive bands on the gel, likely due to low transformation efficiency resulting in the formation of few colonies in some plates.

And subsequently enzyme digestion was carried out using the *Bsl*I restriction enzyme (NEB), with 5-15 min of digestion, and a cut site of CCNNNNN/NNGG, to identify mutations in the target sequence.

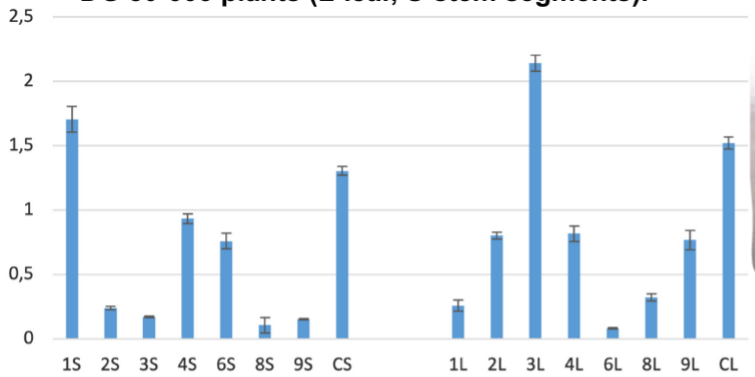
From the electrophoresis results, undigested bands of approximately 226 bp were observed in plant samples 4, 5, 6,8, 13 and 15, while samples 1,2,3,7,9,10,11,12,14 and 16 were digested showing approximately 95 bp (**Figure 12B**). Undigested samples were an indication of nucleotide changes in the target sequence, resulting in alterations of the enzyme cut site. For further analysis, only the undigested samples 4,5,6,8, and 15 were chosen, including 17 and 18 tested with colony PCR (**Figure 11C**).

A) Sequenced and rooted plants

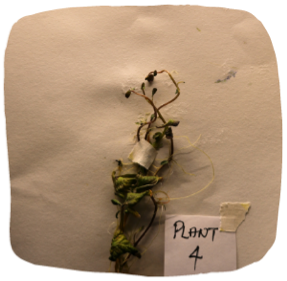


B) Digestion of rooted DG 80-303 plants

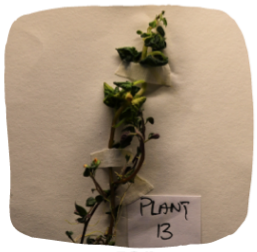
C) Expression analysis of rooted DG 80-303 plants (L-leaf, S-stem segments).




3S/L



4S/L



5S/L

6S/L

9S/L

8S/L

Figure 12: Testing of transformation efficiency for DG 82-330 rooted plants. **A**, Plants and sequence (25 bp) of mutated lines marked in red, PAM green and sgRNA and control sequence in yellow. **B**, enzyme digestion results of rooted plants. **C**, Expression analysis of *StPDS.*

Sanger sequencing was used to further analyze plant samples 5, 8,15, 17 and 18. The results showed nucleotide differences compared to the non-edited DG 82-330 (**Figure 12A**). Plant 5,8,17 and 18 had a one (1) Adenine-based insertion 4 bp downstream of the PAM site. Plant 15 had seven (7) bp deletion at 2 bp downstream of the PAM site. Sample 6 was excluded because plant 6 died.

These findings confirm that the CRISPR-Cas9 system was effective in inducing specific mutations in the *StPDS* gene. The presence of undigested bands in electrophoresis and nucleotide differences in the sequenced plants clearly indicate that the *StPDS* gene has undergone targeted mutations using the CRISPR-Cas9 system. Furthermore, the specificity and precision of the CRISPR-Cas9 system were demonstrated by the observed mutations being close to the PAM site.

4.3. qPCR Analysis

To investigate the effects of targeting the 5'UTR of the *StPDS* gene, we performed qPCR analysis to quantify the expression levels of the gene in the mutated potato plants. Our results indicated that targeting the 5'UTR led to significant changes in the expression of the *StPDS* gene. Specifically, we observed both downregulation and upregulation of the gene expression in the mutated potato plants **(Figure 12C)**.

In plants where the *StPDS* gene was downregulated, such as plants 2 (plant 18), 3, 4, 6, 8, and 9 (plant 15), we observed light green morphology of the stems and leaves (**Figure 12A**). Large oval shape leaves were observed for plant 15, distinct morphology from the other samples and control plant. In contrast, the morphology of the mutated plants where the gene was upregulated, such as plants 1 and 3, the morphology was purple. Interestingly, we also observed that the expression levels differed between the shoots and leaves of the same plant.

For example, in plant 1, the *StPDS* gene was upregulated in the shoots and downregulated in the leaves. In plant 2, we observed variable downregulation in both the shoots and leaves, with the shoots showing a higher rate of downregulation as compared with the leaves. These results suggest that targeting the 5'UTR of the *StPDS* gene also affect tissue-specific expression.

Therefore, the observed light green morphology of the stems and leaves in the downregulated plants, such as plants 2, 3, 4, 6, 8, and 9, suggest a disruption in the carotenoid biosynthesis pathway, in accordance with the role of the *StPDS* gene in the pathway. Conversely, the purple and dark green morphology of the mutated plants where the gene was upregulated, such as plants 1 and 3, may indicate an accumulation of anthocyanins. This observation highlights the importance of considering tissue-specific effects when designing CRISPR-Cas9 experiments aimed at gene regulation in plants.

In comparison with previous studies that targeted the *StPDS* gene (**Table 1**), our study did not result in dwarf or albino plants. However, we did observe light green, purple plants and with big leaves for plant 15, which were distinct from the non-edited DG 82-330 (**Figure 12A, C**). These findings suggest that changes to the 5'UTR sequence of the *StPDS* gene can have a significant impact on potato growth and development. It also highlights the importance of targeting specific regions of the gene, such as the 5'UTR, for maximum impact on gene expression and plant phenotype.

Overall, our results demonstrate the potential of the CRISPR-Cas9 system for targeted gene regulation in plants, with implications for crop improvement in agriculture. The variability in the effects of targeting the 5'UTR of the *StPDS* gene underscores the need for further research to elucidate the underlying mechanisms and optimize the design of CRISPR-Cas9 experiments for specific targets and tissue types

5. Discussion and Conclusion

5.1. Regeneration rate

This study successfully achieved the application of Agrobacterium transformation and CRISPR-Cas9 editing for DG 82-330. The high regeneration of stem segments explants in both protocols as tabulated (**Table 7**) is consistent with previous studies. From P2 and P3 a total of 224 shoots were generated from 61 stem segment explants. Less regeneration vigour of P3 stem segments was due to less concentration of trans-Zeatin riboside in the TZR/GA3.

Whereas P2 leaf explants being particularly successful, generating 52 shoots out of 63 explants. The success of P2 leaf explants was attributed to a good balance between TZR and GA3, with high TZR concentrations. Before the termination of the experiment, 20 leaf explants remained in good condition and showed callus formation around leaf midrib.

Table 7: Explant type, protocol, final number of callus formed and final number of generated shoots for performed experiment 1 and 2

Explant Type	Protocol	Total callus formed	Total generated shoots	Total rooted plants
Leaf	2	15	52	23
Leaf	3	0	0	0
Stem segments	2	58	115	40
Stem segments	3	59	109	18

5.2. Transformation efficiency

Transformation frequency (%) was calculated as the number of kanamycin-resistant plants (total rooted) divided by the number of explants, multiplied by 100%. P2 had transformation frequencies of 36.51% (leaf explants), 106.45% (stem segments), while P3 stem segments had a frequency of 43.33%. These results were attributed to the ability and stability of the regenerated shoots to grow in selection media.

The difference in regeneration rate and transformation efficiency between P2 and P3 can be attributed to several factors other than the ZR/GA3 ratio, such as the inoculation time in the bacterial suspension, the number of explants used, and the frequency of changing the media.

The use of stem segments and leaf explants under different transformation protocols demonstrated the significance of inoculation time, explant type, and the PGRs (ZR/GA3) ratio in influencing regeneration rate and transformation efficiency in diploid genotype, DG 82-330. This findings can be used to optimize regeneration and transformation efficiency of other cultivars, as well.

Additionally, one of the significant findings of this study is the inadvertent targeting of the 5'UTR of exon 1 of the *StPDS* gene, which has not been studied in previous research.

Our findings provide insights into the potential effects of targeting this region on gene expression and morphology of the potato plants. This result highlights the importance of careful design and screening of guide RNAs for CRISPR-Cas9 editing.

The precision of the CRISPR-Cas9 system was demonstrated by the observed mutations being in close proximity to the PAM site. These results support the application of the CRISPR-Cas9 system in targeted gene editing in potato.

The qPCR analysis revealed that targeting the 5'UTR of the *StPDS* gene led to significant changes in gene expression levels.

In conclusion, the findings of this study provide important insights into the application of the *Agrobacterium* transformation protocols and CRISPR-Cas9 editing for diploid potato genotype lines. Targeting of the 5'UTR of exon 1 of the *StPDS* gene provided insights into the potential effects on gene expression and morphology of the potato plants, and can be studied further.

6. Summary

Objectives of the study were achieved, with high regeneration rates in stem segments and leaf explants. The difference in regeneration rates between the two protocols can be attributed to several factors, and mainly to the TZR/GA3 ratio. Transformation frequency was calculated and attributed to the ability and stability of regenerated shoots to grow in selection media.

Further, obtained findings demonstrates the significance of inoculation time, explant type, and the PGRs ratio in influencing regeneration rate and transformation efficiency, which can be useful in optimizing other cultivars.

And the inadvertent targeting of the 5'UTR of exon 1 of the *StPDS* gene provided important insights into the potential effects on gene expression and morphology of potato plants and highlights the importance of careful design and screening of gRNAs for CRISPR-Cas9 editing. Precision of the CRISPR-Cas9 system is also demonstrated and provides insights into gene expression changes. Overall, this study provides valuable contributions to the application of *Agrobacterium* transformation and CRISPR-Cas9 editing in diploid potato genotype lines.

7. Acknowledgement

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8. References

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