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Transient gene editing in Potato (*Solanum tuberosum L.*) with CRISPR/Cas9 to knock-out *F3H*.

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Plant Science

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Abstract

With the continuous desire for crops with superior qualities, the CRISPR/Cas9 gene editing system seems to be a promising, cost-effective, efficient and a much faster method of developing new superior varieties compared to other gene editing and conventional breeding methods. The introduction of gene editing methods, where CRISPR is one of these, has proven to be a game changer in the discussions on GMOs' around the world. The determination and optimization of the transformation methods with higher transformation efficiencies and precise transient gene expression without the addition of foreign genetic material can be compared to mutation breeding methods used for the past 100 years. However, mutation breeding induces random mutations whiles gene editing tools are relatively more precise. When no foreign DNA is added, these methods can be compared biologically, and this is vital for the consideration of whether the resulting plant should be considered a GMO or not.

As a proof-of-concept for the use of CRISPR in potato, this projects aims at knocking-out the flavanone-3-hydroxylase (*F3H*) gene in the anthocyanin pathway of the new red-skin potato variety 'Nansen' for the possible outcome of a yellow-skinned coloured potato. The biolistic transformation techniques (using DNA plasmid and callus) and PEG-mediated transformation methods (using ribonucleoprotein (RNP) and protoplasts) were employed to determine the best method suited for further gene editing applications with 'Nansen'.

Dual guide DNA and RNP were used for gene knock-out in this project. Different polyethylene glycol (PEG) concentrations (25% and 40%) were tested to determine their effect on transformation efficiency. In the determination of the suitable induction and regeneration media to employ for the biolistic transformation, morphological variations were observed based on media composition as well as the condition of growth.

The methods used could not be assessed to determine a suitable protocol for future transformations in 'Nansen' by the end of the project due to severe contaminations of the protoplast cultures and eventually the Corona pandemic with the close-down of the laboratory for students. However, based on previous published studies, both methods seem promising with more regenerated transgene-free plants when ribonucleoproteins with synthetically produced RNA guides (cr-RNP) are used compared to DNA.

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1.0 Introduction

1.1 Potato

Potato (*Solanum tuberosum L.*) belonging to the large Solanaceae family (Nightshade) as tomato *(Solanum lycopersicum)* and eggplant (*Solanum melongena*) with approximately 374.5 million tons being produced globally (Dangol et al., 2019). Its currently the fourth most important crop worldwide, with the majority of its' total production in Europe and Asia (Gillund et al., 2013). It is estimated to still be an important crop in the future for the global populace estimated to increase to 9.7 billion by 2050 (Dangol et al., 2019). Potatoes are either consumed directly or further processed (e.g. to produce flour, chips, spirits, feed, etc.) (Gillund et al., 2013; Zhang et al., 2019).

With around 1000 current potato cultivars, the wild relative of potato exists in polyploid states ranging from diploid to hexaploidy with the most commonly cultivated potato being heterozygous autotetraploid (having 4 different copies of a single genome which occur after duplication) (2n=4x=48) (Dangol et al., 2019; Nicolia et al., 2015; Park et al., 2009). With the reduced sexual fertility, high allelic diversity and complex segregation, potato are vegetatively propagated (Nadakuduti et al., 2019)

In Norway, diverse potato cultivars have been cultivated for almost 250 years in most parts of the country under varied conditions ranging from the marginal sub-arctic climate in the north (70°N) to a temperate climate in the south (58°N) (Gillund et al., 2013; Zhang et al., 2019). Around 314,000 tons of potato are produced annually on 120,000 hectares (ha) with a wholesale value of approximately 500 million NOK (Zhang et al., 2019). Though grown throughout the country, most of the potato production in Norway is located around the south-central area and almost half of the potato produced is located around Norway's largest lake, Lake Mjøsa (Gillund et al., 2013).

Over the years breeding of potato cultivars with attractive qualities such as taste, appearance and disease resistance has been in constant demand on the market for growers (Andersson et al., 2017; Zhang et al., 2019). In the light of the diverse germplasm of potato, genetic improvements either by conventional or biotechnological (gene engineering) techniques have been employed for improvements in desired characteristics (Zhang et al., 2019).

Although genetic improvements through conventional breeding techniques have been successfully employed, the process is relatively slow and require a minimum of 15 years to obtain desired characteristics (Trine Hvoslef-Eide, pers. comm. 2020). Also, with the high heterozygosity and

tetraploid nature of the genome, other major drawbacks faced are allelic suppression (suppression of mutation of a gene by mutations in another gene), intra-species incompatibility, inbreeding depression and transfer of undesired characters along with the selected traits due to genetic linkage (Hameed et al., 2018). The clustered regularly interspaced short palindromic repeats (CRISPR) with its associated proteins (Cas9) collectively known as (CRISPR/Cas9) system is a current breakthrough in gene editing technologies with high versatility, efficiency and faster means of obtaining transformants compared to conventional breeding (Liang, Z. et al., 2017; Qi et al., 2019). Currently, two (2) newly developed potato varieties ('Nansen' and 'Hassel'), bred by the Norwegian plant breeding company 'Graminor' have been released on the Norwegian and Nordic markets (Graminor, 2017). These have been developed using conventional cross-breeding for Nordic growing conditions and are known to possess better qualities compared to the already existing potatoes on the market. 'Hassel' is a yellow-skinned potato variety and known to be early maturing and high yielding, having a good flavour and resistant to common scab. 'Nansen', on the other hand, is a red-skin potato which is semi-early and high yielding. It also has a very good flavour, smooth skin, an attractive appearance when boiled and a stronger than average resistance to late blight which caused the famine in 1840 (Graminor, 2017; Lal et al., 2018). However, despite the better qualities and late blight (*Phytophthora infestans*) resistance of 'Nansen', its demand on the market has been relatively low due to its red-skin coloured tubers compared to yellow varieties such as 'Hassel'. One reason for this could be the increase in demand for ready-made potatoes for cooking and any remaining yellow skin is less visible on the product compared to the red-skin varieties (Anders Wulff-Vester, pers. comm. 2020).

With the role of anthocyanins in pigment regulation in tissues, obtaining a yellow-skinned potato could be made possible through the regulation of the gene encoding for transcription factors such as basic helix-loop-helix (*bHLH)* in potato, as well as the flavanone-3-hydroxylase (*F3H*) enzymes of the anthocyanin pathway (Klimek-Chodacka et al., 2018; Strygina et al., 2019; Zhang et al., 2017).

In the light of the consumer preference to yellow-skin coloured potato, the use of CRISPR/Cas9 gene editing system seems to be a promising means to regulate the anthocyanin pathway. Through this technique, we could obtain a yellow skin-coloured 'Nansen' tubers for increased market acceptance within a short time compared to the classical/conventional breeding techniques (Peng et al., 2016; Qi et al., 2019), and without the cumbersome back-crossing needed to get rid of

unwanted characters in a cross-breeding scheme. When using the right techniques, we could obtain this goal, without adding foreign DNA to 'Nansen' and thus, possibly in the future, be given a possibility of a fast-track regulatory procedure.

The Norwegian consumers in a recent survey conducted through an industry-led project GENEinnovate (Gene editing to innovate Norwegian breeding Industries), have indicated that, using CRISPR to obtain late blight resistance to reduce the chemical usage in potato production would be a valid reason for choosing gene editing approach compared to trait such as skin colour (Bratlie et al., 2019; Bratlie et al., 2020). However, we regard the skin colour as a good marker for a proof-of-concept for the various techniques in gene editing for potatoes.

1.2 Anthocyanins

Anthocyanins are plant pigments belonging to a diverse family of phenolic compounds known as flavonoids (Glover & Martin, 2012). The biosynthesis of flavonoids (secondary metabolites) have been one of the most studied pathways (Shirley, 1996) starting from the mid-1800s with Mendel's experiment on the colouration in peas (Holton & Cornish, 1995; Martín et al., 2017). Anthocyanins are also known to play key roles in the colouration of the peel (pink, red, dark purple and blue), pulps (red and violet, solid or patchy), eyes and flowers, leaves and stems of plants (Strygina & Khlestkina, 2017).

Anthocyanins are composed of anthocyanidins backbones with sugar and acyl conjugates. With only six anthocyanidins prevalent to plants out of 20 discovered, Pelargonidin (orange/red), cyanidin (red/magenta), and delphinidin (violet/blue) are the primary anthocyanidins and chemically differ from each other by the number of hydroxyl groups at their B-rings (Liu et al., 2018), giving the difference in colour.

Figure 1 shows the anthocyanin biosynthetic pathway starting with the synthesis of naringenin chalcone from 4-coumaroyl-CoA and malonyl-CoA mediated by chalcone synthase (*CHS*) and after isomerized to naringenin by chalcone isomerase (Liu et al., 2018). The produced naringenin is converted into dihydrokaempferol by Flavanone 3-hydroxylase (*F3H*) which is the gene of interest to knock-out in this project. The dihydrokaempferol produced could be further hydroxylated by flavonoid 3′,5′-hydroxylase (*F3′5′H*) or flavonoid 3′-hydroxylase (*F3′H*) into two other dihydroflavonols namely dihydromyricetin or dihydroquercetin, respectively. These three

dihydroflavonols are further converted to converted into colourless leucoanthocyanidins by dihydroflavonol 4-reductase (*DFR*) and subsequently to coloured anthocyanidins by anthocyanidin synthase (*ANS*). Finally, flavonoid 3-O-glucosyltransferase (*UFGT*) attaches sugar molecules to the anthocyanidins resulting in the specific anthocyanins (Holton & Cornish, 1995; Liu et al., 2018)

With the diverse possibility of altering the anthocyanin pathway, Holton and Cornish, (1995) indicated that most changes in the anthocyanin pathways are very visible and have no negative effects on the growth and development of the plants. This was evident via the reduction in the purple colour of carrot callus after the knockout of the (*F3H*) gene (Klimek-Chodacka et al., 2018). Liu et al., (2018) however indicated that, although the biosynthesis of the pathway of the anthocyanin has been well studied, more research is needed to understand the inhibition of these regulators and genes.

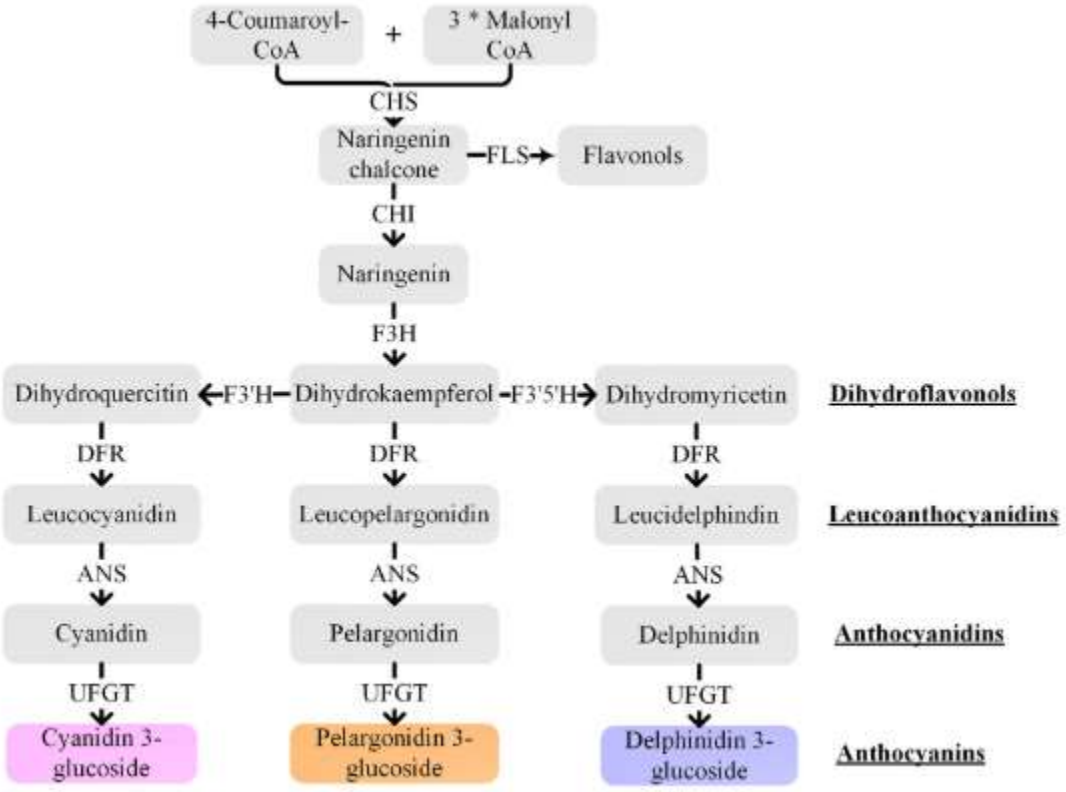


FIGURE 1. Diagrammatic representation of the anthocyanin. CHI, chalcone isomerase; CHS, chalcone synthase; *F3H*, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, flavonoid 3-O-glucosyltransferase; FLS, flavonol synthase (Liu et al., 2018)

1.3 Gene editing techniques with emphasis on CRISPR/Cas9

The introduction of gene editing techniques have resulted in the tremendous improvement in achieving desired characteristics, though conventional breeding methods such as mutation breeding and crossbreeding have been used for the creation and selection of crop with desirable qualities for years (Qi et al., 2019; Salimath et al., 2007). The zinc finger nucleases (ZFNs), transcriptor activator-like effector nucleases (TALENs) and the CRISPR/ Cas9 system have been the most widely used gene editing techniques (LaFountaine et al., 2015; Qi et al., 2019). These techniques require restriction enzymes and guides to induced DNA breaks at target sites (LaFountaine et al., 2015).

The guides of the ZFNs complexes are composed of a sequence of 3 to 6 zinc finger proteins with each zinc finger recognizing a three base pair (bp) sequence, whiles that of the TALENs are composed of transcription activator-like effectors (TALEs) (LaFountaine et al., 2015). These guides are linked to endonuclease such as the same Fok1 which operates as a dimer to induce double-stranded break (DSB).

The use of CRISPR/Cas9 system for genetic editing for molecular biologists was discovered in 2012 (Peng et al., 2016), and currently has two major competing applicants for patents concerning CRISPR/Cas9 in Europe and the United States (Emmanuelle Charpentier and Jennifer Doudna at the Helmholtz Centre for Infection Research in Germany, and the University of California, Berkeley respectively); and Feng Zhang, at the Massachusetts Institute of Technology (MIT) (Peng, 2016). Though conventional breeding technologies have been used for crop improvement for over 100 years (Smith et al., 2005; van Harten, 1998), the rapid and increased precision achieved using the CRISPR/Cas9 gene editing technology, compared to other gene editing technologies (ZFNs and TALENs) has increased the preference of CRISPR/ Cas9 (Qi et al., 2019).

The CRISPR/Cas9 system which was discovered in 1987 (Peng et al., 2016), is an integral part of the bacterial adaptive immune defense mechanism against plasmids and phages (Martin et al., 2014; Peng et al., 2016; Qi et al., 2019). The CRISPR/Cas system has been classified into three categories namely: Type I, Type II and Type III. The most used system today is the type II system which requires only one Cas protein to recognize and cleave target sites, whereas type I and type III CRISPR systems requires the assembly of a set of Cas proteins (Peng et al., 2016).

With the varied number and function of nucleolytic proteins identified in various bacterial species, current editing systems predominantly utilize *Staphylococcus pyogenes* Cas9 protein (Klimek-Chodacka et al., 2018; Peng et al., 2016). This is a single Cas protein with two nuclease domains, HNH and RuvC, which cleaves the target DNA and non-target strands, respectively (Dangol et al., 2019; Klimek-Chodacka et al., 2018).

The CRISPR type II locus includes the trans-activating CRISPR RNA (tracrRNA) gene, CRISPR RNA (crRNA) spacer array gene and the Cas9 gene which are transcribed into tracrRNA, pre-crRNA and Cas9 proteins (after translation) respectively (Peng et al., 2016). The pre-crRNA (composed of sequences complementary to the target) also has a sequence that enables the interaction with the tracrRNA via complementary base pairing to form a single guide RNA (sgRNA) with usually has an 18-20 nucleotide complementary base to the target sequence (Qi et al., 2019). The tracrRNA also interacts with the Cas9 protein to form the CRISPR/Cas9 complex for DNA cleavage which only occur at target site having a short protospacer adjacent motif (PAM) with a 5'-NGG-3' sequence for SpCas9 (Klimek-Chodacka et al., 2018; Peng et al., 2016).

The cleavage of the double-stranded DNA at the target locus by the Cas9 enzyme triggers either nonhomologous end-joining (NHEJ) or homology-directed recombination (HDR) DNA repair processes in targeted cells (Liang, X. et al., 2017) (Figure 2). NHEJ being the predominant repair process is error-prone and produces random nucleotide insertion, substitutions, and deletions at the repaired sites (Klimek-Chodacka et al., 2018; Liang, X. et al., 2017). The HDR repair process, on the other hand, is exploited for nucleotide-substitution or gene replacement with a donor DNA template that contains the desired genetic change (Klimek-Chodacka et al., 2018).

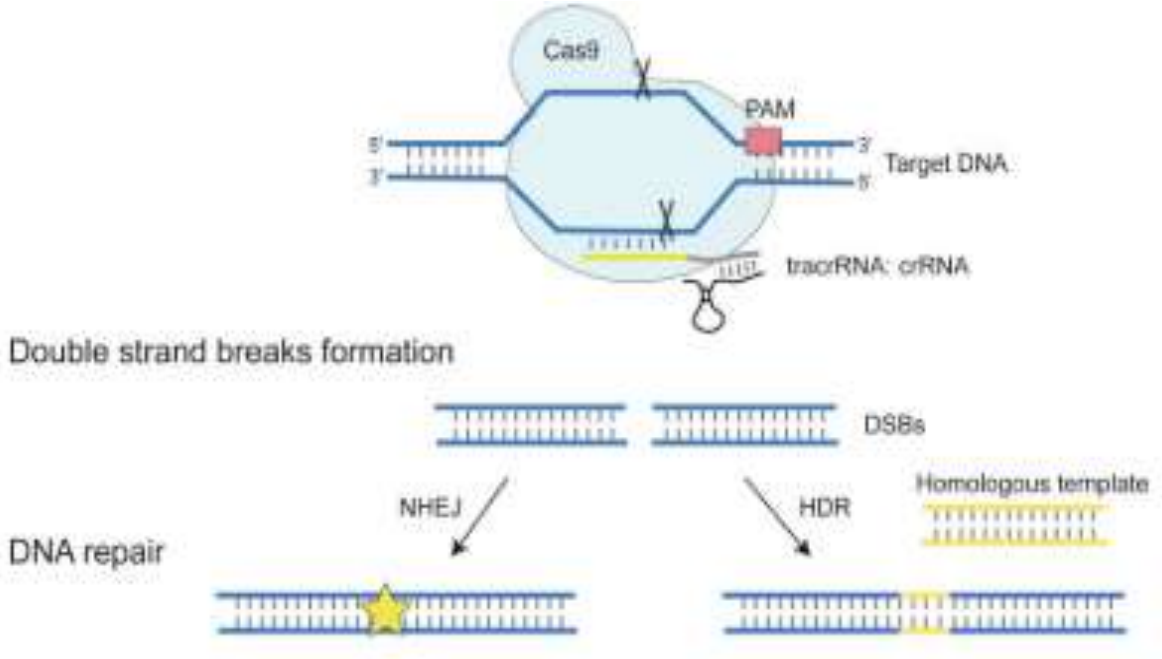


Figure 2. Diagrammatic representation of the CRISPR/Cas9 system. The gRNA binds to Cas9 (blue) through the scaffold (black). Genomic DNA (deep blue) is opened by Cas9 and the complementary sequence base pairs with the spacer sequence (yellow). The target sequence is cut (black scissors) 3 bp upstream of the PAM DSBs can be repaired through the NHEJ or the HDR pathway (Peng et al., 2016)

1.4 Genetically modified organisms (GMOs) legislation in the EU and Norway

In the EU legislation, GMOs are defined as "organisms in which the genetic material have been altered in a way that does not occur naturally by mating and/or natural recombination" (Ricroch et al., 2016). This indicates that the methods used are the main focus and not the end product or phenotype (Bratlie & Borge, 2018). Over the years, several questions have been raised with regards to the adoption of genetically modified crops in the European Union (EU) as well as the feasibility of growing both GM and non-GM crops (Devos et al., 2009). However, the strict regulations and requirements in the permits have been a huge obstacle for small companies and research institutions, while huge companies having the economic muscles explore the avenues in the gene editing of organisms (Huesing et al., 2016).

In Norway, the EU definition of the 'GMO' was perceived to be "too wide", resulting in a more detailed characterization of methods involved as either conventional breeding methods or genetic engineering techniques (Figure 3) (Bratlie & Borge, 2018; Bratlie et al., 2019). The Norwegian

Gene Technology Act (1993) was made to ensure producing GMOs' in an ethical and societally responsible manner according to the principle of sustainability and without harmful effects on health and the environment (Bratlie & Borge, 2018; Bratlie et al., 2019).

With some current gene editing techniques resulting in transient gene expression using CRISPR, foreign DNA is not stably integrated whiles obtained genetic changes are similar as in nature as well as using conventional methods such as mutagenesis (Bratlie & Borge, 2018; Peng et al., 2016; Qi et al., 2019).

As part of the proposed amending for the legislation, the Gene Technology Act should include the differentiation of various types of GMOs based on genetic change using the tiering system though applications are case dependent with variations in type and scope of the planned release (Bratlie & Borge, 2018). Within the 4 main levels of the tiering system (Figure 4), only a notification is required on level 1, where changes made are similar to what happens in nature or using conventional breeding methods (Bratlie & Borge, 2018). This creates the avenue for trial involving transient gene expression with CRISPR employing various delivery mechanisms such as biolistic and protoplast transformation with RNPs and DNA (Andersson et al., 2018; Qi et al., 2019).

There is currently an avenue of increased acceptance by the young generation since transgene-free genome-edited plants only display a very small change in their genome with no added foreign DNA and antibiotic resistance. (Gillund & Myhr, 2016).

Conventional breeding methods that are not regulated by the Gene Technology Act
Crossbreeding
Mutagenesis (using radiation or chemicals to induce mutations)
Triploidisation (applying thermal or pressure shocks to fertilised fish eggs to produce an extra set of chromosomes in order to make the fish sterile)
Cell fusion within the same species (fusion of cells results in extra copies of the genetic material - used within plant breeding)

Genetic engineering techniques that are regulated by the Gene Technology Act
Inserting novel genes from the same or a different species ("classical genetic modification")
Gene editing which is used to make targeted changes to the genetic material of an organism, with or without inserting new DNA
Temporary transfer of nucleic acids (e.g. RNA/DNA-vaccines)
Regulating gene expression (e.g. using RNAi or epigenetic changes, where nucleic acids are used to change gene expression, but not the actual DNA sequence)
Cell fusion between different species

Figure 3. Classification of conventional breeding and genetic engineering techniques (Bratlie & Borge, 2018).

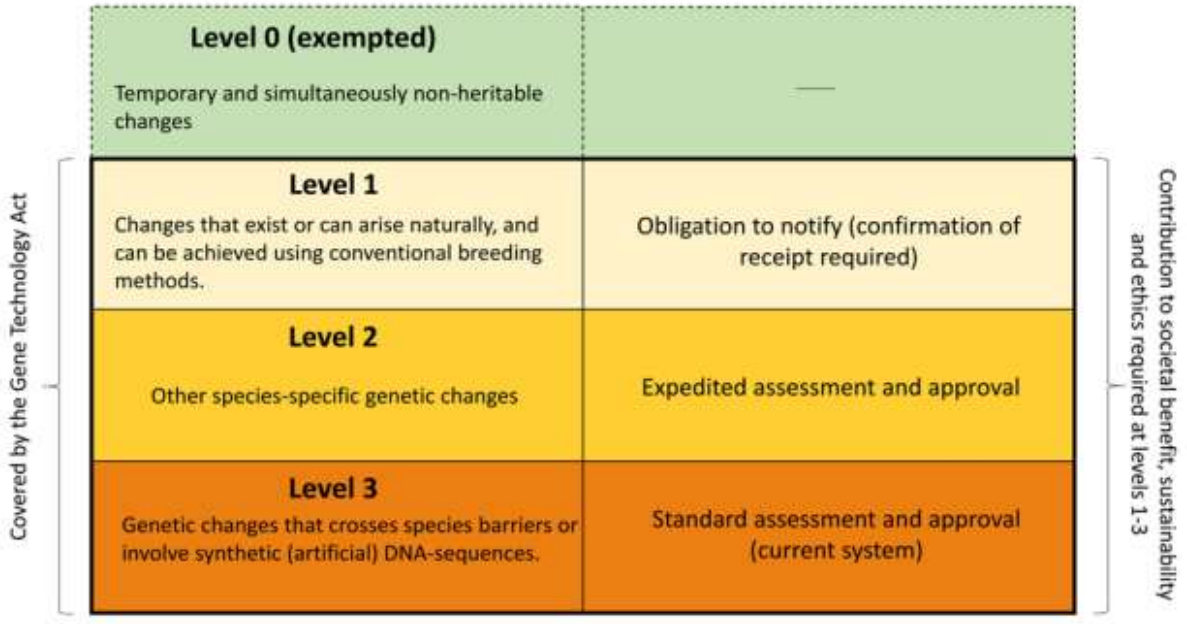


Figure 4:The classification levels based on the nature of genetic change in an organism according to the Norwegian Gene Act (Bratlie & Borge, 2018).

1.5 Methods of transformation

With the numerous gene editing methods employed in crop improvement, one of the aims of the GENEinnovate (Gene editing to innovate Norwegian breeding Industries) program is to establish an effective protocol for the introduction of novel gene and mutation in potato (Anders Wulff-Vester, pers. comm. 2020).

Currently, the methods used for the delivery of CRISPR/Cas9 constructs in gene editing include *Agrobacterium*-mediated transformation, direct transfer of genetic (using a variety of techniques such as biolistics, electroporation, electrophoresis or microinjection) as well as PEG mediated transformation which is a chemical-based method (Qi et al., 2019). Though optimization of a method with regards to species is vital for increased transformation efficiency, an efficient shoot regeneration protocol is a prerequisite to facilitate success with the genetic transformation methods (Dalla Costa et al., 2019; Kumlay & Ercisli, 2015; Peng et al., 2016; Tycko et al., 2016).

Amongst these methods, the biolistic transformation and the PEG-mediated protoplast transformation methods were the selected methods to be compared in this project. Other methods will be compared in the GENE-innovate project as comparing all methods would be too large for a master thesis, even for a one year's work of (60ects).

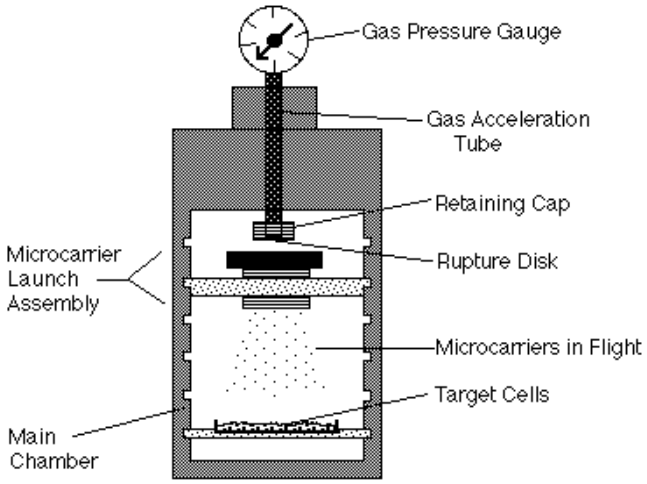


Figure 5. Overview of biolistic transformation. The high-pressure helium gas causes the rupturing of the rupture disk (at a specific pressure) resulting in the launch of the microcarriers (minute gold particles coated with the desired genes) on the microcarrier. The microcarriers penetrate the plant cells and the genes cargo is released for transcription and translation (Kikkert, 1993).

The biolistic transformation technique developed in the 1980s' has been the most used physical transformation method besides the *Agrobacterium*-mediated transformations (Dalla Costa et al., 2019; Qi et al., 2019). The biolistic technique enables the delivery of gold coated particles with genetic cargo (DNA, RNAs, RNPs) which are shot under high velocity into cells through their cell wall barrier (Dalla Costa et al., 2019) (Figure 5).

This could result in nuclear, mitochondrial and plastidial transformation due to random integration of genetic cargo, unlike the *Agrobacterium*-mediated transformation where the nuclear genome is the target due to the transfer DNA (T-DNA) (Dalla Costa et al., 2019; Ziemienowicz et al., 2001). The introduction of particles at high velocities could result in damage to target tissues as well as gene silencing since the multiple inserts are sometimes prone to recombination. With callus being used mostly for biolistic transformation, a range of potato induction and regeneration media protocols have been established (Al-Hussaini et al., 2015; Turhan, 2004). However, with callus induction and regeneration being genotype/ variety dependent, varied response have been observed

due to the varied media composition as well as conditions of growth (light and darkness) used (Al-Hussaini et al., 2015; Islam, 2015; Kumlay & Ercisli, 2015; Omidi & Shahpiri, 2003; Taiz et al., 2015; Turhan, 2004)

Despite the drawbacks of using the biolistic transformation, it is one of the most used transformation methods since it's not limited to species as compared to other methods such as *Agrobacterium*-mediated transformation (Dalla Costa et al., 2019). Also, the increased possibility of transiently expressing gene, especially with multiple biolistic events has rendered its use in various plant species using different plant material (cell suspension culture, leaf sections and somatic embryos) (Dalla Costa et al., 2019; Hafiza et al., 2010; Liang et al., 2019; Prakash & Varadarajan, 1992; Romano et al., 2001).

On the other hand, the PEG-meditated transformation being a chemical-based method functions by destabilising the permeability of the cell membrane of protoplasts (cells with a degraded cell wall) (Dalla Costa et al., 2019; Liang et al., 2019; Taiz et al., 2015). This promotes protoplast fusion as well as the exchange/uptake of genetic material (DNA, RNA, RNP) into the cytoplasm of the cells (Al Abdallah et al., 2017; Dalla Costa et al., 2019)

With PEG being amphiphilic (having both hydrophilic and hydrophobic parts), its hydrophilic segments bind to the negatively charged backbone of DNA resulting in a DNA-PEG-complex. With the method not fully understood, it is proposed that the complex formed results in the suppression of the negative charge of the DNA enabling its diffusion through the hydrophobic membrane of the cell (Karp et al., 1987; Selga, 2017).

Though PEG-mediated mechanism is not fully understood and the regeneration protocols are also genotype/ variety dependent (Hu et al., 1999; Selga, 2017), its function is also not limited to certain genotypes or species and also an alternative to obtain transgene-free plant (Andersson et al., 2018; Dalla Costa et al., 2019; Malnoy et al., 2016).

1.6 Aims of this thesis

This project aims at comparing the biolistic transformation (using plasmid DNA) with the PEG mediated protoplast transformation (using RNP) methods in order to:

1. Produce yellow skin-coloured ‘Nansen’ potatoes by knocking out the *F3H* gene via transient gene expression as a proof-of-concept
2. Determine which method is better for transient gene expression in the ‘Nansen’ potato variety.

2.0 Materials and methods

This chapter is divided into sections of which include: Gene identification and primer testing for the gene of interest (*F3H)* in 'Nansen', Callus induction and regeneration (trial), Biolistic transformation, PEG-mediated protoplast transformation, Microscopy and lastly, Analysis.

2.1 Gene identification and Primer testing in 'Nansen'

2.1.1 Plant materials

In-vitro virus-free 'Nansen' potatoes were obtained from the "potato bank" at NIBIO, Ås, Norway through Sissel Haugslien and Dag-Ragnar Blystad. The plant materials were sub-cultured every 3-6 weeks on full Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) with 3% sucrose, 0.8% agar and a pH of 5.7. They grew in a growth chamber with 16 hours day length at an intensity of 30 µmol/m^2/s and temperatures 23 ± 2°C before used for callus initiation and protoplast isolation for the biolistic and PEG-mediated transformation, respectively. In-vitro plants were screened during the experiments using the media for infection detection from the laboratory ('LLB' media) to obtain disease-free plants after contaminations.

2.1.2 Plant DNA isolation for primer testing

To isolate DNA, young leaves were harvested and frozen in liquid nitrogen. Frozen leaves were homogenized using the Retsch MM301 mixer mill (25hz for 30 seconds) and DNA isolated using QIAGEN DNeasy Plant Mini Kit according to its protocol (Appendix 1). The plasmid yield and purity were determined using the nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.1.3 Gene of interest and Primers testing

The sequence of the Flavanone-3-hydroxylase (*F3H),* as well as two designed primer pairs were obtained from Anders Wulff-Vester (Table 1). Two primer pairs were used in the amplification of the gene of interest (*F3H*) in 'Nansen' to verify its presence within its genome. A gradient polymerase chain reaction (PCR) with an annealing temperature ranging from 50 ℃ to 60 ℃ was conducted to verify the function of the primers as well as their optimal annealing temperature to minimize non-specific binding (Appendix 2). The reaction conditions for amplification of the gene

was as follows: denaturation at 94℃ for 3 min., then 30 cycles of 94℃, 30 sec.; gradient annealing for 30 seconds followed by 1 min. extension at 60 ℃, then a 10 min. final extension at 68 ℃ and kept at 4 ℃ after completion of the reaction.

The PCR products were run on a 1% agarose gel containing gel red for 40 min. at 80 volts. The resulting bands were visualized after the gel electrophoresis using the transilluminator to determine the optimal primer annealing temperatures based on the visibility of bands in the gel.

The PCR products of the selected lanes with the most visible bands in the gels were sequenced three times, to determine nucleotide sequence at GATC, Germany for Sanger sequencing. The obtained sequence reads were aligned to the initial sequence used in primer design and blasted for in the National Center for Biotechnology Information (NCBI) for sequence alignment percentages and e-value determination. This was done to determine the specificity of the primer to the gene of interest (*F3H*) and the consistency of the reads. An optimal annealing temperature of 53℃ was selected for the '*StF3H* 2' primer pair based on the sequence results to be used in later PCR analysis.

Table 1. Primer pairs tested to verify the presence of the gene of interest (*F3H*) in the genome of 'Nansen'

Primers name	**Forward primer**	**Reverse primer**	**Fragment length in base pair (bp)**
StF3H 1	tctccacccttatttcacc	gtcgtgacatgcatatctctatc	486
StF3H 2	tgttagttggcacgaagggt	tttctaaatgatgacatgcacct	590

2.2 Callus induction and regeneration

With 'Nansen' being a new variety with no recorded regeneration protocol, different callus induction and shoot regeneration media were tested before transformation, to increase success in the regeneration of gene edited plants. Only stem cuttings were used in the callus induction since more starting materials could be obtained from the stem of the in-vitro culture.

This section describes the basal media composition used for both different induction and regeneration media as well as the growing conditions. The viability of the callus induced was determined before regeneration by 2% acetocarmine.

2.2.1 Basal media composition for callus induction and regeneration

Media tested were based on variations of different media compositions for callus induction and regeneration of potato protocols. All media had a basal composition of full MS, 3% sucrose, 3.5 g/L gelrite and pH adjusted to 5.7 after the addition of plant growth regulators and autoclaved.

2.2.2 Callus induction media and viability/embryogenic callus test

To determine the suitable callus induction media, a total of 40 stem cutting of 1-2 mm (with ten stem cuttings per petri dish) were used for each media treatment. The nine different potato callus induction media were kept under both light and dark conditions at 23 ± 2 ℃ (Table 2). Light treated stem cuttings were exposed to a light intensity of 10 µmol/m^2/s for 16 hours and 8 hours darkness while dark treated had no light. The explants and formed calli were transferred to fresh media, with the same composition as before, every 2-3 weeks.

Due to thrip invasion and contamination of the callus induction set up in light, three main media compositions were selected to repeat the callus induction in the light. The following media were chosen, based on the callus size results obtained under the dark condition: media 2 (2 mg/L 2,4-D), media 5 (5 mg/L 2,4-D) and media 6 (0.5 mg/L Kinetin + 5 mg/L NAA). The callus induction on media 2 (2 mg/L 2,4-D) in the light was selected for biolistic transformation since it had greener, friable and embryogenic callus with possible reduced somaclonal variation due to the low 2,4-D concentration (Gupta & Holmstrom, 2005; Hoque & Morshad, 2014).

Table 2. Composition of potato callus induction medias tested.

Media name	**Hormone concentrations.**
Media 1 (control)	No hormone added
Media 2	2 mg/L 2,4-D
Media 3	3 mg/L 2,4-D
Media 4	4 mg/L 2,4-D
Media 5	5 mg/L 2,4-D
Media 6	0.5 mg/L Kinetin + 5 mg/L NAA
Media 7	2 mg/L 2,4-D + 2 mg/L BAP
Media 8	2 mg/L 2,4-D + 1 mg/L BAP
Media 9	1 mg/L 2,4-D + 1 mg/L BAP
Media 10	1 mg/L 2,4-D + 2 mg/L BAP

2.2.3 Preparation of 2% (w/v) Acetocarmine

45% acetic acid solution was prepared by pipetting 22.5 ml of glacial acetic acid into 27.5 ml distilled water in a glass beaker flask. 1.0 g of carmine was added to the 45% acetic acid solution and heated on a hot plate in the fume hood to gently boil for 5 minutes. The supernatant was obtained and stored at room temperature until use as according to Gupta & Holmstrom (2005).

2.2.4 Embryogenic callus (cells) staining

Two drops of the 2% acetocarmine solution were added to a small piece of callus (2-5mm) placed on a glass slide. The callus was gently divided into small parts to increase contact with the callus (cells) and the solution. The glass slide was held with forceps and heated for 5 sec. before washing off the staining solution. The coverslip was then placed on the calli and observed under the Lecia M205 stereo microscope.

2.2.5 Regeneration media

Callus induced under the selected condition (2 mg/L 2,4-D in light) was placed on two different regeneration (without Media C) (Table 3). This was hindered due to contamination and exposure to high light intensity 50 µmol/m^2/s. The second round of callus regeneration test using three different regeneration at 30 µmol/m^2/s was performed. The day length and temperature were 16 hours and 23 ± 2 ℃ respectively.

Table 3. Composition of induction media tested.

Media name	**Hormone concentrations**
Media A	0.22mg/L TDZ + 0.49 mg/L NAA
Media B	3mg/l BAP + 0.5 mg/l GA3 + 0.03mg/l NAA
Media C	0.9 mg/l Thiamine-HCl, 0.8mg/L zeatin, 2mg/L GA3

2.3 Biolistic Transformation

A modified callus transformation protocol of the laboratory where callus was bombarded twice at a nine cm distance, using the 1 µm gold particle at a pressure of 1100 psi was used in this project This section entails the plasmids composition, subcloning of plasmids, prior preparation before transformation, biolistic transformation protocol and growth, kanamycin dosage selection followed by the determination of transformant using selection media.

2.3.1 Plasmid composition for biolistic transformation

The two plasmids namely pCRISPR 1 and pCRISPR 2 were designed and assembled by Anders Wulff-Vester, NMBU, Ås, Norway and Mark Smedley, John Innes Centre, Norwich, UK, using the Golden Gate cloning technique. The sequences outside the right and left borders of the RK2 plasmid were the origin of replication (*oriV*) and a trans-acting gene (*trfA)*, whose gene product, the TrfA protein, binds to and activates *oriV*.

A total of 4 different gRNA sequences were designed with each plasmid containing 2 different sgRNA sequences (Table 4). These sequences have the recognition sites located within the first exon of the (*F3H)* gene, encoding the F3H enzyme, which is vital for the biosynthesis of the primary anthocyanidins namely pelargonidin, cyanidin, and delphinidin.

The sgRNA sequences had the *Solanum tuberosum U6* (StU6) promotor to promote the expression. Double (2x) 35S promoter from the cauliflower mosaic virus (CaMV 35S) was used in the expression of the kanamycin resistance (KanR) by the neomycin phosphotransferase II (NPT II) gene for selection of transformed cells. The translation of the KanR was enhanced using a tobacco mosaic virus (TMV-Omega). The cassava vein mosaic virus (CsVMV) promoter was also used for the expression of the Cas9 Protein to enable the interaction of the gRNA and the Cas9 protein for the formation of the CRISPR/Cas9 complex for genome editing (Figure 6).

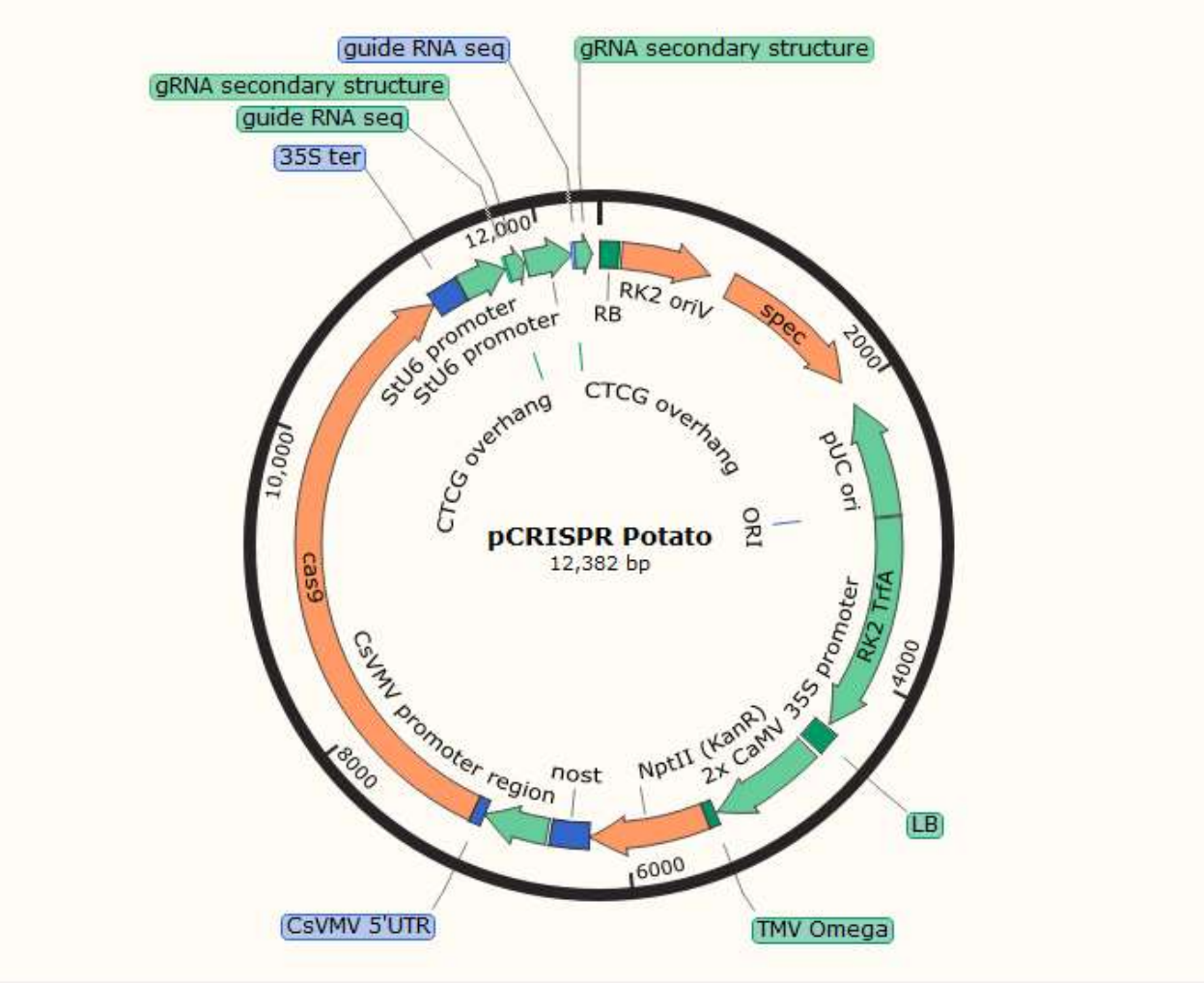


Figure 6. The plasmid map containing the 2 different gRNA, 2x 35S promoter for the Neomycin phosphotransferase II (*NPT II*) gene and the Cassava vein mosaic virus (CsVMV) promoter for the expression of the Cas9 Protein.

Table 4. Paired sgRNA sequences used in each plasmid design

Plasmids	**Guide RNA**	**Sequences**
Plasmid 1	Guide 1	ttcaaacaagttttattagg (reverse)
	Guide 2	ttttcaggtaactgatcatg
Plasmid 2	Guide 3	tgaagaagaacgtccaaaag
	Guide 4	atgtctggtggcaagaaagg

2.3.1.1 GUS plasmid

The plasmid (pWRG1515) for GUS expression stored at -20°C was obtained from Anders Wulff-Vester (Figure 7). The cauliflower mosaic virus (CaMV 35S) was used in the expression of both the gusA gene which encodes the ß-glucuronidase enzyme (GUS) and the hygromycin phosphotransferase (hpt) gene which confers resistance to hygromycin B. The transcriptions were both terminated by the Nos polyA which also protects the mRNA molecule from enzymatic degradation in the cytoplasm. The GUS enzyme functions by cleaving the chromogenic (colour-generating) substrate X-gluc (5-bromo-4-chloro-3-indolyl ß-D-glucuronic acid) resulting in the production of an insoluble blue colour in plant cells displaying GUS activity (Liang & Opsahl-Sorteberg, 2019).

The GUS plasmid was used to determine the transformation efficiency of single and double bombardment due to its visible results (blue colouration of transformants). Although double bombardment was used in the main transformation using pCRISPR 1 and pCRISPR 2 to increase the number of transformants based on literature, the GUS plasmid was used to also provide an estimation of the possible number of transformants to expect.

Figure 7. Schematic representation of the GUS plasmid with kanamycin resistance used in for the determination of transformation efficiency.

2.3.1.2 GUS staining protocol

GUS histochemical staining buffer using X-Gluc was evenly distributed onto the transformed calli in a petri dish without media and incubated overnight at 37°C (Table 5). Transformants (with blue spots) were observed under the Leica M205 stereo microscope.

Table 5. Components for GUS histochemical staining buffer with X-Gluc:

Volumes added	**Concentrations of stock**
100 µl	1 M $NaPO_4$ pH 7.0
5 µl	Triton X-100 (0.5%)
50 µl	X-Gluc 20 mg/ml in Dimethylformamide
845 µl	sterile water
1000 µl (total)	

2.3.2 Plasmid subcloning

For bacterial transformation and rapid replication of the plasmids, the plasmid stocks were subcloned in the DH5α Competent Cells (*Escherichia coli (E. coli))* according to the Invitrogen Subcloning Efficiency™ DH5α™ Competent Cells protocol (Appendix 3).

To enable single colony growth, 50µl, 100µl, and 200µl of stab *E.coli* cultures with the respective plasmid were streaked onto plates containing solid LB media supplemented with 50 µg/ml kanamycin as the antibiotic for selection of transformed bacterial cells. The plates were inverted and incubated at 37 ℃ overnight without shaking for the growth of the transformed bacteria cell and later stored at 4℃.

2.3.2.1 Plasmid DNA isolation from E. coli

Single colonies of the transformed *E. coli* were transferred into glass flask with 50ml of Liquid LB media with the kanamycin concentration of 50 µg/ml for selection. The flasks were then incubated in a shaking incubator at 37 ℃ overnight and 290 rpm for replication of the transformed bacteria.

Plasmid DNA was isolated from the final 50 ml *E. coli* stab culture the Qiagen Plasmid Midi Kit according to the protocol and eluted in 1ml (Appendix 4). The plasmid yield and purity were determined using the nanodrop ND-1000 spectrophotometer and visualized on the 1% agarose gel.

2.3.3 Prior preparation before transformation

The step used for gold particle sterilization, plasmid concentration and coating of the gold particles are described below.

2.3.3.1 Gold particle sterilization and storage

For sterilization, 1 ml absolute ethanol (abs EtOH) was added to 60 mg gold (Au)-particles of in an Eppendorf tube and vortexed for 2 min. The mixture was centrifuged at 10000 rpm 1min and the supernatant discarded to obtain the sterilized gold pellets. Gold pellets were resuspended in 1ml sterile water and stored at -20 °C.

2.3.3.2 Plasmid concentration and storage 1 µg/µl

The plasmids were precipitated by adding sodium acetate ($1/10^{th}$ the volume of isolated plasmid) and ice-cold isopropanol (twice the isolated plasmid volume) and centrifuged at 10000 rpm for 20 min at 4°C. The supernatant was decarded and the pellet was then washed with abs. EtOH and centrifuged at 10000 rpm for 10 mins at 4°C. The supernatant was then pipetted off and the pellets left to air dry for 10 min. The pellets were resuspended in $1/10^{th}$ of its initial volume using the Buffer AE and the yield determined to by the nanodrop ND-1000 spectrophotometer. It was the adjusted to 1 µg/µl and stored at -20 °C.

2.3.3.3 Coating of gold particles with plasmid

The added components were finger vortex for 3 min and left for 5 min on ice (Table 6). The mixture was then centrifuged for 60 seconds at 1000rpm and the supernatant pipette off after which the pellets were washed with 200µl of abs. EtOH. The tube was left of ice for 5 min and centrifuged for 60 seconds at 1000rpm. The supernatant was then pipetted off and coated particles resuspended in 150µl of abs. EtOH. The coated gold particles were kept on ice for shooting.

Table 6. Volumes and stock of components for biolistic transformation.

Volumes of components for 15 biolistic events	**Stock concentrations**
170 µl Au	(1 µm, 60 mg/ml)
40 µl DNA	(1 µg/µl)
170 µl $CaCl_2$	(2.5 M)
70 µl Spermidine	(0.1 M)

2.3.4 Biolistic transformation protocol and growth

The Biolistic ™ Particle Delivery System Model PDS-1000 (DuPont) was used following the protocol of the laboratory. The rupture disc (1100 psi), macro carrier and stop plate, gene gun chamber and its components sterilized with 70% EtOH and left to dry in the flow hood. The helium gas cylinder was adjusted to 1100 psi.

The gold coated particles were resuspended by dipping the eppendorf tube into a sonicator for a few seconds. 10µl of the gold particle solution was pipetted onto the middle section of the macro carrier in its holder and air-dried for 5 min in the flow hood. The rupture disc was dipped in isopropanol and assembled in the holder for sterilization and to ensure proper sealing to deliver the exact pressure.

The 'Nansen' calli were spread on a filter paper and left for 3-5 min after which they were transferred onto a 9 cm petri dish containing the induction media for shooting. With the components assembled, the petri dish was placed at the 9 cm distance in the particle gun chamber and shoot after a vacuum over 25 mmHg was created. The petri dish was rotated before the second round of transformation to increase the contact with other calli cells. Four independent transformation events were carried out with each plasmid in all three transformation rounds. The gold particles used in the control were without plasmid (DNA).

The petri dish was covered and sealed using a parafilm and kept in the dark for at 23 ℃ for seven days to recover from the shooting stress before transfer to the light conditions. The calli were

grown in the absence of antibiotic selection for a month since the test for the best regeneration was interrupted by contaminations

2.3.5 Kanamycin dosage testing for selection

The kanamycin concentration of 50,100, 150 and 200 mg/L was tested to determine the best concentration of to use for selection. 50 mg/L of kanamycin was selected as the minimum lethal dose for selection based on the concentrations tested. Sealed petri dishes of callus on kanamycin media with 2 mg/L 2,4-D were placed under the same condition as callus induction.

2.3.6 Callus growth and selection of transformants

Since the calli of the first and second transformation events grew larger due to contamination of the regeneration set-up, replica samples (samples from the same calli lump) were respectively placed on selection media (with kanamycin) and non-selection (without kanamycin) as described by Sharma et al., (2018) (Figure 8). This was done as an initial step to determine transformants with stable integration from those with possible transient expression. Since dying calli lumps on the selective media may not have stable integration (for resistance to kanamycin), their replica calli on the non-selective media would be selected for analysis of transient gene transformants. On the other hand, calli lump surviving on the selection media would not have their replica on the non-selective media analysed since they may have stable integration.

In the light of the possibility of losing calli (cell) with transiently expressed genes on the selection media, all callus from the three rounds of biolistic transformation were kept for possible regeneration into plants and sample leaves screened to determine transformants

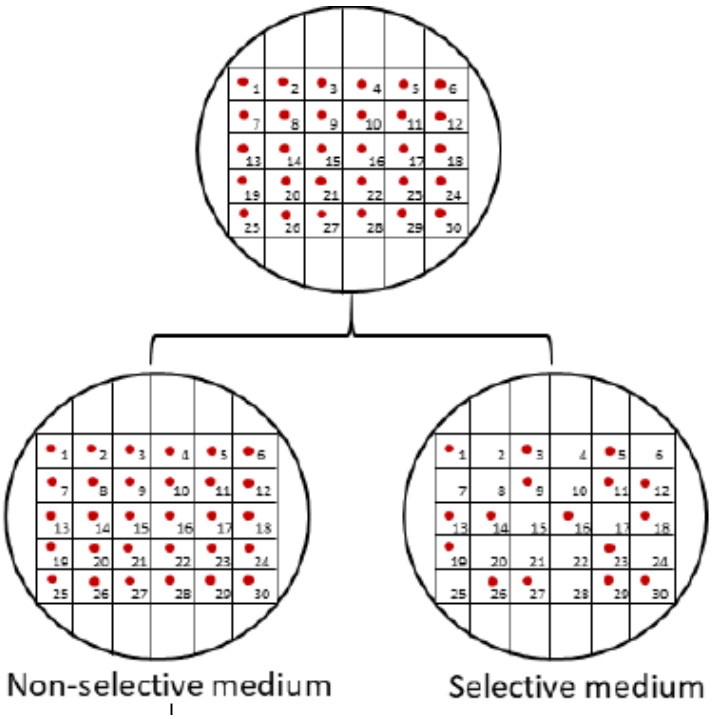


Figure 8. Diagrammatic representation of the grid system employed for the identification of transformants (callus/cells) with or without stable integration of genes (Sharma et al., 2018).

2.4 PEG-mediated transformations (Protoplast isolation, transfection and regeneration)

The dual RNP complex in transient gene expression as described by Al Abdallah et al., (2017) was employed. However, the RNP concentrations and quantities used for transfection were based on the potato transformation protocols as described by Andersson et al., (2018).

Leaves from the in-vitro 'Nansen' shoots cultures were cut under sterile conditions with all media composition and protoplast isolated steps followed as described by Nicolia et al., (2015) (Appendix 5). The glass jars were used instead of the petri dish with 15ml of media G and F to fully cover the protoplast lenses and reduce possible leakage of the sugar-rich liquid media from the petri dish in the growth chamber. Protoplast viability was assessed using propidium iodide (PI) and Fluorescein diacetate (FDA) as described Lin et al., (2018). The individual steps employed are further described below.

2.4.1 Ribonucleoprotein (RNP) construct for protoplast transfection

Four (4) crRNA was customised and ordered from the Integrated DNA Technology (IDT) with the same sequences as the plasmid sgRNAs used for the biolistic transformation (Table 4). A modified protocol for the assembly of RNP components as described in the IDT RNP protocol was used (Appendix 6) without further dilution with PBS buffer to have an increased concentration (Mariette Andersson, pers. comm. 2019). 2.0 nmol of crRNA and 2.0 nmol of tracrRNA together with 0.03 nmol (5µg) of Cas9 were assembled to form the single CRISPR/Cas9 RNP complex (Andersson et al., 2018). The assembly of the dual CRISPR/Cas9 RNP complex was achieved by adding equal volumes (11.6 µl) of the respective single CRISPR/Cas9 RNP complex. The resulting dual CRISPR/Cas9 RNP complexes were gently mixed and incubated for 5 min. at room temperature prior to transfection.

2.4.2 PEG concentration for transfection

The transfection was done using either 40% or 25% PEG for 30 min. per transfection with the dual RNP complexes. This was to determine the best PEG-4000 concentration for 'Nansen' since 40% and 25% PEG were used for 30 min. and only 3 min. respectively during the protoplast transfection set up of Andersson et al., (2017).

2.4.3 Protoplast viability test

A stock of 0.5mg/ml propidium iodide (PI) and 5 mg/ml Fluorescein diacetate (FDA) were prepared by dissolving in 0.65 M mannitol and acetone respectively. A freshly staining solution was prepared by mixing 20 µl of PI and 20 µl FDA in 1 ml 0.65 M mannitol (Lin et al., 2018).

For the staining, 10 µl of staining solution was added to 20 µl of isolated protoplast cells and kept in the dark for 2 min. The images were visualized with the Leica 5000B microscope. An excitation/ emission wavelength of 495nm/ 517nm and 538nm/ 617nm were used for FDA and PI, respectively. The observed green fluorescence (FDA) and red fluorescence (PI) indicates whether the cell is viable or non-viable (dead) respectively.

2.4.4 Culture and protoplast isolation protocol

Twenty to thirty (20-30) leaves of the 4- 6 weeks old in-vitro plant were cut under sterile condition and placed on 20ml of Medium B on the abaxial side. The petri dishes were sealed and incubated at 4 °C overnight in the dark. After, Medium B was then pipetted off and leaves were leaves sliced into (1-2 mm) using a new scalpel blade and incubated for 30 min in 20 ml of the Plasmolysis solution in the dark at room temperature. The Plasmolysis solution was then pipetted off and substituted with 25 ml of Medium C, sealed and incubated overnight in the dark at 25°C without shaking.

The overnight culture was gently shaken on the shaker for 10 min. The resulting green solution containing the released protoplast is sieved through sterile filter papers (100µm and 70µm) into a 50 ml tube after per wetting with 5ml of the Washing solution. The remaining protoplast in the sieve is washed using 5 ml of the Washing solution.

Eight (8) ml of the protoplast suspension was transferred into a sterile 15ml tube and was topped to the 15ml mark by adding 7ml of the Washing solution. The suspension was centrifuged at 50 x *g* (minimum acceleration and deceleration) for 5 min. The resulting supernatant was discarded, and protoplasts gently re-suspended in 2 ml of Washing solution. Six (6) ml. of the resulting protoplast suspension were gently layered on top of the 6 ml Sucrose solution in a sterile 15ml tube without disrupting the interface. The tube was centrifuged at 50 x *g* for 15 min (minimum acceleration and deceleration) and the thick dark band of protoplasts at the interface of the two solutions were pipetted (with a cut tip) into a fresh sterile 15 ml tube containing 3 ml of the Transformation buffer 1 and kept at 4°C in the dark during counting and staining.

The protoplast density (protoplast/ml) and viability were determined by the haemocytometer and the staining, respectively. The protoplast suspension was then centrifuged at 50 x *g* for 10 min (minimum acceleration and deceleration), the supernatant was then discarded, and protoplasts gently re-suspended in Transformation buffer 2 at the concentration of 1.5 x 10^6 protoplasts/ml.

The transfection was performed by adding 20 µl of dual RNP complex to 100 µl of the protoplast, gently flicked. 110 ul of the different PEG-4000 concentrations (40% and 25%) were then added, finger flicked gently and incubated on ice for 30 min. The transfection reaction was stopped by adding 5ml of the washing solution which was centrifuged at 50 x *g* for 5 min (minimum acceleration and deceleration) and the supernatant discarded. The pelleted protoplasts were

resuspended and gently mixed with 1 ml of Alginate solution concentration. 2-4 drops of the mixture were placed on the solid Setting agar for a maximum of 2 hours. The protoplast lenses were then released with 2-3 ml of the Floating solution and moved to 15 ml of medium E in a sterilized jar. The jars were sealed with parafilm and incubated at 25 °C for 3 days in a dark incubator. Light was gradually introduced by placing the jar on a shelf without light in the growth chamber at 23±2°C for 3 weeks until the protoplast mini calli were visible to the naked eye. The protoplast lenses were then transferred into medium F after mini calli formation and placed on the shelf with a light intensity of 10 µmol/m^2/s. Medium F was changed every 7-10 days until completely hindered by contaminations before the required period of 6 weeks to transfer into medium G as described by in the protocol of Nicolia et al., (2015) (Appendix 5).

2.4.5 Treatment of yeast and bacterial contamination

Protoplast lenses containing protoplasts were washed with autoclaved water and transferred into sterile (autoclaved) jars containing 15 ml of fresh media every 7-10 days supplemented with the antibiotic mix to curb the bacterial growth as described by Coelho et al., (2012) (Table7). No fungicides were added to yeast contaminated media.

2.4.6 Antibiotic mix composition

Both penicillin G and streptomycin were dissolved in 90 ml of water and added to the 10 ml of chloramphenicol after dissolving with abs. EtOH. The mixture was filter sterilized, and 5 ml aliquots of the antibiotic mix were stored at -20°C. 5 ml of the antibiotic mix was added to a liter of Medium F before use (Table 7).

Table 7. Antibiotic mix for bacterial infection in media containing protoplast (Coelho et al., 2012).

Antibiotic	**Quantity (for 100ml)**
Penicillin 4G	1g
Streptomycin	0.5g
Chloramphenicol	0.1g

2.5 Microscopy

The callus induction and regeneration images were observed using the Leica M205 stereo microscope. Protoplast images were also observed through the Leica 5000B microscope. Z-stack images were captured using the Leica DFC42 camera and the LAS v 4.3 software.

2.6 Analysis

Transformants were not analysed at the end of the project due to the full stop in the laboratory and the closure of the University due to the Corona pandemic. However, transformed calli were kept alive on the induction media for possible regeneration later. Callus induction and Gus staining results were analysed using the R-commander.

In order to determine transformants, calli would have been freeze-dried (since it contains more water than leaves) for 3 days to ensure efficient water removal or leaves of the regenerated plants frozen using liquid nitrogen. Dried samples would have been homogenized using the Retsch MM301 mixer mill at 25hz for 30 seconds and DNA isolated using QIAGEN DNeasy Plant Mini Kit according to its protocol.

The selected primer set *'StF3H2'* would have been used in the digital droplet polymerase chain reaction (ddPCR) or PCR to amplify the region of interest. PCR products would be run on a 1% agarose gel (gel electrophoresis) and also sequenced to determine transformants. The probes (fam and hex) for the ddPCR reaction were designed using the Bio-Rad ddPCR™ NHEJ Genome Edit Detection Assays software.

3.0 Results

3.1 Callus Induction and regeneration

3.1.1 Callus induction

Calli were produced on all the different media compositions with the exception of the control media (hormone-free media) under both the light and dark conditions (Figure9, Appendix 7).

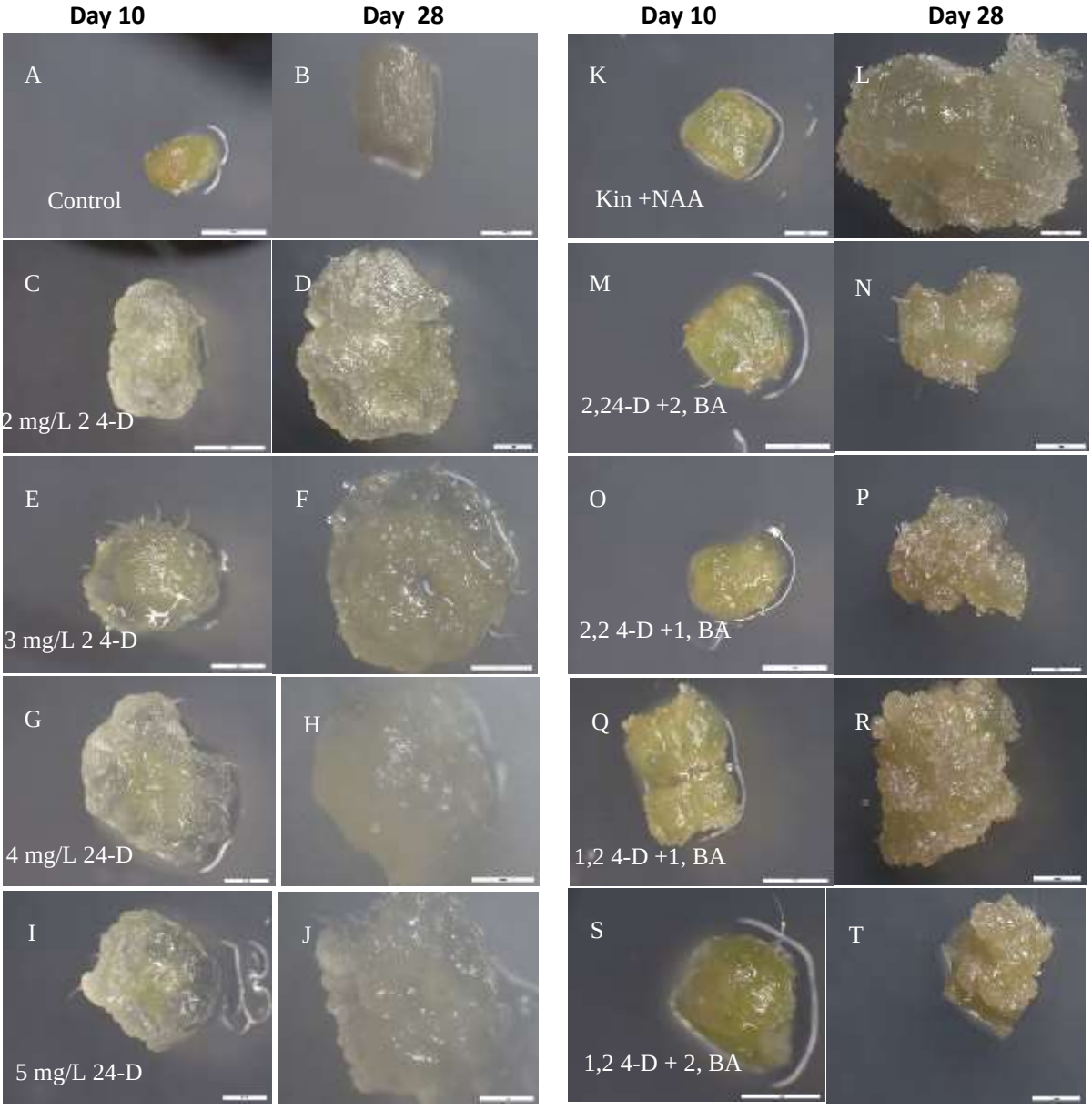


Figure 9. Potato callus induction under dark condition in different media composition. 10 days after induction (A,C,E,G,I,K,M,O,Q, S) and 28 days after induction. (B,D,F,H,J,L,N,P,R,T). Induction media tested include: hormone free media (control) (A,B), 2 mg/L 2,4-D (C,D); 3 mg/L 2,4-D (E,F), 4 mg/L 2,4-D(G,H), 5 mg/L 2,4-D (I,J), 0.5 mg/L Kinetin + 5 mg/L NAA (K,L), 2 mg/L 2,4-D + 2 mg/L BAP (M,N), 2 mg/L 2,4-D + 1 mg/L BAP (O,P), 1 mg/L 2,4-D + 1 mg/L BAP (Q,R), 1 mg/L 2,4-D + 2 mg/L BAP (S,T). With the exception of images B and C having a scale bar of 500µm and 2 mm respectively, all the scale bars are 1mm.

Morphological differences in calli sizes, colour and texture was also observed due to the variation in media composition used as well as condition of growth (Figure 9 and 13). Under the dark conditions, whitish and glossy coloured calli were obtained on media with only 2,4-D while darker calli were observed on media containing cytokinin (BAP and Kinetin). All the calli were friable (suitable for suspension cultures) except the callus produced on 0.5 mg/L Kinetin + 5 mg/L NAA which also had some whitish crystallizations along the edges.

With the few selected media compositions for the second light setup (after the first/initial setup was massively contaminated by thrips invasion (Figure 10)), greener friable calli were produced on the media with 2 mg/L and 5 mg/L 2,4-D (Figure11A-F). The explants on the media with 0.5 mg/L Kinetin + 5 mg/L NAA produced whitish crystallized calli along its edges while the central part remained green and compact (Figures 11G-I).

The calli induced under the various conditions also showed a bright red circular colouration (the nucleus) within the white translucent cells after staining during the embryogenic test (Figure 12).

However, though large calli were produced on both 0.5 mg/L Kinetin + 5 mg/L NAA and 5 mg/L 2,4-D larger calli compared to 2 mg/L 2,4-D under both conditions, callus for the biolistic transformation were induced under the light condition using 2mg/L 2,4-D.

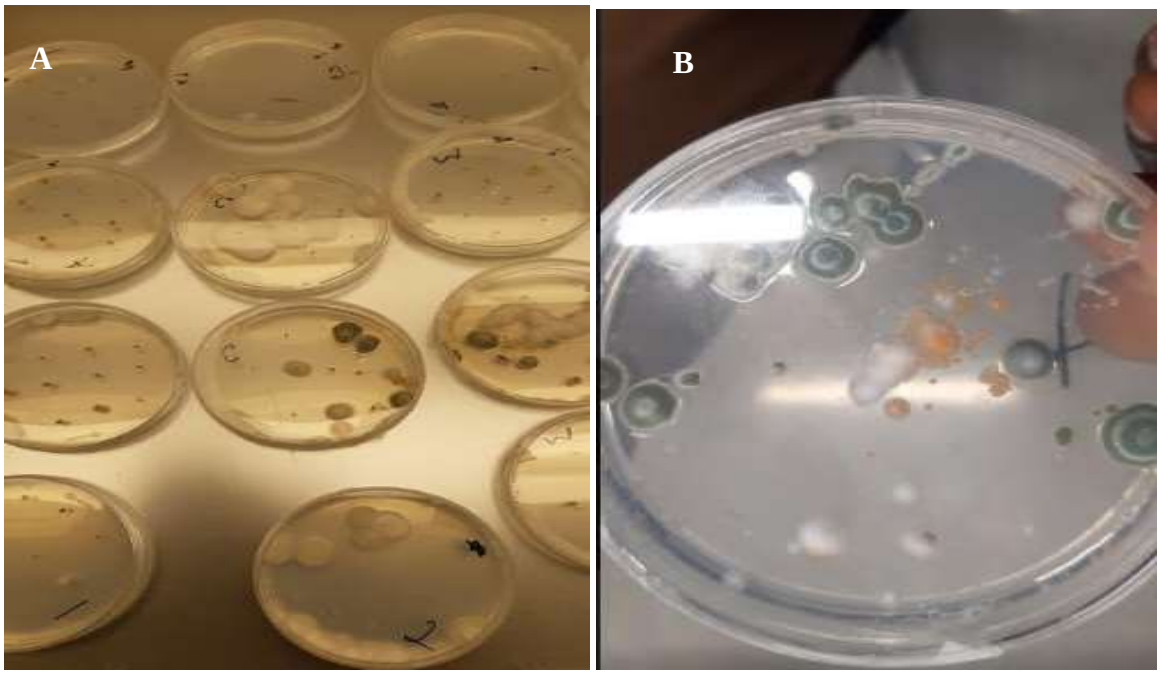


Figure 10. Thrips infestation/contamination in first round of potato callus induction using all 9 different media compositions in the light. Media contamination after 10 days (A). The visible trail of left behind by thrips (B).

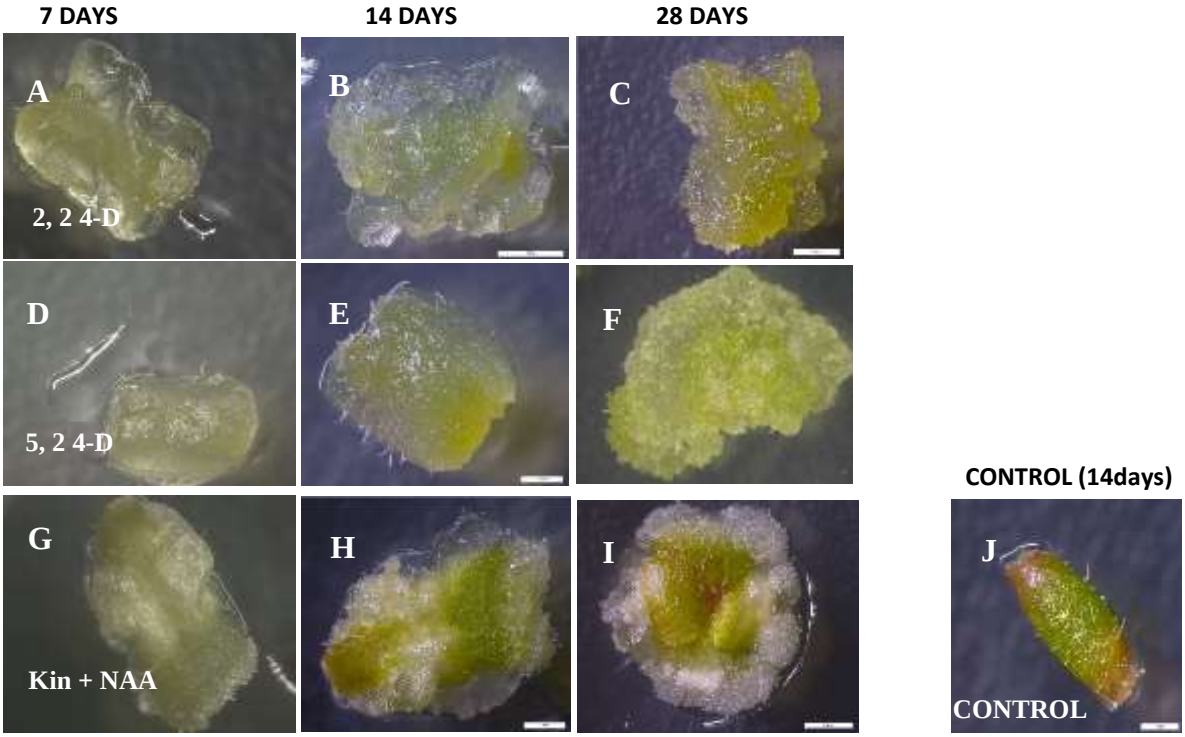


Figure 11. Second round of potato callus induction under light using selected media compositions. 7 days after induction (A,D,G) 14 days (B,E,H, J) 28 days (C,F,I). Induction media used tested include: 2 mg/L 2,4-D (A,B,C); 5 mg/L 2,4-D (D,E,F), 0.5 mg/L Kinetin + 5 mg/L NAA (G,H,I) and Control (media without) after 14 days (J). Scale bar of 1 mm for figures (E,H,J) and 2 mm for (B,C,I)

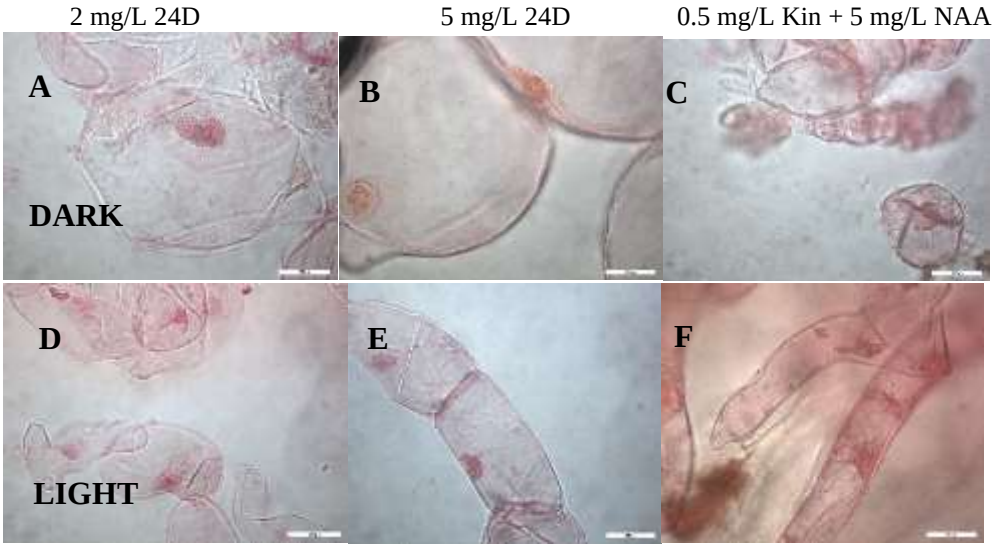


Figure 12. Embryonic test of potato callus induced under dark (A,B,C) and light (D,E,F). Media types include: 2 mg/L 24D (A,D); 5 mg/L 24D (B,E), 0.5 mg/L Kinetin + 5 mg/L NAA (C,F). (Callus images for other dark media types were lost and not included but showed a bright red colouration of the nucleus). Scale bar :200µm.

3.1.2 Callus regeneration

The initial (first) regeneration test without the media (0.9 mg/l Thiamine-HCl, 0.8mg/L zeatin, 2mg/L GA3) was hindered by contamination as wells as exposure to the extremely light intensity (50 µmol/m^2/s) (due to wrong labelling on the shelf) after 6 weeks. No sign of regeneration was then observed at that time. (Figure 13, Table 8).

After the repetition (second) of the regeneration under the preferred light intensity (30 µmol/m^2/s) with the inclusion of the new media (0.9 mg/l Thiamine-HCl, 0.8mg/L zeatin, 2mg/L GA3), possible signs of regeneration were observed after 2 months (Figure11, D, E, F, G). Although the percentage of regeneration was low in all the media compositions, the media with (0.22mg/L TDZ + 0.49 mg/L NAA) had a relatively higher regeneration percentage (40%) (Table 8). Unfortunately, samples could not be rescued after contamination and had to be discarded to prevent further spread of the contamination in the laboratory and growth rooms. (Figure 18 D).

Figure 13 Potato shoot regeneration from callus in different media compositions. First round of regeneration (A,B,C) and the second /repeated regeneration (D,E,F,G). Scale bar: 1mm (A and B) and 2 mm for the others.

Table 8. Percentages of potato callus regeneration in the different media composition. The First round was only 6 weeks due to thrips invasion and subsequent contamination, the second round lasted 2 months.

Round	**Media name**	**Hormone concentrations**	**Number of callus used**	**Number of regenerated**	**Percentage of formation**
First round (6 weeks)	Media A	0.22mg/L TDZ + 0.49 mg/L NAA	12	0	0 %
	Media B	3mg/l BA+ 0.5 mg/l GA3 + 0.03mg/l NAA	12	0	0 %
	Control	No hormone	6	0	0 %
Repeated round (2 months)	Media A	0.22mg/L TDZ + 0.49 mg/L NAA	10	4	40 %
	Media B	3mg/l BA+ 0.5 mg/l GA3 + 0.03mg/l NAA	10	2	20 %
	Media C	0.9 mg/l Thiamine-HCl, 0.8mg/L zeatin, 2mg/L GA3	10	1	10 %
	Control	No hormone	6	1	16 %

3.2 Biolistic transformation

3.2.1 Transformation efficiencies of single and double events of biolistic transformation (GUS)

The transformant were observed to be localized in the single event and more spread in the double transformation event. An average of 10 transformants was obtained after a single shoot compared to 27 transformants after double shooting using the Gus plasmid. This showed an approximately a 63% increase in the rate of transformation after the double bombardment (Figure 14, Table 9, Appendix 8)

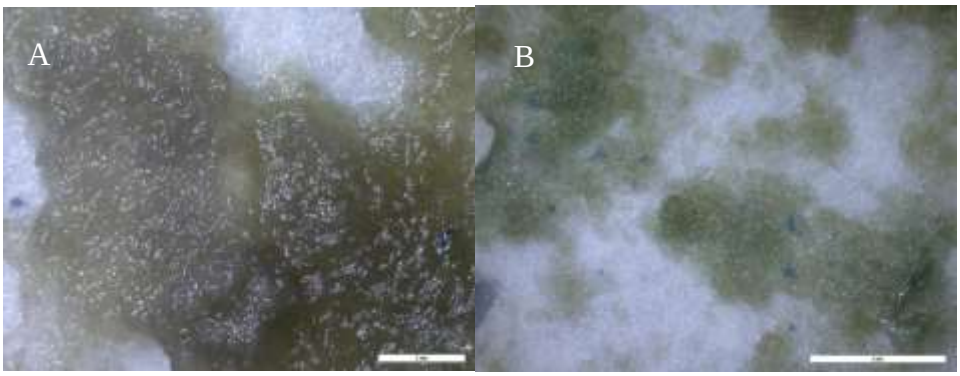


Figure 14. Transient gene expression after single (A, scale bar =2mm) and double (B, scale bar=3mm) shooting per calli with the GUS.

Table 9. Average number of transformants after single and double biolistic transformation events

Number of shots	Shoot samples	Average transformants	Standard deviation
Control (no plasmid)	1	0	NA
single	3	10	2.0
Double	3	27	3.6

3.2.2 Dosage of kanamycin dosage response

After 3 weeks, all the explants on the different kanamycin concentrations (50,100, 150 and 200 mg/L of kanamycin) had darker/dying callus compared to the control which remained green (Figure 15). With 100% dark/dying calli observed on all the kanamycin containing media, 50 mg/L, being the lowest kanamycin concentration was selected as the concentration for determining between the transformed and untransformed calli.

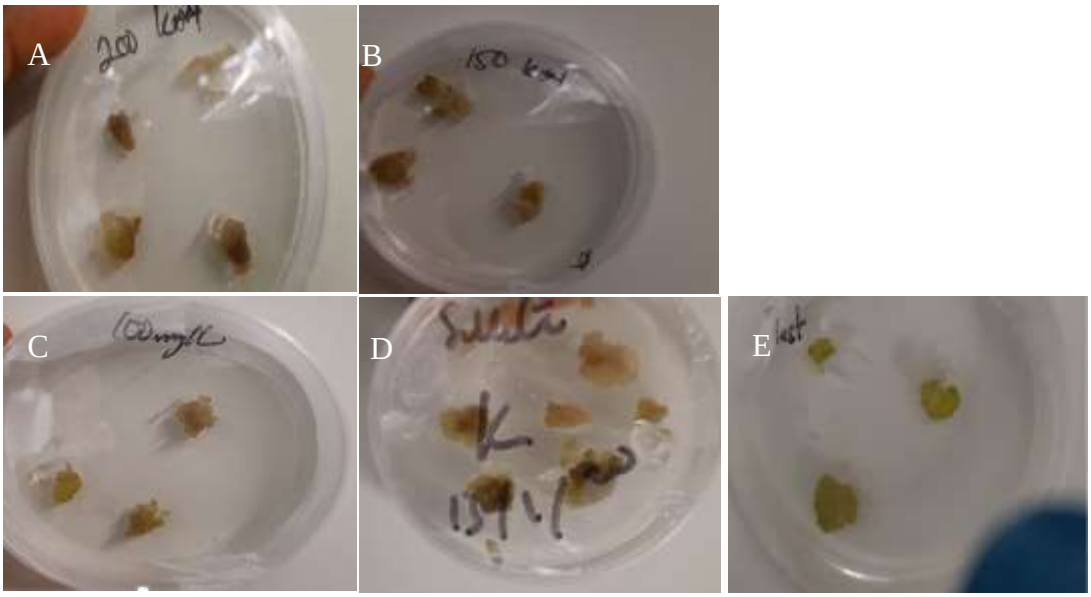


Figure 15: Potato callus on different kanamycin concentrations after 3weeks. 200 mg/L (A),150 mg/L (B) and 100 mg/L (C), 50 mg/L (D) and E (control / no kanamycin).

3.3 Protoplast isolation and viability test

Protoplast isolation was successful with approximately 37 x10^8 isolated protoplasts from 2 petri dishes. The visualization of the protoplast after staining showed that the protoplasts isolated were viable due to the observed green fluorescence from the FDA stain (Figure 16).

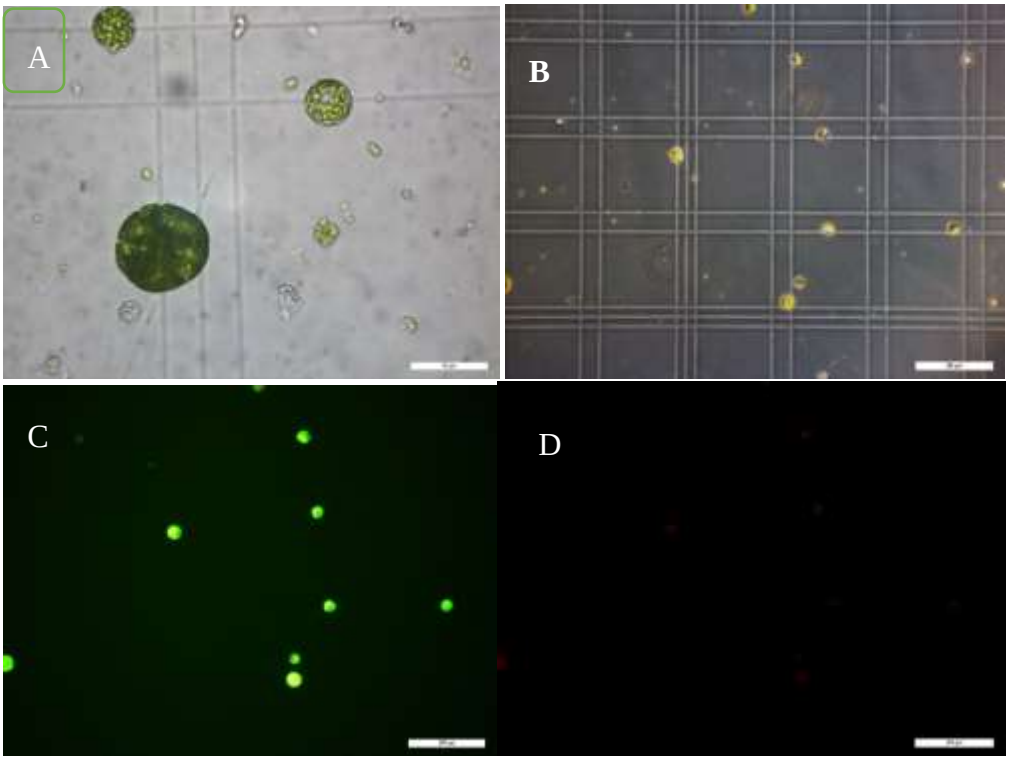


Figure 16. Potato protoplast image under bright field (A, scale bar 50µm), phase contrast (B, scale bar 200µm), fluorescence protoplast stained using FDA (C, scale bar 200µm) and fluorescence stained using Propidium iodide (D, scale bar 200µm)

3.3.1 Protoplast regeneration and contamination

Though visible signs of protoplast growth were observed within the protoplast lenses, the regeneration media surrounding the protoplast lenses turned cloudy due to contaminants (Figure 17; A, B). The control media (media without protoplast lenses) remained clear with no contamination.

For the first and second transformation and regeneration, the contaminants were identified to be yeast due to the budding structure observed and the fermented scent of the media were whiles the third (done in a different room) had highly mobile bacteria contaminants (Figure 17; C, D).

With the continuous breaking of the fragile protoplast lenses due to contamination as well as the weekly washing (to reduce contamination) and media change, the protoplast regeneration events were hindered. Though antibiotics were included in the regenerating media in the third (3rd) round, the colour of the media still became cloudy (though not as deep as before) indicating the presence of the contaminants.

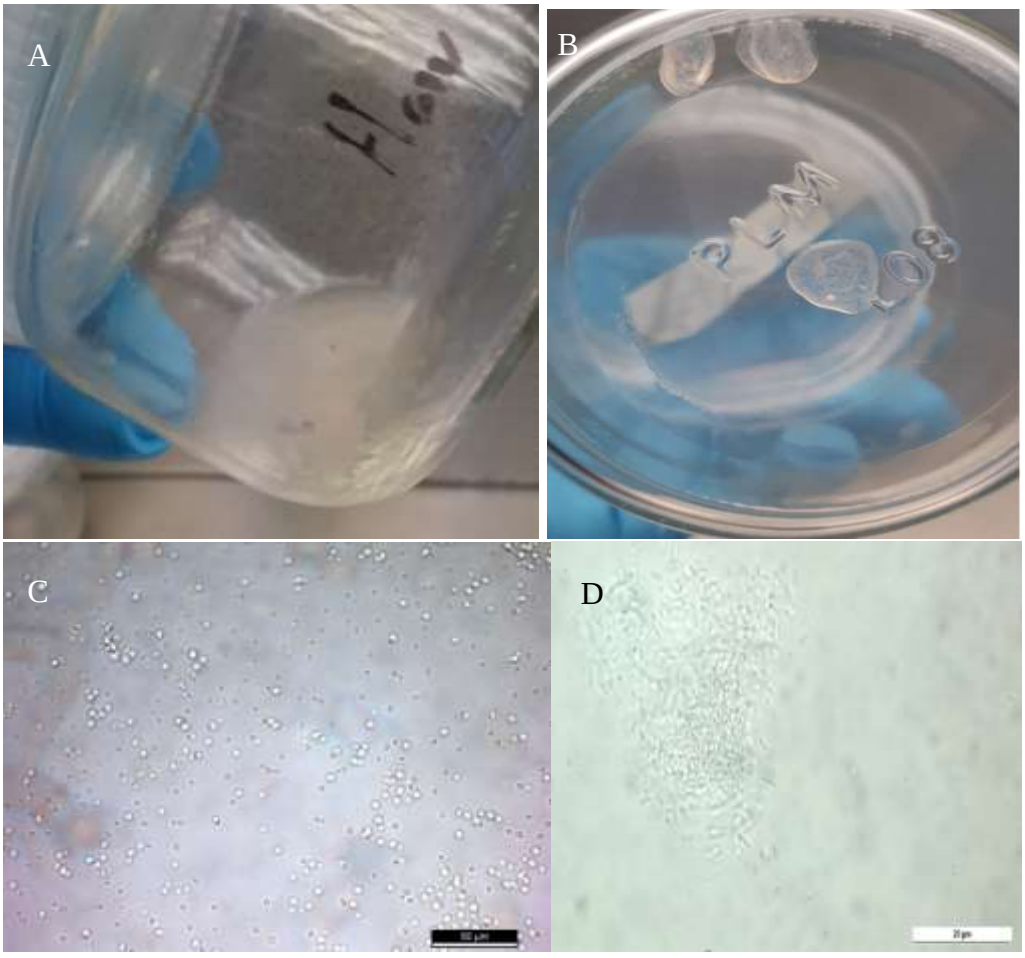


Figure 17. Contamination of transformed potato protoplast during regeneration. Cloudy media due to contamination (A), transformed protoplast in the alginate lenses before media change (B), the image of yeast infestation in the first and second regeneration events (C, bar =50 µm) and bacterial contamination in the thirds round of regeneration (in constant motion) (D, bar=20 µm). Images of the yeast and bacterial contamination were taken using the Leica M205 stereo microscope.

3.4 Further explanation of the contamination

With the consistent contamination starting around sub-cultured in-vitro explants in the media, the few remaining uncontaminated explants were screened on the 'LLB' media to obtain clean (contaminant-free) in-vitro material to sub-culture and use during the experiment (Figure 18, A,B, C). With the observed trails observed in the media, the yellow sticky insect trap used showed that a major cause of the contaminations was caused by thrips, though other causes could be due to overlooked/poor aseptic technique (Figure 18, D, E)

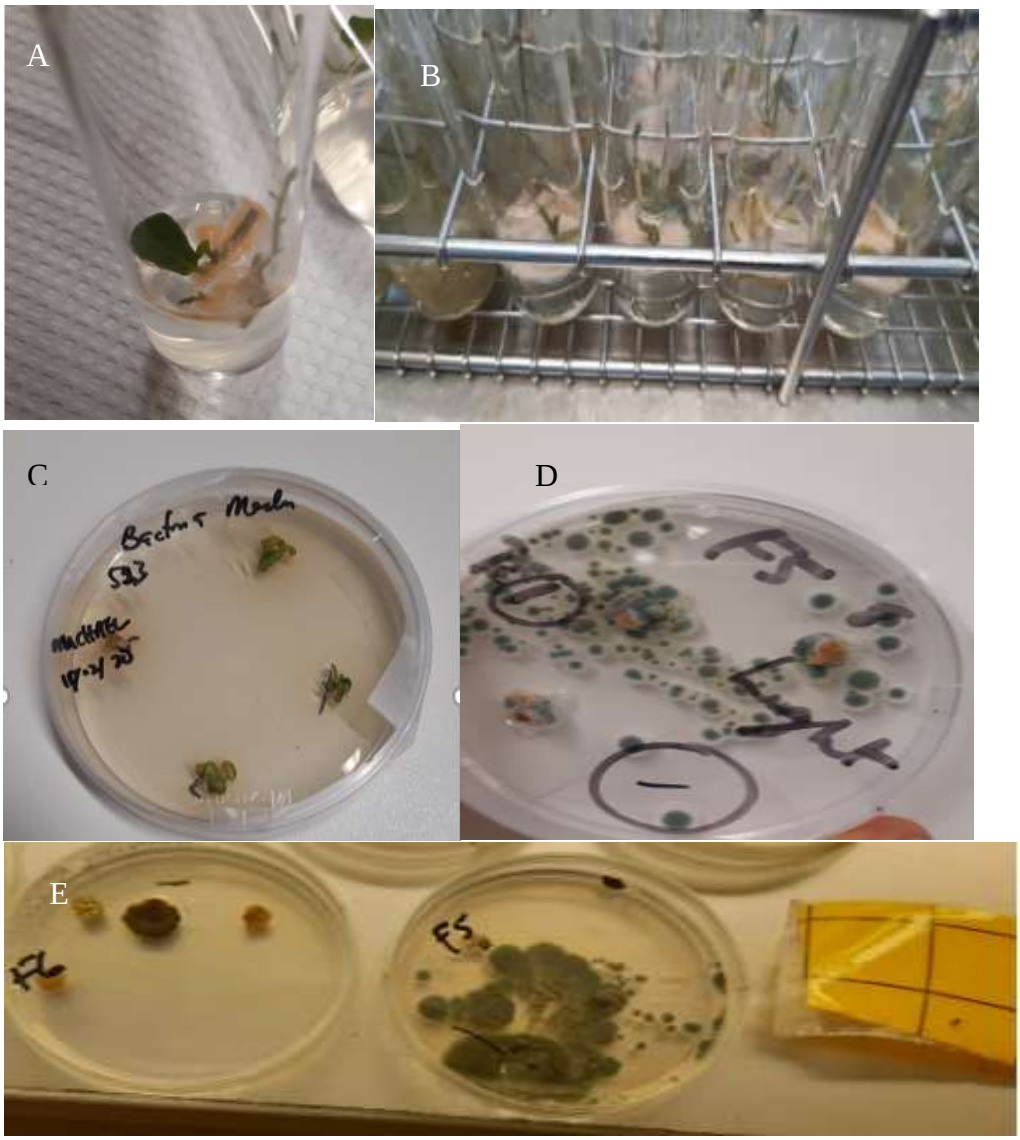


Figure 18. Contaminations and screening for contaminant free in-vitro potato explants. Contamination of media around the explant (A), growth of contaminates after 3 weeks (B), screening of un-contaminated explants for multiplication of explants after (2 weeks) and contamination due to thrips and poor aseptic techniques (D, E). Footprints of the thrips can be clearly seen as distinct dot not connected with any potato plant tissue (D, E).

3.5 Primer testing and sequencing results

After the gradient PCR and running of the PCR products on the gel (gel electrophoresis), it was observed that the fifth lane showed clearer visible bands (indicating high PCR products) and the corresponding temperature 53°C was selected as the optimum annealing temperature (Figure 19). An additional faint band was observed above the expected band in the gel run using the PCR products obtained with '*StF3H* 1' primer pair (Figure 19, A). A barrier due to uneven gel setting was also observed in the gel with the amplified regions using the '*StF3H* 2' primer pair resulting in the poor band formation/ separation (Figure 19, B). After 3 independent sequencing and read alignment of the selected PCR products, poor/ noisy reads (peaks) were obtained from using primer '*StF3H* 1' primer pair set while identical and consistent reads (peaks) were obtained using the '*StF3H* 2' (Figure 20, A,B, C; Appendix 9). Variation in nucleotides were also observed after alignment with the *F3H* gene used in primer design (Figure 20, C). The blasted results of the sequenced reads obtained using the '*StF3H* 2' primer pair showed a 99% identity and an expectation value of (e-value) of (0.0) for all 3 sequence results to the gene of interest (*F3H*) compared to the different identities (99%, 98%, 97% and respective e-values (1e-176, 3e-169, 4e-174) obtained using '*StF3H* 1'primer pair (Appendix 10). Though a repetition to get clearer images were not possible due to the corona pandemic, the '*StF3H* 2' primer pair was selected to use for the analysis of transformants.

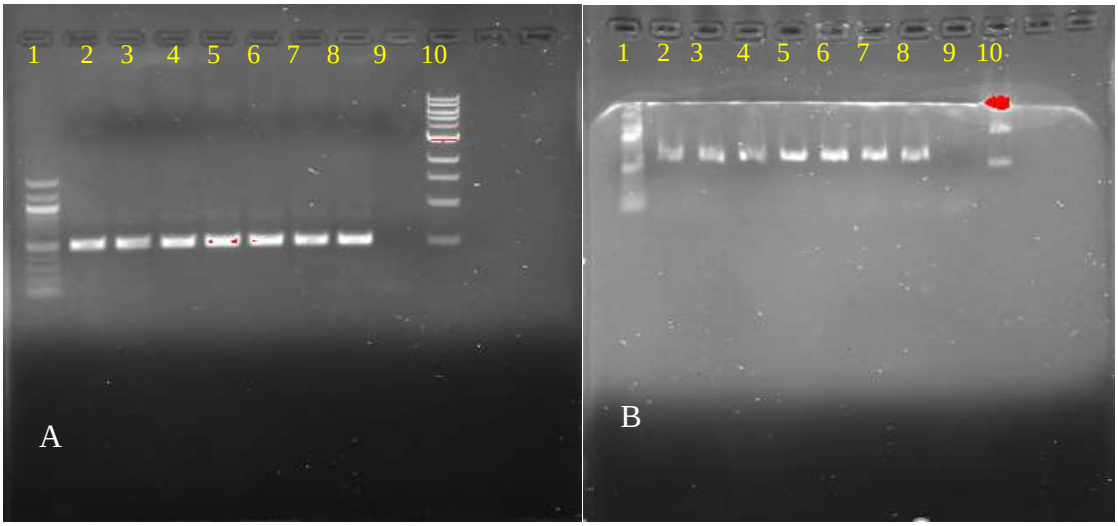
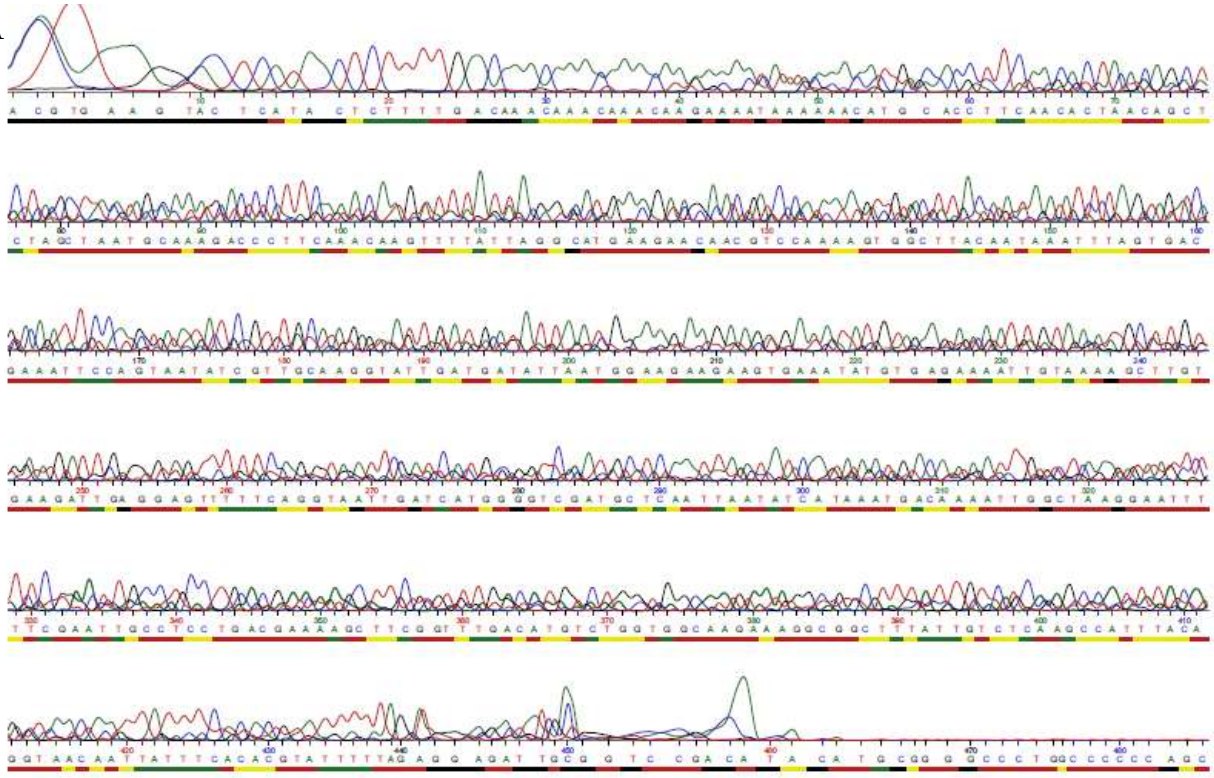
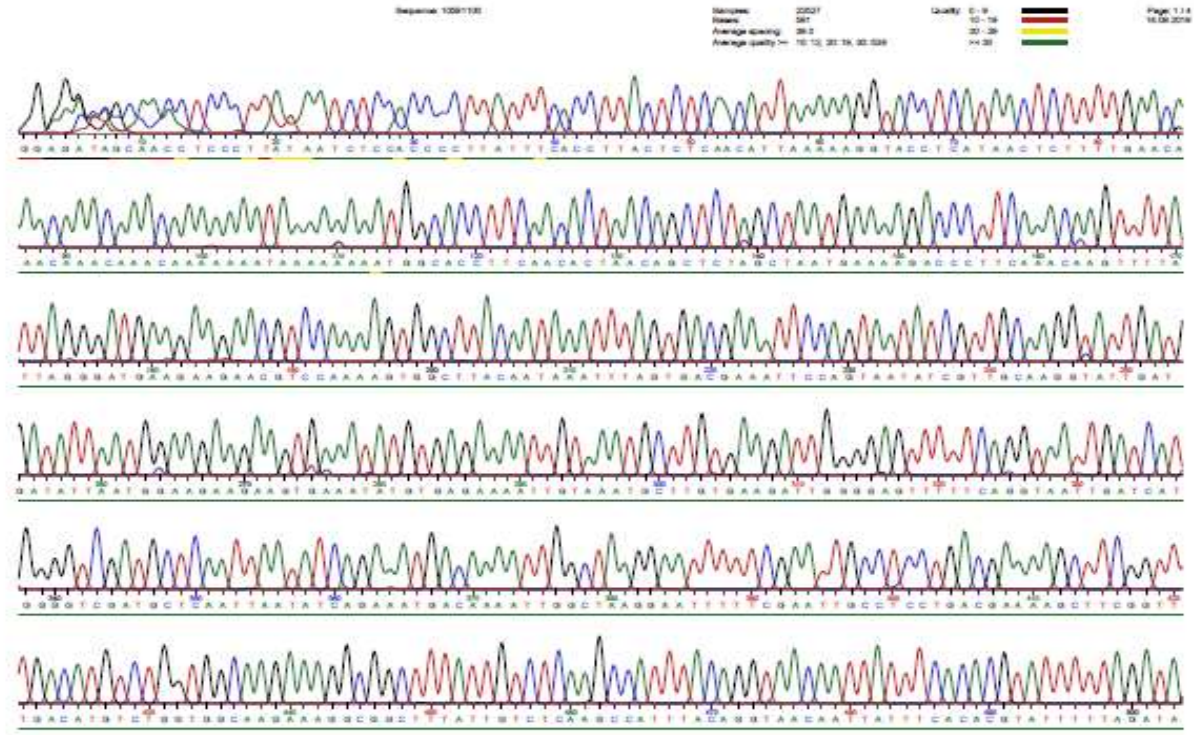


Figure 19. Results after gel electrophoresis of gradient PCR products from Nansen with *StF3H* 1 primer pair (A) and *StF3H* 2 (B). 100bp ladder (1), Gradient PCR annealing temperatures (2 to 8), negative control with no template (9) and 1kb ladder (10). The selected lane (5) with an annealing temperature of 53 °C based on the visible bands was selected.

A



B



C

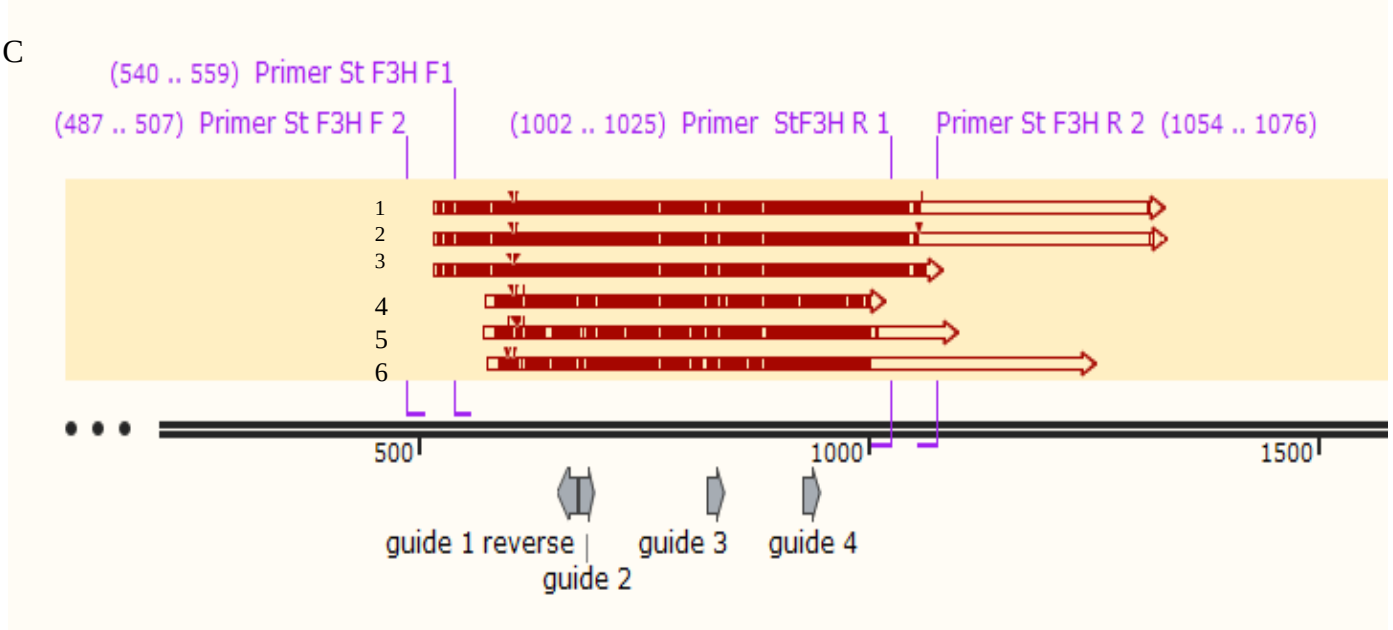


Figure 20. Sequencing results of amplified sequence using the 2 different primer pairs. Poor/noisy sequencing reads obtained using the '*StF3H* 1' primer set (A), distinct sequence reads with no noise using '*StF3H* 2' primer set (B), aligned sequence read using primer set '*StF3H* 2' (1,2,3) and '*StF3H* 1' (4,5,6) to the gene used in the design of primers (C)

4.0 Discussion

4.1 Callus Induction and regeneration

The exogenous application of auxin and cytokinin in intermediate levels have been used in callus induction for a number of plant species, though little is still known at the molecular level (Ikeuchi et al., 2013; Pan et al., 2010). Diverse morphological and physiological characteristics of potato callus due to media composition and genotype has been documented in several potato experiments (Al-Hussaini et al., 2015; Iqbal et al., 2014; Kumlay & Ercisli, 2015). These variations in observed characteristics have been attributed to factors of which include variations in endogenous levels of phytohormones within individual cultivar and conditions of in-vitro growth (light and temperature) (Kumlay & Ercisli, 2015). As expected in this experiment, the different media composition and light conditions (light and dark) resulted in observed variations in morphological characteristics of calli. These variations could be a result of the complex molecular response mechanism resulting in the observed phenotypic changes.

In light of diverse potato callus induction media compositions, varying concentrations of 2,4-D have been employed for callus induction (Khalafalla et al., 2010; Omidi & Shahpiri, 2003; Turhan, 2004). However, while higher 2,4-D concentrations (4.0 mg/L) have been reported to increase somaclonal variation causing cell death or regeneration of abnormal potato plantlets, lower 2,4-D concentrations (2.0 mg/L) are reported to enable the regeneration of normal healthy plantlets (Hoque & Morshad, 2014). Pan et al., (2010) also reported that, the increase in 2,4-D concentrations (6.0 mg/L) induces osmotic stress and inhibits the initiation of somatic embryos.

Furthermore, with the limited reports on the effect of light and dark on potato callus induction, potato callus has been induced under varied conditions light conditions in several experiments (Al-Hussaini et al., 2015; Iqbal et al., 2014; Nadakuduti et al., 2019). According to Islam (2015), tobacco callus (a model plant species) is known to induce better and more embryonic callus under light compared to darkness, probably due to the development of the photosynthetic pigments (chlorophyll) that influence growth and development. Omidi & Siddique (2003) also indicated that, more embryogenic potato calli are obtained under the light conditions compared to darkness. According to Taiz et al., (2015), the development of the light-harvesting pigments (chlorophyll) of photosynthetic plants/cells results in the characteristic green colouration which is less developed in an etiolated plant (long, skinny and white plants produced in the absence of light).

With the observed characteristic green calli produced under the light condition compared to dark condition in this experiment, the colour change could be attributed to the relative development of the photosynthetic pigments of the cell under the prevailing conditions of growth.

Gupta & Holmstrom (2005) also indicated that, embryogenic cells are characterized as having a dense cytoplasm and a large nucleus which shows a bright red coloration after acetocarmine staining. However, though a bright red colouration of the nucleus was observed after the staining with acetocarmine in this experiment, the embryogenic of the callus could not be confirmed due to the relatively smaller nuclear sizes and require further trials where regeneration is observed.

Based on the observed results in these experiments , though 5.0 mg/L of 2,4-D produced bigger and more friable callus under both light conditions compared to 2.0 mg/L of 2,4-D, the lowest concentration (2.0 mg/L) was selected to reduce the risk of somaclonal variation to a minimum, to regenerate a high number of healthy plantlets after the transformation.

According to Al-Hussaini et al. (2015), regeneration is a complex process and is affected by several external and endogenous factors and could have a cumulative effect. Despite the low regeneration percentage observed, medium A (0.22mg/L TDZ + 0.49 mg/L NAA) had the highest regeneration percentage (40%) compared to the other media (30% and 10%). However, though a 40% frequency with signs of regeneration was observed after callus induction with 2mg/L 2 4-D in this experiment, the finding of Al-Hussaini et al. (2015) showed a 0% and 25% callus regeneration percentage after callus induction with 2mg/L 2,4-D and 2mg/L 2,4-D + 2mg/L BA respectively, in the cultivar they used. The observed differences could be attributed to variation in cultivar/varietal response due to the endogenous hormones concentration (Kumlay & Ercisli, 2015). However, based on the observed increase in regenerated callus after induction with 2mg/L 2,4-D + 2mg/L of BA, there could be a possibility of having more regeneration percentage of the 'Nansen' calli in the regeneration medium A after induction in media with 2mg/L 2,4-D + 2mg/L of BA.

With the low regeneration percentage obtained in medium A in this experiment, there is the possibility of having few regenerated transformants which could falsely indicate a reduction in transformation efficiency of the transformation method (biolistic). Since the success of callus induction and regeneration is dependent on genotype/ variety, media composition and prevailing conditions (Kumlay & Ercisli, 2015), as well as mother plant treatment mother plant treatments

(Hvoslef-Eide, 1990; Hvoslef-Eide, 1991a; Hvoslef-Eide, 1991b), it would be beneficial to primarily obtain an optimized regeneration protocol for 'Nansen' before transformation to obtain unbiased transformation efficiency.

Though this was not part of the main objectives of the thesis, it was conducted as a preliminary study to determine a suitable induction and regeneration protocol for 'Nansen' prior to the biolistic transformation as well as foundation for further detailed qualitative and quantitative experimentation. I would, however, suggest the inclusion of the potato induction and regeneration protocol as described by Nadakuduti, et al., (2019) in subsequent optimization experiments though variation in response should be expected due to the genotypic/varietal differences.

4.2 Contamination of in vitro cultures

Contamination in tissue culture is unfortunately commonly faced either through faulty laboratory procedures or through the presence of microorganisms outside or within the tissues of explants (Cassells, 1991). However, with culture vesicle having closed but loose-fitting caps to allow gaseous exchange with the external environment, mites and thrips are known to travel though larger pores as well as loose/poorly sealed vesicles (Bhojwani & Dantu, 2013; Blake, 1988). Their invasion also results in bacterial and fungal contamination as they carry fungal spores and bacteria within and on their bodies (Blake, 1988). Moreover, since bacterial and fungal contaminants often grow faster, the introduction of these organisms on sugar-rich media enhances their growth compared to explants (Bhojwani & Dantu, 2013; Blake, 1988).

With the source of the thrip unknown, the massive invasion resulting in the contamination in the callus induction and regeneration encountered during this project could (to a low extent) be attributed to faulty/poor aseptic techniques (cracked/ poorly sealed vessels, poor sterilization). Moreover, though the observed contaminants around the sub-cultured explants could have originated from within the explants, faulty aseptic procedure during sub-culturing could have contributed to the introduction and spread of contaminants around explants. In retrospect, NIBIO has admitted that their laboratories have been infested with thrips around the time when the Plant Cell Laboratory (PCL) also was infected. Since the only transfer between these laboratories have been the potato cultures of 'Nansen' from NIBIO to PCL, it is highly likely that NIBIO's laboratory were the first to be infected (Trine Hvoslef-Eide, pers. comm. 2020).

Also, the hindered protoplast regeneration due to the high infection rate could be partly attributed to the sugar-rich liquid media used, which is highly favourable for rapid growth of micro-organism. With no contamination in some control media (media without protoplast), the source of contamination was not certain. However, since the first and second regeneration events performed in the same room showed only yeasts contaminants whiles the third regeneration event, done in a different room showed bacteria contaminates, the source of contamination was determined to have been from the environment (external). Furthermore, with the lengthy protoplast isolation protocol requiring frequent movement out of the flow hood, a poor aseptic technique could have contributed to the introduction of these contaminants. To add to the problem, the lack of the initial sterilization of the (believed to be) aseptic plant material obtained from NIBIO in the followed protocol could have been a cause of contamination and should be included in future events, when transferring cultures between laboratories.

Although the use of antibiotics has been reported to be an effective means of bacterial control in tissue culture (Coelho et al., 2012), it is known to either result in the development of resistant bacterial strains or just suppress the growth and development of bacteria (Fogh, 2012). With the continuous observation of a cloudy media after the addition of antibiotics, a thorough identification of the bacterial would have been better to determine the best antibiotics to use to control contaminants but this was not done, as it would have been beyond the time frame of this master thesis.

4.3 Transformation methods and comparisons

Since the pandemic resulted in an abrupt halt in the laboratory work, and the transformation methods could not be compared as originally planned. However, the section below discusses the possible outcomes of the transformation methods based on the preliminary observations during the project and a literature study.

4.3.1 Biolistic transformations

With several reports having indicated varied effects of the number of bombardment events for efficient transient GUS expression, multiple bombardment events have been reported to be beneficial for increased transient GUS expression (Hafiza et al., 2010; Parveez et al., 1998; Petrillo et al., 2008; Romano et al., 2001) . Moreover, the number of observed transients GUS expressions are reported to be significantly reduced after 3 days of calli growth (Parveez et al., 1998).

Based on the preliminary results obtained during this project, a 63% increase in transient GUS expression (blue colouration) was observed after the double bombardment event compared to the single bombardment. The low number of observed transformants in both the single (10) and double (27) bombardment events could be due to the prolonged recovery period (7 days) before the determination of transformants. With the observed increase in transformed cells after the double bombardment, more transformants are expected with either transiently expressed genes, stable integrations or both within the genome of the target cells.

With no selection marker required during transient gene expressions, increasing the number of biolistic transformations increases the tendency of having more transformed plants for screening. Moreover, with current reports indicating the high tendency of obtaining transiently transformed plant using ribonucleoproteins with synthetically produced RNA guides (cr-RNP) compared to plasmid DNA and in-vitro transcriptionally produced RNP (IVT-RNP) (Andersson et al., 2018), multiple bombardment events using cr-RNPs could increase the chances of obtaining transiently transform plants.

Although the transformation protocol of the laboratory was followed in this experiment, the pre-treatment of the calli with both 0.1 M sorbitol and 0.1 M mannitol to induce plasmolysis in future

biolistic transformation should be considered to further increase the transformation efficiency (Romano et al., 2001). The induction of plasmolysis promotes the contraction of the plasma membrane from the cell wall due to of loss of water from the cell (Taiz et al., 2015) and could reduces the effect of the direct impact of the projected particle during the biolistic transformation. However, sorbitol and mannitol concentrations and treatment duration before transformation should be considered since increased concentrations (0.4M) are detrimental to cells due to extreme water loss from the cells (Romano et al., 2001).

To reduce the cost and labour of analysis for identification of transformants, the method employed (division of callus lumps and having their replica on selection and non-selection media) in the project was not optimum since some transiently transformed calli (cells) may be lost. However, this could have reduced the sample size for analysis since only callus lumps on the non-selective media with their other half (replica) dying on the selection media would be analysed callus as they may either be untransformed, or have transiently expressed the gene. An optimum method would have been to regenerate all transformed cells without selection to increase the number of regenerated plants to analyse and determine transformants. Also, a random selection of 3-4 seedlings derived from each callus lump could be employed to reduce the cost and labour in analysing all the samples (Liang, Z. et al., 2017).

However, the biolistic transformation technique still remains an important transformation method since it enables the delivery of more and diverse genetic cargo (Dalla Costa et al., 2019; Hafiza et al., 2010; Liang et al., 2019; Prakash & Varadarajan, 1992; Romano et al., 2001) with increasing application for transient gene expression using RNP (Liang, Z. et al., 2017; Svitashev et al., 2016).

4.3.2 PEG-mediated transformations

Though limited studies of PEG-mediated transformation in potatoes using the CRISPR/Cas9 have been conducted (Andersson et al., 2018; Dangol et al., 2019), this method has been employed in both transient gene expression and stable integrations based on the genetic cargo (DNA or RNP) for gene editing (Andersson et al., 2017; Andersson et al., 2018; Dangol et al., 2019; Liang, Z. et al., 2017).

According to the findings of Andersson et al. (2017), 2% of the regenerated lines of the tetraploid potato ‘Kuras’ showed knockouts in all four alleles of the granule-bound starch

synthase (*GBSS*) gene after PEG-mediated transformations using naked DNA. With 2–12 % of regenerated shoots having at least one allelic mutation, 67% of the mutant lines had multiple alleles mutation. Most of these mutations resulted in indels of 1-10 bp as well as vector DNA inserts of 34-236 bp.

Andersson et al. (2018) also reported that, the use of cr-RNP resulted in a 9 % induced mutation with transformants being transgene-free. On the other hand, though a mutation frequency of 25% was observed using the IVT-RNP, 80% of the regenerated shoots had unintended inserts at the cut sites with DNA template from the in-vitro transcription. Also, 2-3% of the regenerated shoots from the RNP-experiment had mutation is all the four alleles resulting in the complete knockout of the granule-bound starch synthase (GBSS) enzyme function. Liang et al., (2017) indicated that, the use of RNP in gene editing in wheat resulted in a lower off-target of 5.7 % mutations in the mutant lines compared to the 35.6% when using plasmid DNA.

The extrapolated results of Craig (2005) indicated that, the extended use of low PEG concentrations (12.5% for 15 min) results in an increase in the number of transformants compared to higher concentrations over a shorter time (25% for 5 min). Andersson et al., (2018) also indicated that, relatively higher number of mutant lines could be obtained at a higher PEG concentrations with extended duration (40% PEG for 30 min), compared to lower concentrations for a shorter time (25% PEG for 3 min). This could be attributed to incomplete transfection though high PEG concentrations could be toxic to cells (Andersson et al., 2018). With 420 regenerated shoots after using 25% PEG compared to the 33 regenerated shoots after using 45% PEG, it is evident that, both the duration and the PEG concentration affects the rate of transformation and number of regeneration plant. Based on the setup in this project, the use of different PEG concentrations (40% and 25%) for the same time duration (30 min) would have aided in determining an optimal protocol for ‘Nansen’.

The delivery of cr-RNP using the CRISPR/Cas9 tools as a method for potato breeding has good potential in yielding transgene-free commercial lines compared to using DNA (Andersson et al., 2018). However, though a developed protoplast isolation, transfection and regeneration protocol could be employed for other potato varieties during gene editing (Andersson et al., 2018; Nicolia et al., 2015), continuous optimization of a protocol seems to be vital for improved success in future

gene editing applications with higher transgene-free knock-outs ratios (Andersson et al., 2018; Dangol et al., 2019; Sakamoto et al., 2020; Tuncel et al., 2019; Veillet et al., 2019).

4.3.3 Comparison of biolistic and PEG transformation

Since transformants were unfortunately not analysed within the project timeframe due to loss of protoplast by contaminations and coronavirus outbreak, much cannot be said about the transformation efficiencies of the used methods. However, both transformation methods seemed to be promising for transient gene expression since they are not restricted to certain species though all tissue culture protocols require optimized protocols due to observed genotypic/ varietal response. Moreover, the use of the cr-RNP seems to be a better means to obtain transgene-free mutant lines and should be tested again using both methods in further studies.

4.4 Primer testing and sequencing results.

The macromolecule separation achieved in a gel matrix during electrophoresis is influenced by the electrical current forcing the charged macromolecules (negative charge of the DNA) to migrate from the negative electrode to the positive electrode according to their charge, size, as well as density of the gel (Maurye et al., 2017). At a constant voltage, the pace at which the DNA fragments move are dependent on size and density of the gel matrix, though movements are faster in less dense gel matrix (Lee et al., 2012). However, uneven casting of the gel could contribute to the formation of barriers within the gel matrix restricting the flow of larger fragments (Lee et al., 2012; Maurye et al., 2017) as observed during this project resulting in the poor band separation in Figure 19B.

Moreover, successful sequencing reactions are characterised as having distinct and evenly separated single peaks with no background signals, which are indicating of excellent primer binding and optimised primer design (Genomics, 2020). The poor and varied reads obtained using '*StF3H* 1' primer pair set could be attributed to several factors of which include; poor primer binding, unspecified binding due to unoptimized annealing temperature as well as contamination. However, with the high percent identities and low e-values of the sequences obtained with the

'*StF3H* 1' primer pair set to the *F3H* gene (Appendix 10), the observed background noise observed could be attributed to contamination by a foreign DNA or amplification of a different gene. These sequences could have been confirmed by excising the bands and sequencing rather than the PCR products, but there was no time to repeat and check this.

With reports of possible conserved sequences in potato varieties (Bernal-Galeano et al., 2020; Li et al., 2018), the observed nucleotide variation after alignment with the *F3H* gene used in primer design indicates the existence of specific nucleotide sequences identified only in 'Nansen'. It would have been beneficial to repeat the gradient PCR and gel to confirm the annealing temperatures of both primer pairs, but this could not be done due to the corona pandemic. In light of the consistency of sequenced reads with high identity and an expectation value as well as longer length of the amplified region, the '*StF3H* 2' primer pair was selected to use for the analyses of transformants with an annealing temperature of 53°C.

4.5 Analysis of transformants.

With the use of dual sgRNA complex for both the biolistic and PEG-mediated transformation, fragment length of 150bp and 250 bp were expected to be excised respectively (plasmid 1 and 2. Table 2) after PCR and visualization on the products gel electrophoresis.

However, a low simultaneous cut frequency of 0.7% has however been observed with the use of dual sgRNA with relatively higher single cut frequency (Veillet et al., 2019). This indicates that single cuts with possible nucleotide insertions, substitution or deletions would be mostly observed along the cut sites due to the imprecise DNA repair mechanism (Klimek-Chodacka et al., 2018). Also, due to the high DNA insertion rate of 2.2% (Veillet et al., 2019) compared to the cr-RNP with no insertion (Andersson et al., 2018), more of transiently transformed plants could be observed from PEG-mediated transformation using RNP compared to the Biolistic transformation methods where DNA plasmids could result in some stable integrations. To add to this, there is an increased possibility of having single cuts from the PEG-mediated methods since the RNP were assembled individually and combined before transformation compared to the DNA plasmid that has both sgRNA in one plasmid. The increased possibility of single cuts for PEG-mediated

methods could be attributed to the uneven distribution and introduction of dual RNP into a single cell.

However, by the end of the experimental period, the plants could not be regenerated to obtain large enough leaf samples for analysis. Though DNA could be extracted from 100 mg of freeze dried calli using the Dneasy kit according to the protocol, the obtained DNA would not be from cells with identical genome (from a single cell). They would have been from over a million different cells (composed of either transformed and untransformed cells) with each cell having the potential of developing into a single plant. Also, since transformed cells tend to initially grow quite slower than wild type/ untransformed cell cultures due to the tissue damage and recovery period after the biolistic transformation (Dalla Costa et al., 2019; Romano et al., 2001), the ratio of the transformed compared to the wildtype/ untransformed cells would be relatively low due to exponential multiplication of callus (Dalla Costa et al., 2019; Santos et al., 2017).

Even with the newly acquired ddPCR in our laboratory, having the capacity to screen 20,000 droplets/cell at a time (Bio-rad, 2020), the possibility of some positive cell reaction would be extremely low. This challenge would also be experienced using the normal PCR and running of the gel since the transformed cells might be overshadowed by the wildtype /untransformed cells. It would, however, be beneficial to regenerate plants and obtain leaf samples for analysis to increase the chance of detecting transformants as well as visually observing the colour change of tubers formed in time.

5.0 Conclusions

With no transformants analysed due to the slow progress because of contaminations as well as the outbreak of the coronavirus during the timeframe of the experiments, the success and frequency of the intended genetic change (knocking out of the *F3H* gene) could not be assessed within the timeframe. To determine the best method for transient gene expression is therefore impossible at this point. Nevertheless, though media test for callus induction and regeneration was not the main focus of this experiment, optimizing callus induction and regeneration protocol for 'Nansen' is also a vital step prior to transformation to increase regeneration percentages or obtain an increase number of shoots to identify putative transformants.

However, with the increased possibility of having transgene-free transformants with ribonucleoprotein (cr-RNP), its application in practical breeding should be considered in further trials for accelerating precision in crop improvements. Also, with the new trend of public acceptance to improved transgene-free crops, the acceptance and the implementation of the proposed tier-based system for GMO in Norway would be vital for further research and for practical implementations in the Norwegian plant breeding.

6.0 References

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7.0 Personal communication

Anders Wulff-Vester (2020)

Andersson Mariette (2019)

Trine Hvoslef- Eide (2020)

8.0 Appendices

Appendix 1 QIAGEN DNeasy Plant Mini Kit according to its protocol

1. Disrupt samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the TissueRuptor®, the TissueLyser II or a mortar and pestle.
2. Add 400 µl Buffer AP1 and 4 µl RNase A. Vortex and incubate for 10 min at 65°C. Invert the tube 2–3 times during incubation. Note: Do not mix Buffer AP1 and RNase A before use.
3. Add 130 µl Buffer P3. Mix and incubate for 5 min on ice.
4. Recommended: Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm).
5. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x g.
6. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.
7. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x g (≥8000 rpm). Discard the flowthrough. Repeat this step with the remaining sample.
8. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 1 min at ≥6000 x g. Discard the flow-through.
9. Add another 500 µl Buffer AW2. Centrifuge for 2 min at 20,000 x g. Note: Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.
10. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
11. Add 100 µl Buffer AE for elution. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g.
12. Repeat step 11.

Appendix 2 PCR component

Table 10. PCR components used in Reaction

	PCR components	**Master mix for 10 reactions**	**For 20µl per PCR reaction**
Master mix	5x Taq buffer	40 µl	4 µl
	10mM dNTPs	4.0 µl	0.4 µl
	10µM forward primer	4.0 µl	0.4 µl
	10µM reverse primer	4.0 µl	0.4 µl
	Nuclease free water	127µl	12.7 µl
	One Taq polymerace	1.0 µl	0.1 µl
	DNA template	----	2

Appendix 3 Invitrogen Subcloning Efficiency™ DH5α™ Competent Cells protocol

1. Thaw on ice one tube of DH5α™ cells. Place 1.5 ml microcentrifuge tubes on wet ice.

2. Gently mix cells with the pipette tip and aliquot 50 µl of cells for each transformation into a 1.5 ml microcentrifuge tube.

3. Refreeze any unused cells in the dry ice/ethanol bath for 5 minutes before returning to the -80°C freezer. Do not use liquid nitrogen.

4. Add 1 to 5 µl (1-10 ng) of DNA to the cells and mix gently. Do not mix by pipetting up and down. For the pUC19 control, add 2.5 µl (250 pg) of DNA to the cells and mix gently.

5. Incubate tubes on ice for 30 minutes.

6. Heat shock cells for 20 seconds in a 42°C water bath without shaking.

7. Place tubes on ice for 2 minutes. 8. Add 950 µl of pre-warmed medium of choice to each tube.

9. Incubate tubes at 37°C for 1 hour at 225 rpm.

10. Spread 20 µl to 200 µl from each transformation on pre-warmed selective plates. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, plate 100 µl on an LB plate containing 100 µg/ml ampicillin.

11. Store the remaining transformation reaction at +4°C. Additional cells may be plated out the next day, if desired. Incubate plates overnight at 37°C.

Appendix 4 Qiagen Plasmid Midi Kit according to the protocol

Quick-Start Protocol March 2016

QIAGEN® Plasmid Mini, Midi and Maxi Kits

The QIAGEN Plasmid Mini Kit (cat. nos. 12123 and 12125), the QIAGEN Plasmid Midi Kit (cat. nos. 12143 and 12145), the QIAGEN Plasmid Maxi Kit (cat. nos. 12162, 12163 and 12165) and the Plasmid Buffer Set (cat. no. 19046) can be stored at room temperature (15–25°C) for up to 2 years if not otherwise stated on label.

Further information

- QIAGEN Plasmid Purification Handbook: **www.qiagen.com/HB-1193**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Add RNase A solution to Buffer P1, mix and store at 2–8°C.
- **Optional**: Add LyseBlue® reagent to Buffer P1 at a ratio of 1:1000.
- Prechill Buffer P3 at 4°C. Check Buffer P2 for SDS precipitation.
- Isopropanol and 70% ethanol are required.
- Symbols: ● QIAGEN Plasmid Mini Kit; ▲ QIAGEN Plasmid Midi Kit; and ■ QIAGEN Plasmid Maxi Kit.

Table 1. Recommended LB culture volumes

Kit	High-copy plasmid	Low-copy plasmid
QIAGEN Plasmid Mini	3 ml	Not recommended
QIAGEN Plasmid Midi	25 ml	100 ml
QIAGEN Plasmid Maxi	100 ml	500 ml

1. Harvest overnight bacterial culture by centrifuging at 6000 x *g* for 15 min at 4°C.
2. Resuspend the bacterial pellet in ● 0.3 ml, ▲ 4 ml or ■ 10 ml Buffer P1.

Sample to Insight

3. Add ● 0.3 ml, ▲ 4 ml or ■ 10 ml Buffer P2, mix thoroughly by vigorously inverting 4–6 times and incubate at room temperature (15–25°C) for 5 min. If using LyseBlue reagent, the solution will turn blue.
4. Add ● 0.3 ml, ▲ 4 ml or ■ 10 ml prechilled Buffer P3, mix thoroughly by vigorously inverting 4–6 times. Incubate on ice for ● 5 min, ▲ 15 min or ■ 20 min. If using LyseBlue reagent, mix the solution until it is colorless.
5. ●: Centrifuge at 14,000–18,000 x *g* for 10 min at 4°C. Re-centrifuge if the supernatant is not clear. ▲ and ■: Centrifuge at ≥20,000 x *g* for 30 min at 4°C. Re-centrifuge the supernatant at ≥20,000 x *g* for 15 min at 4°C.
6. Equilibrate a QIAGEN-tip ● 20, ▲ 100 or ■ 500 by applying ● 1 ml, ▲ 4 ml or ■ 10 ml Buffer QBT, and allow column to empty by gravity flow.
7. Apply the supernatant from step 5 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
8. Wash the QIAGEN-tip with ● 2 x 2 ml, ▲ 2 x 10 ml or ■ 2 x 30 ml Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow.
9. Elute DNA with ● 0.8 ml, ▲ 5 ml or ■ 15 ml Buffer QF into a clean ● 2 ml, ▲ 15 ml or ■ 50 ml vessel. For constructs larger than 45 kb, prewarming the elution buffer to 65°C may help to increase the yield.
10. Precipitate DNA by adding ● 0.56 ml, ▲ 3.5 ml or ■ 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA and mix. Centrifuge at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.
11. Wash the DNA pellet with ● 1 ml, ▲ 2 ml or ■ 5 ml room-temperature 70% ethanol and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant supernatant.
12. Air-dry pellet for 5–10 min and redissolve DNA in a suitable volume of appropriate buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris-Cl, pH 8.5).

Figure 21. Qiagen Plasmid Midi Kit according to the protocol.

Appendix 5 Media composition and protoplast isolation protocol (Nicolia et al. et al (2015).

1. Internodes containing a single auxiliary bud of potato Cv. Desirée are propagated *in vitro* using plastic boxes containing 50 ml of Medium A and sealed with micropore tape. Growing conditions are: 24°C/20°C for 16h light / 8h dark.

2. About 20-30 leaves (1 g) from 4-6 weeks old plants are excised in sterile condition and placed with the abaxial side down in plastic petri dishes containing 20 ml of Medium B, then sealed with parafilm, covered with aluminum foil and incubated at 4 °C for 24 h.

3. Leaves are subsequently cut in slices (1-2 mm) using a sterile blade in a glass petri dish containing few ml of Medium B and collected in a fresh plastic petri dish containing 10 ml of medium B.

4. Medium B is removed and slices incubated in 20 ml of Plasmolysis solution for 30 min, petri dish is kept covered with an aluminum foil and at room temperature (RT).

5. Plasmolysis solution is removed and substituted with 25 ml of Medium C, petri dish is sealed with parafilm, wrapped in aluminum foil and incubated overnight (ON) at 25°C without shaking.

6. The next day the petri dish is incubated for 10 min at RT with very gentle shaking; the solution will turn green due to released protoplasts.

7. Two sterile filters of 100µm and 70µm are mounted together on a 50 ml tube and pre-wetted with 5 ml of Wash solution. The solution containing released protoplasts is gently aspirated with a pipette and sieved trough the filters, remaining protoplasts are washed from the filters using other 5 ml of Wash solution.

8 The sieved protoplasts suspension is transferred to sterile 15 ml centrifuge tubes (8 ml per tube), and the tubes toped up to 15 ml with additional Wash solution. The suspension is centrifuged at 50 x *g* (minimum acceleration and deceleration) for 5 min. Supernatant is subsequently discarded and protoplasts gently re-suspended in 2 ml of Wash solution.

9. Fresh sterile 15 ml centrifuge tubes each containing 6 ml of Sucrose solution are prepared and a maximum of 6 ml of re-suspended protoplasts slowly layered on top with a sterile Pasteur pipette, taking care that the interface is not disrupted. The tubes are subsequently centrifuged at 50 x *g* for 15 min (minimum acceleration and deceleration), a thick dark band of protoplasts should appear at the interface of the two solutions.

10. A fresh sterile 15 ml centrifuge tube containing 3 ml of Transformation buffer 1 is prepared. Using a pipette with a cut tip, the floating protoplasts are gently transferred from the previous step in the tube. A small amount (10-20 ul) of protoplasts is used to quantify density (protoplasts/ml) using a haemocytometer; protoplasts in Transformation buffer 1 are stored at 4°C in the dark during counting.

11. Protoplasts are centrifuged at 50 x *g* for 10 min (minimum acceleration and deceleration), supernatant is subsequently discarded and protoplasts gently re-suspended in Transformation buffer 2 at the concentration of 1.6 x 10^6 protoplasts/ml.

12. Fresh sterile 15 ml centrifuge tubes are prepared for each transfection or control (i.e. PEG + / pDNA +; PEG + / pDNA -; PEG - / pDNA -). About 10µg of plasmid DNA (from 10 µl up to 20 µl) are pipetted in each tube followed by 100 ul of protoplasts in Transformation buffer 2 (approximately 160,000 protoplasts).

13. A volume ranging from 110µl to 120 µl of PEG solution, accordingly to the volume of plasmid DNA used, is gently added to each tube (tubes are gently flicked before and after adding the PEG solution). Samples are incubated at RT for 3 min.

14. Transfection reactions are stopped adding carefully 5 ml of Wash solution to each tube and subsequently centrifuged at 50 x *g* for 5 min (minimum acceleration and deceleration)

15. Supernatant is discarded and transfected protoplasts or controls gently re-suspended in 1 ml of Medium E. The same volume of Alginate Solution is added to give a final density of 8 x 10^4 protoplasts/ml.

16 The two solutions are gently mixed inverting the tubes and the solution is transferred in aliquots (usually 4 big drops) to the surface of solid Setting agar. The drops are left at RT for maximum of 2 h to allow solidification of alginate.

17 The alginate lens are subsequently released from the surface of Setting agar with the help of 2-3 ml of Floating solution and moved to fresh petri dishes containing 10 ml of Medium E. Petri dishes are sealed with parafilm, covered with aluminium foil and incubated at 25 °C for 3 days.

18. After 3 days light is gradually increased replacing aluminium foil with a white paper foil. Once protoplast mini calli are visible to the naked eye (usually after 3 weeks) Medium E is replaced with 10 ml of Medium F and calli exposed to full light by this stage (fresh Medium F is provided every week).

19. After 4-6 weeks in Medium F, calli are released from alginate drops adding 5 ml of Releasing solution and incubating for a maximum of 10 min; a forceps or a tip can be gently used to help releasing. The Releasing solution is carefully aspirated and calli washed with 10 ml of Medium F, released calli are then incubated in 10 ml of Medium G for other 4-6 weeks (fresh Medium G is provided every week).

20. Large green calli are then briefly dried on a sterile filter paper, moved individually on petri dishes containing solid Medium H and incubated in the same conditions used for potato propagation.

21. Calli are moved on fresh Medium H each 10-15 days, shoots usually emerge after 3 months of culture.

22. Mature shoots are moved to solid Medium I for rooting and plantlets moved to Medium A

Table 12. Media and solutions recipes

Medium A	**For 1 L**
½ MS salts and organics (Duchefa)	2.2 g
Vit. MS stock	0.5 ml
Sucrose	15 g
Agar	8 g
pH	5.6 (KOH)
* filter sterilize in ½ of the volume, then add to autoclaved agar in the other half	

Medium B*	**For 1 L**
MS modif. No. 4 (Duchefa)	2.7 g
Vitamins NN stock	0.1 ml
Casein hydrolysate	100 mg
NAA	2 mg
BAP	0.5 mg
pH	5.8 (KOH)
* filter sterilize	

Plasmolysis solution*	**For 1 L**
D-Sorbitol	91.1 g (0.5 M)
* filter sterilize	

Medium C *	**For 1 L**
Macro stock	10 ml
$CaCl_2$ stock	3 ml (6 mM)
Iron stock	10 ml
Micro stock	1 ml
Vit. mix 1 stock	5 ml
Vit. mix 2 stock	5 ml
Vit. mix 3 stock	5 ml
Sugars stock	20 ml
Organic acids stock	10 ml
Casein hydrolysate	500 mg
Glucose	36.95 g (0.205 M)
Mannitol	37.35 g (0.205 M)
PVP 10	20 g
NAA	1 mg

BAP	0.4 mg
Cellulase RS (Yakult)	10 g
Macerozyme (Yakult)	2 g
pH	5.6 (KOH)
* add all the components with the exception of $CaCl_2$, then check the pH and incubate at 55°C for 10 min. Let to cool down at RT, add $CaCl_2$ and filter sterilize. Prepare fresh.	

Wash solution*	**For 1 L**
Macro stock	10 ml
$CaCl_2$ stock	3 ml (6 mM)
Iron stock	10 ml
Micro stock	1 ml
NaCl	14.03 g (0.24 M)
NAA	2 mg
BAP	0.5 mg
pH	5.6 (KOH)
* filter sterilize	

Sucrose solution*	**For 1 L**
Sucrose	147.19 g (0.43 M)
* filter sterilize	

Transformation buffer 1*	**For 250 ml**
Mannitol	8.65 g (190 mM)
$CaCl_2 \cdot 2H_2O$	3.67 g (100 mM)
MES	1.25 g (0.5 % w/v))
pH	5.6 (KOH)
* filter sterilize	

Transformation buffer 2*	**For 50 ml**
Mannitol	4.55 g (0.5 M)
$MgCl_2 \cdot 6H_2O$	152 mg (15 mM)
MES	50 mg (0.1 % w/v))
pH	5.6 (KOH)
* filter sterilize	

PEG solution*	**For 10 ml**
PEG 4000 (Fluka)	2.5 g (25 % w/v)
Mannitol[a]	5 ml (0.4 M)
$Ca(NO_3)_2$[b]	500 ul (0.1 M)
*filter sterilize, prepare fresh	

[a] use a 0.8 M filter sterilized stock solution
[b] use a 2 M filter sterilized stock solution

Medium E *	**For 1 L**
Macro stock	10ml
$CaCl_2$ stock	1.25 ml (2.5 mM)
Iron stock	10 ml
Micro stock	1 ml
Vit. mix 1 stock	5 ml
Vit. mix 2 stock	5 ml
Vit. mix 3 stock	5 ml
Sugars stock	20 ml
Organic acids stock	10 ml
Casein Hydro lysate	500 mg
Glucose	33.7 g (0.17 M)
Mannitol	30.92 g (0.17 M)
BSA	1 g
NAA	1 mg
BAP	0.4 mg
pH	5.6 (KOH)
*filter sterilize	

Alginate solution*	**For 500 ml**
Alginic acid-Na salt	14 g (2.8% w/v))
Sorbitol	36.44 g (0.4 M)
* autoclave and store at 4°C	

Setting agar*	**For 1 L**
Sorbitol	72.88 g (0.4 M)
$CaCl_2 \cdot 2H_2O$	7.351 g (50 mM)
Phyto agar	8 g
* filter sterilize in ½ of the final volume, then add to autoclaved agar in the other half	

Floating solution*	**For 1 L**
Sorbitol	72.88 g (0.4 M)
$CaCl_2 \cdot 2H_2O$	7.351 g (50 mM)
* filter sterilize	

Medium F*	**For 1 liter**
MS modif. No. 4 (Duchefa)	2.70 g

NH_4Cl	107 mg
Vit. NN stock	1 ml
Adenine sulphate	40 mg
Casein hydrolysate	100 mg
Sucrose	2.5 g
Mannitol	54.7 g
NAA	0.1 mg
BAP	0.5 mg
pH	5.8 (KOH)
*filter sterilize	

Releasing solution*	**For 100 ml**
Na-Citrate	588.2 mg (20 mM)
Sorbitol	9.11 g (0.5 M)
* filter sterilize	

Medium G*	**For 1 L**
MS modif. No. 4 (Duchefa)	2.70 g
NH_4Cl	267.5 mg
Vit. NN stock	1 ml
Adenine sulphate	80 mg
Casein hydrolysate	100 mg
Sucrose	2.5 g
Mannitol	36.4 g
IAA	0.1 mg
Zeatin	2.5 mg
pH	5.8 (KOH)
* filter sterilize	

Medium H *	**For 1 L**
MS salts and organics (Duchefa)	4.4 g
Sucrose	10 g
Zeatin	2 mg
NAA	0.01 mg
GA_3	0.1 mg
Gelrite (Duchefa)	2.5 g
pH	5.8 (KOH)
* filter sterilize in ½ of the final volume, then add to	

autoclaved gelrite in the other half

Medium I*	**For 1 L**
MS salts and organics (Duchefa)	4.4 g
Sucrose	20 g
GA3	0.1 mg
Gelrite (Duchefa)	2.5 g
pH	5.8 (KOH)
* filter sterilize in ½ of the final volume, then add to autoclaved gelrite in the other half	

Vitamins MS stock*	**For 50 ml**
Thiamine-HCl	5 mg
Pyridoxine-HCl	25 mg
Nicotinic acid	25 mg
Glycine	100 mg
Myo-Inositol	5000 mg
* filter sterilize. Store aliquots at -20°C	

Macro stock*	**For 1 l**
KNO_3	74 g
$MgSO_4 \cdot 7H_2O$	49.2 g
KH_2PO_4	3.4 g
* filter sterilize. Store at 4°C	

$CaCl_2$ stock*	**For 100 ml**
$CaCl_2 \cdot 2H_2O$	29.4 g (2M)
* filter sterilized. Store at 4°C	

Iron stock*	**For 100 ml**
Na_2EDTA	140 mg
$FeSO_4 \cdot 7H_2O$	190 mg
* filter sterilized. Store at 4°C	

Micro stock*	**For 100 ml**

H_3BO_3	150 mg
$MnSO_4 \cdot H_2O$	500 mg
$ZnSO_4 \cdot 7H_2O$	100 mg
$Na_2MoO_4 \cdot 2H_2O$	12 mg
$CuSO4 \cdot 5H_2O$	1,2 mg
$CoCl_2 \cdot 6H_2O$	1,2 mg
KI	38 mg
* filter sterilized. Store at 4°C	

Vit. mix 1 stock*	**For 100 ml**
Pantothenoic acid	50 mg
Choline Chloride	50 mg
Ascorbic acid	100 mg
p-Aminobenzoic acid	1 mg
Nicotinic acid	50 mg
Pyridoxine-HCl	50 mg
Thiamine-HCl	500 mg
* filter sterilize. Store aliquots at -20°C	

Vit. mix 2 stock*	**For 100 ml**
Folic acid	20 mg
Biotin	0,5 mg
Cyanocobalamin (Vit. B12)	1 mg
* filter sterilize. Store aliquots at -20°C	

Vit. mix 3 stock*	**For 100 ml**
Cholecalciferol (Vit. D)	0.5 mg
* filter sterilize. Store aliquots at -20°C	

Sugars stock*	**For 100 ml**
Sorbitol	625 mg
Sucrose	625 mg
D(-)Fructose	625 mg
D(-)Ribose	625 mg
D(+)Xylose	625 mg
D(+)Mannose	625 mg
L(+)Rhamnose monohydrate	625 mg
D(+)Cellobiose	625 mg
Myo-Inositol	250 mg

* filter sterilized. Store at 4°C

Organic acids stock*	**For 100 ml**
Pyruvic acid	100 mg
Fumaric acid	200 mg
Citric acid monohydrate	200 mg
DL-Malic acid	200 mg
* filter sterilized. Store at 4°C	

Vitamins NN stock*	**For 50 ml**
Glycine	100 mg
Myo-Inositol	5000 mg
Thiamine-HCl	25 mg
Pyridoxine-HCl	25 mg
Nicotinic acid	250 mg
Folic acid	25 mg
Biotin	2.5 mg
* filter sterilize. Store aliquots at -20°C	

Appendix 6 Protocol for RNP complex formation

1. Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA, tracrRNA, sgRNA) in IDTE buffer to a stock concentration of 100 µM.
2. If you are using sgRNA, dilute it to a working concentration of 10 µM (1:10 dilution) in IDTE Buffer, then go to the next section: Create the RNP complex.
3. Mix the crRNA and tracrRNA oligos in equimolar concentrations in a sterile microcentrifuge tube to a final duplex concentration of 10 µM. The following table shows an example of a 10 µL final volume duplex:

Component	Amount (µL)
100 µM Alt-R CRISPR-Cas9 crRNA	1
100 µM Alt-R CRISPR-Cas9 tracrRNA	1
Nuclease-Free Duplex Buffer	8
Total volume	10

4. Heat the duplex at 95°C for 5 min.
5. Remove from heat and allow to cool to room temperature (15–25°C).

Create the RNP complex

1. Combine the guide RNA and Cas9 enzyme in equimolar amounts.

Component	Amount (µL)
10 µM Alt-R guide RNA [From Prepare the guide RNA, step 2 (sgRNA) or step 5 (crRNA:tracrRNA)]	10
Alt-R S.p. Cas9 enzyme (62 µM stock)*	1.6
PBS†	88.4
Total volume	100

* All Alt-R S.p. Cas9 enzymes are provided at a stock concentration of 62 µM.

† Cas9 RNP complexes can be made in PBS or in Cas9 dilution buffer (30 mM HEPES, 150 mM KCl, pH 7.5).

2. Incubate at room temperature for 5–10 min for optimal formation of the RNP complex.

Figure 22. Protocol for RNP complex formation

Appendix 7 Sum, mean and statisitical difference between medias.

	mean	sum	sd	0%	25%	50%	75%	100%	data:n
Media 1(control)	0.00	0	0.0000000	0	0	0.0	0.0	0	2
Media 1(control) light	0.00	0	0.0000000	0	0	0.0	0.0	0	2
Media 10	10.00	40	0.0000000	10	10	10.0	10.0	10	4
Media 2	10.00	40	0.0000000	10	10	10.0	10.0	10	4
Media 2 light	10.00	40	0.0000000	10	10	10.0	10.0	10	4
Media 3	9.50	38	0.5773503	9	9	9.5	10.0	10	4
Media 4	10.00	40	0.0000000	10	10	10.0	10.0	10	4
Media 5	9.25	37	0.5000000	9	9	9.0	9.5	10	4
Media 5 light	10.00	40	0.0000000	10	10	10.0	10.0	10	4
Media 6	9.50	38	0.5773503	9	9	9.5	10.0	10	4
Media 6 light	10.00	40	0.0000000	10	10	10.0	10.0	10	4
Media 7	10.00	40	0.0000000	10	10	10.0	10.0	10	4
Media 8	10.00	40	0.0000000	10	10	10.0	10.0	10	4
Media 9	9.50	38	1.0000000	8	9	10.0	10.0	10	4

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
MEDIA.TYPE	13	359.1	27.621	182.5	<2e-16 ***
Residuals	38	5.8	0.151		

```
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 23. Statistical results from callus induction.

Appendix 8 Means and statistical difference from GUS staining

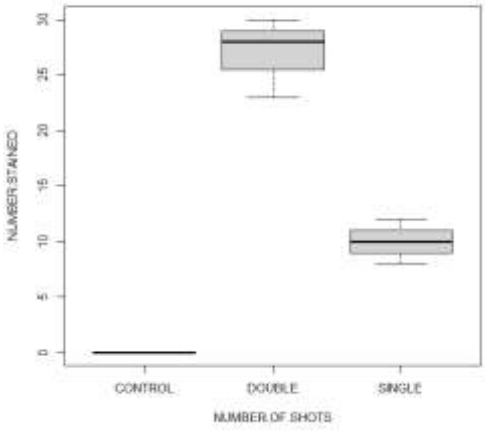


Figure 24. Box plot of the of the Gus transformed

```
Response: Gus.stains
                Sum Sq Df F value   Pr(>F)
Number.of.shots 726.86  2  42.756 0.001997 **
Residuals        34.00  4
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

	mean	sd	data:n
control	0	NA	1
double	27	3.605551	3
single	10	2.000000	3

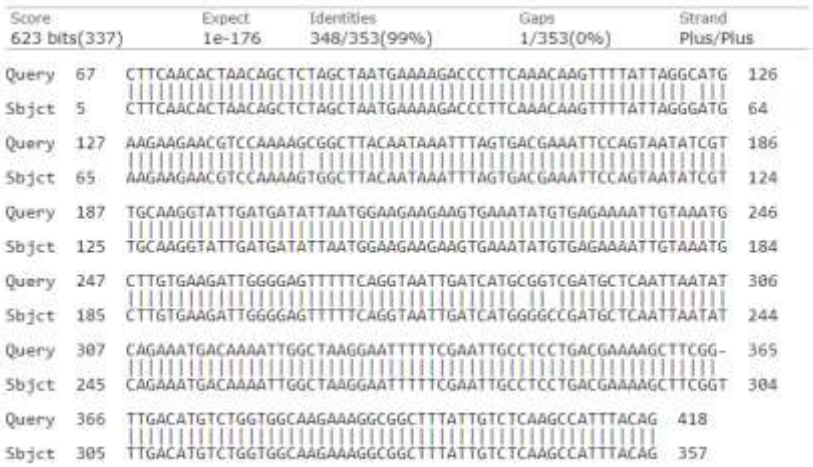
Figure 25. Statistical results from the Gus transformation.

Appendix 9 Sequenced reads images/ Electropherogram results

Figure 26. Sequenced images (electropherogram results)f of the *F3H* gene in 'Nansen' using primer set '*StF3H 1'* and '*StF3H 2'* from 3 independent reactions. Noisy Reads obtained using the primer set '*StF3H 1'* (A,B,C) and distinct and clear results obtained using primer set '*StF3H 2'*(D,E,F)

Appendix 10 Blast results on NCBI.

A



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Sbjct  5    CTTCAACACTAACAGCTCTAGCTAATGAAAAGACCCTTCAAACAAGTTTTATTAGGGATG  64

Query  127  AAGAAGAACGTCCAAAAGCGGCTTACAATAAATTTAGTGACGAAATTCCAGTAATATCGT  186
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Query  187  TGCAAGGTATTGATGATATTAATGGAAGAAGAAGTGAAATATGTGAGAAAATTGTAAATG  246
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Sbjct  125  TGCAAGGTATTGATGATATTAATGGAAGAAGAAGTGAAATATGTGAGAAAATTGTAAATG  184

Query  247  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGCGGTCGATGCTCAATTAATAT  306
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Sbjct  185  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGCCGATGCTCAATTAATAT  244

Query  307  CAGAAATGACAAAATTGGCTAAGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGG-  365
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Sbjct  245  CAGAAATGACAAAATTGGCTAAGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGGT  304

Query  366  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAG  418
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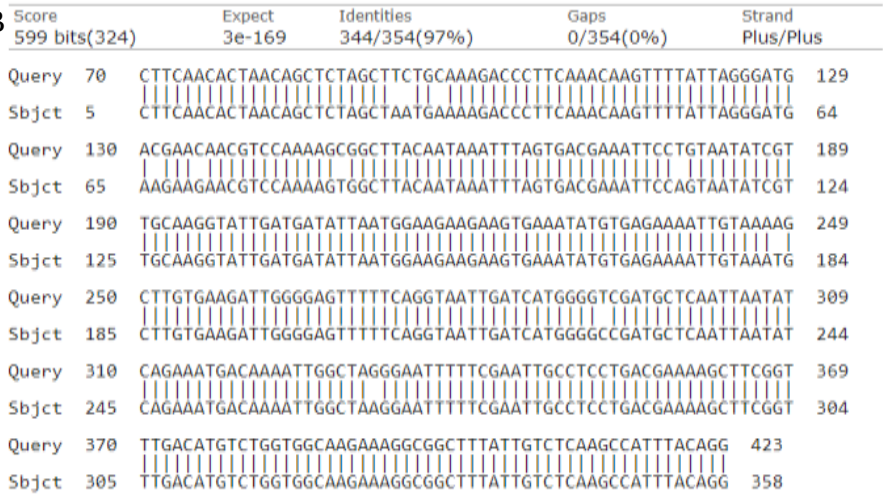
Sbjct  305  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAG  357
```

B



Score	Expect	Identities	Gaps	Strand
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Sbjct  125  TGCAAGGTATTGATGATATTAATGGAAGAAGAAGTGAAATATGTGAGAAAATTGTAAATG  184

Query  250  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGTCGATGCTCAATTAATAT  309
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Sbjct  185  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGCCGATGCTCAATTAATAT  244

Query  310  CAGAAATGACAAAATTGGCTAGGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGGT  369
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C

Score	Expect	Identities	Gaps	Strand
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Query  120  AAGAACAACGTCCAAAAGTGGCTTACAATAAATTTAGTGACGAAATTCCAGTAATATCGT  179
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Sbjct  185  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGCCGATGCTCAATTAATAT  244

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Sbjct  305  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAGG  358
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D

Score	Expect	Identities	Gaps	Strand
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Query  300  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGTCGATGCTCAATTAATAT  359
Sbjct  185  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGCCGATGCTCAATTAATAT  244

Query  360  CAGAAATGACAAAATTGGCTAAGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGGT  419
Sbjct  245  CAGAAATGACAAAATTGGCTAAGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGGT  304

Query  420  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAGG  473
Sbjct  305  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAGG  358
```

E

Score	Expect	Identities	Gaps	Strand
649 bits(351)	0.0	353/354(99%)	0/354(0%)	Plus/Plus

```
Query  120  CTTCAACACTAACAGCTCTAGCTAATGAAAAGACCCTTCAAACAAGTTTTATTAGGGATG  179
Sbjct  5    CTTCAACACTAACAGCTCTAGCTAATGAAAAGACCCTTCAAACAAGTTTTATTAGGGATG  64

Query  180  AAGAAGAACGTCCAAAAGTGGCTTACAATAAATTTAGTGACGAAATTCCAGTAATATCGT  239
Sbjct  65   AAGAAGAACGTCCAAAAGTGGCTTACAATAAATTTAGTGACGAAATTCCAGTAATATCGT  124

Query  240  TGCAAGGTATTGATGATATTAATGGAAGAAGAAGTGAAATATGTGAGAAAATTGTAAATG  299
Sbjct  125  TGCAAGGTATTGATGATATTAATGGAAGAAGAAGTGAAATATGTGAGAAAATTGTAAATG  184

Query  300  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGTCGATGCTCAATTAATAT  359
Sbjct  185  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGCCGATGCTCAATTAATAT  244

Query  360  CAGAAATGACAAAATTGGCTAAGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGGT  419
Sbjct  245  CAGAAATGACAAAATTGGCTAAGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGGT  304

Query  420  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAGG  473
Sbjct  305  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAGG  358
```

F

Score	Expect	Identities	Gaps	Strand
649 bits(351)	0.0	353/354(99%)	0/354(0%)	Plus/Plus

```
Query  120  CTTCAACACTAACAGCTCTAGCTAATGAAAAGACCCTTCAAACAAGTTTTATTAGGGATG  179
Sbjct  5    CTTCAACACTAACAGCTCTAGCTAATGAAAAGACCCTTCAAACAAGTTTTATTAGGGATG  64

Query  180  AAGAAGAACGTCCAAAAGTGGCTTACAATAAATTTAGTGACGAAATTCCAGTAATATCGT  239
Sbjct  65   AAGAAGAACGTCCAAAAGTGGCTTACAATAAATTTAGTGACGAAATTCCAGTAATATCGT  124

Query  240  TGCAAGGTATTGATGATATTAATGGAAGAAGAAGTGAAATATGTGAGAAAATTGTAAATG  299
Sbjct  125  TGCAAGGTATTGATGATATTAATGGAAGAAGAAGTGAAATATGTGAGAAAATTGTAAATG  184

Query  300  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGTCGATGCTCAATTAATAT  359
Sbjct  185  CTTGTGAAGATTGGGGAGTTTTTCAGGTAATTGATCATGGGGCCGATGCTCAATTAATAT  244

Query  360  CAGAAATGACAAAATTGGCTAAGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGGT  419
Sbjct  245  CAGAAATGACAAAATTGGCTAAGGAATTTTTCGAATTGCCTCCTGACGAAAAGCTTCGGT  304

Query  420  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAGG  473
Sbjct  305  TTGACATGTCTGGTGGCAAGAAAGGCGGCTTTATTGTCTCAAGCCATTTACAGG  358
```

Figure 27. Blast results obtained for 'Nansen' after amplification of *F3H* gene using the '*StF3H* 1'Primer pair (A,B,C) and '*StF3H* 2'Primer pair (D,E,F).

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