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# Gene Editing of Cultivated and Wild Strawberry (*Fragaria x ananassa* and *Fragaria vesca*) with CRISPR/Cas9 Knocking out *F3H*.

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## Abstract

The goal of this thesis was to transform the cultivated strawberry, *Fragaria x ananassa*, and the wild strawberry, *Fragaria vesca*, using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) alongside CRISPR associated protein 9 (Cas9). The CRISPR/Cas9 system would be used to induce a knock-out mutation in the *F3H* gene of both the cultivated and wild strawberry. The *F3H* gene is an important gene in the anthocyanin pathway and, if knocked-out, it is postulated that the production of anthocyanins would cease. This could result in strawberries that are unable to produce color pigments, yielding white berries or berries with different shades of pigmentation. This would serve as a proof-of-concept for CRISPR/Cas9 in these strawberries.

The strawberry is an important horticultural crop in Norway. However, environmental challenges and disease control are challenging aspects of the production of this crop. Due to reduced availability of legal fungicides, the challenges increase each time a fungicide is taken off the list of approved pesticides in Norway. Climate changes, such as more rain and wet weather during the maturation phase of strawberries, add to the possibility of fungal diseases and hence, yield losses. To face these challenges, the development of new cultivars is important. The CRISPR/Cas9 system allows the precise introduction of mutations in the plant genome, as well as the deletion and insertion of larger fragments of DNA. These attributes allow for a more precise and stable deletion, or introduction, of traits to a cultivar than traditional breeding techniques, which is a long and winding road toward success. The CRISPR/Cas9 system could, therefore, be an important tool when breeding new cultivars. This could also be another tool to introduce novel genes for resistance from wild strawberries through cis-genesis of wild characters into the gene pool of cultivated strawberries.

The octoploid nature of the cultivated strawberry makes it an interesting test of the capabilities of the CRISPR/Cas9 system. When this thesis was planned, few attempts at CRISPR/Cas9 mediated mutation of the strawberry had been done. The goal of this thesis was, therefore, to develop a protocol for the CRISPR/Cas9 mediated transformation of the cultivated and wild strawberry. The goal of this thesis was not achieved, due to technical challenges coupled with the closure of available laboratory facilities because of the Covid19 pandemic. However, an extensive troubleshooting process of the seamless assembly method presented by Li et al. (2015) explains potential challenges of plasmid construction. Also, successful attempts at direct regeneration of strawberry explants show that it is possible to circumvent the use of callus when using CRISPR/Cas9, thus possibly avoiding the increased risk of somaclonal variation from the use of callus.



Keywords: CRISPR/Cas9, seamless assembly, Plant breeding, cultivated strawberry, wild strawberry.

## Sammendrag

Målet med denne oppgaven var å genredigere hagejordbær, *Fragaria x ananassa*, og markjordbær, *Fragaria vesca*, med CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9). CRISPR/Cas9 systemet ville bli brukt til å indusere mutasjoner i *F3H* genet til både hage- og markjordbær. Hypotesen var at dette ville føre til en forstumming (silencing) av *F3H*. *F3H* spiller en viktig rolle i syntesen av anthocyaniner og en forstumming av dette genet kan føre til at produksjonen av anthocyaniner opphører. Dette kan resultere i jordbær uten evne til å produsere fargepigmenter og dermed bær som er hvite eller har en uventet pigmentering. Disse resultatene ville bevist at CRISPR/Cas9 mediert mutasjon kan brukes i disse jordbærene.

Jordbær er den viktigste frukt- og bærveksten i Norge. Dessverre er det flere utfordringer assosiert med dyrking av denne veksten, blant annet, skader påført av hardt vær og sykdom. Ettersom flere plantevernmidler ikke lenger er tillat for bruk i Norge, blir disse utfordringene større. Klimaendringer, som regn og vått vær under modningsfasen til jordbær øker også risikoen for soppinfeksjon og derav, tap av avling. For å møte disse utfordringene er det viktig å utvikle nye kultivarer. CRISPR/Cas9 systemet gjør det mulig å presist introdusere mutasjoner i planters genom. I tillegg gir det også mulighet til å fjerne eller sette inn større fragmenter av DNA. Disse egenskapene gjør det mulig å presist og stabilt, innføre eller fjerne egenskaper hos en kultivar. Dette kan også gjøres mer effektivt enn det som er mulig når en bruker tradisjonell planteforedling hvor veien mot suksess er lang og smal. CRISPR/Cas9 systemet kan derfor være et viktig verktøy når en foredler fremtidens kultivarer. Det kan også gi en ny måte å introdusere nye gener fra markjordbær gjennom cis-genesis av villtype trekk i genforrådet (gene pool) til hagejordbær.

Hagejordbær er oktoploid, noe som gjør veksten gunstig for testing av CRISPR/Cas9 systemet. Da denne oppgaven ble planlagt hadde det blitt gjennomført få studier hvor CRISPR/Cas9 ble brukt i jordbær. Målet med oppgaven var derfor å utvikle en protokoll for CRISPR/Cas9 mediert mutasjon av hage- og markjordbær. På grunn av tekniske problemer i tillegg til stenging av tilgjengelige laboratoriefasiliteter som følge av Covid19 pandemien ble dette målet ikke nådd. Istedenfor presenteres en omfattende feilsøking av seamless assembly-metoden, utviklet av Li et al. (2015). Feilsøkingen viser at seamless assembly har mulige utfordringer i plasmid konstruksjon. I tillegg viser et vellykket forsøk med direkte regenerering av jorbærvev at det er mulig å unngå bruk av calluskultur når en bruker CRISPR/Cas9. Dette kan gi mulighet til å unngå somaclonal variasjon, som kan oppstå gjennom bruk av callus.



Nøkkelord: CRISPR/Cas9, seamless assembly, planteforedling, hagejordbær, markjordbær.

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# 1 Introduction

New techniques in plant biotechnology have made it possible to make precise changes in an organism's DNA. One of these fields, known as gene editing, has important advantages compared to traditional breeding. The main advantages include the high specificity with which genomic alterations can be made and the speed in which new cultivars can be produced.

In particular, the use of the CRISPR/Cas9 (Clustered Regularly Interspersed Palindromic Repeats/CRISPR associated protein 9) system has been instrumental in the practice of gene editing. Since the first publication in 2012 (Jinek et al., 2012), more than 116,000 peer reviewed papers have been published through Google Scholar. Of these, ~18,500 have been on plants. The CRISPR/Cas9 system allows the user to make precise cuts in double stranded DNA. This function makes it possible to induce the knock-out of a gene's function, exchange a gene, or facilitate the insert of foreign DNA.

New techniques like CRISPR/Cas9 could be an asset when facing environmental changes, such as extreme weather or the exacerbation of infectious diseases. The CRISPR/Cas9 system may be used to produce cultivars better suited to extreme environments. Horticultural crops are an important part of Norwegian agriculture. Despite this, so far no one has published studies on successful gene editing in Norway or produced gene edited cultivars. Strawberry is an example of an important crop that could be improved using gene editing.

The strawberry is produced in all Norwegian counties (Døving et al., 2017), and is the most important horticultural crop in Norway (Alsheikh et al., 2009). However, there are challenges associated with production of strawberry for commercial consumption. Common problems faced by farmers who produce strawberries are infectious diseases, frost damage, and competition from weeds and pests (Davik et al., 2000; Døving et al., 2017). The production of new cultivars is an important aspect of facing these challenges. Especially concerning diseases and frost damage. In this respect, the CRISPR/Cas9 system can prove to be an invaluable tool (Abdelrahman et al., 2018).

This thesis uses the CRISPR/Cas9 system to attempt to genetically edit two species of strawberry: the cultivated strawberry (*Fragaria x ananassa*) and the wild strawberry (*Fragaria vesca*).

## 1.1 Production and genetic composition of the cultivated and wild strawberry

The cultivated strawberry is of the genus *Fragaria* in the Rosaceae family. This strawberry is one of the most important horticultural crops cultivated in Norway (Alsheikh et al., 2009). It is an octoploid (2n=8x=56) hybrid of *F. virginiana* x *F. chiloensis,* that appeared in Europe between 1714-1759 (Hancock et al., 1991). This hybrid is known for "its large fruit size, high yields and strong vigor" (Hancock et al., 1991). Even if this hybrid is a product of a cross between *F. virginiana* and *F. chiloensis*, the most plausible progenitor is the wild strawberry (Yuan et al., 2019).

The wild strawberry is of the *Fragaria* genus in the Rosaceae family. There is no commercial production of the wild strawberry in Norway, but the plant is found in the wild in most parts of Norway (Bele et al., 2015). The genome of this diploid (2n=2x=14) plant is small (240 Mb) (Shulaev et al., 2010). However, there are distinct genomic similarities between the wild and cultivated strawberry (Shulaev et al., 2010) The wild strawberry was sequenced by a research group in 2010 (Shulaev et al., 2010). This provides an excellent genomic reference also when working with the octoploid cultivated strawberry.

The cultivated strawberry is harvested for its fleshy shoot tip. This is dotted with small achene which are small, dry, one-seeded fruits that, anatomically, are nuts (Shulaev et al., 2010). Commercial propagation of strawberries is done through vegetative propagation, and new progeny is acquired from stolons or runners (Døving et al., 2017). In 2018, Norway produced 7,970 tonnes of strawberries over 14,170 decares (Fig. 1) (SSB Norway, 2020). Strawberries are grown in all Norwegian counties, but the bulk of the production is done in the Hedmark, Vestfold, Buskerud, and Oppland areas (Statens landbruksforvaltning, 2012).

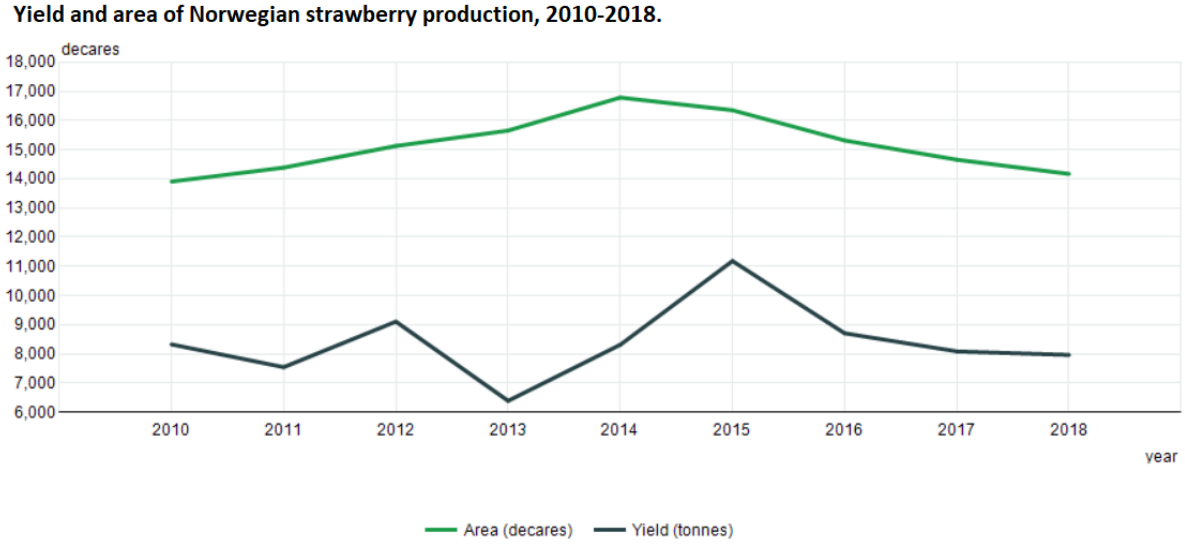



*Figure 1: The graph shows the yield of strawberries (in tonnes) in Norway from 2010 to 2018 (shown in dark blue) as well as how many decares are used to produce strawberries (green). Graph taken from Statistics Norway's website (SSB Norway, 2020).*

The cultivated strawberry is susceptible to many different diseases. In Norway, some of the most prominent are: gray mold, caused by the necrotic fungus *Botrytis cinereal*; powdery mildew, caused by *Sphaerotheca macularis;* and red steele, caused by *Phytophthora fragariae* (Døving et al., 2017). Gray mold causes the most economic losses in the Norwegian strawberry production. One main reason for this is that it easily develops resistance to fungicides (Døving et al., 2017). In addition, strawberry production in the Nordic countries is challenged by harsh weather conditions, as well as competition from foreign strawberry producers (Davik et al., 2000).

The production of new strawberry cultivars is imperative to face harsh weather conditions and pesticide-resistant diseases (Alsheikh et al., 2009). However, breeding such new cultivars using traditional techniques is challenging on account of the strawberry's high ploidy level. The multi-allelism of the octoploid strawberry alters the segregation and inheritance patterns that can be expected in diploids (Acquaah, 2012). If a polyploid acts as a diploid during meiosis, such as tobacco, a normal biometric analysis of inheritance can be applied (Kearsey et al., 1998).

The cultivated strawberry is not fully diploidized. Rather, it is a mix of polysomic (has at least one more chromosome than normal) and disomic (having one or more chromosome present twice, without a doubling of all chromosomes) (Lerceteau-Köhler et al., 2003). It is, therefore, necessary to account for differences in gamete structures when breeding the octoploid strawberry (Katepa-Mupondwa et al., 2002). This makes the conventional breeding of the a more complicated and time-consuming process

than that of a diploid species. The use of CRISPR/Cas9 could be a way to circumvent these challenges and continue to provide novel cultivars with the desired characteristics.

1.2 CRISPR/Cas9

CRISPR was originally discovered in prokaryotes and constitutes an important family of DNA sequences (Barrangou, 2015). Along with CRISPR associated proteins (Cas), the CRISPR/Cas system contributes to the antiviral defense system in many prokaryotes (Barrangou et al., 2007; Makarova et al., 2006; Pourcel et al., 2005). In most cases, the CRISPR/Cas immune system functions in three stages (Fig. 2) (Barrangou, 2015; Reis et al., 2014). In the acquisition stage, DNA from invasive genetic elements (Fig. 2-A1) are sampled and integrated into the CRISPR loci (Fig. 2-A2) (Barrangou et al., 2007). These are known as spacers, "Non-transcribed nucleotide sequences between genes (IGS) in a cell" (Rédei, 2008), and become part of the prokaryotes adaptive immune system (Fig. 2-A2) (Barrangou et al., 2007). During the expression process, the spacers are transcribed and processed (crRNA biogenesis), making small interfering CRISPR RNAs (Fig. 2-B) (crRNA or guide RNA) (Brouns et al., 2008).

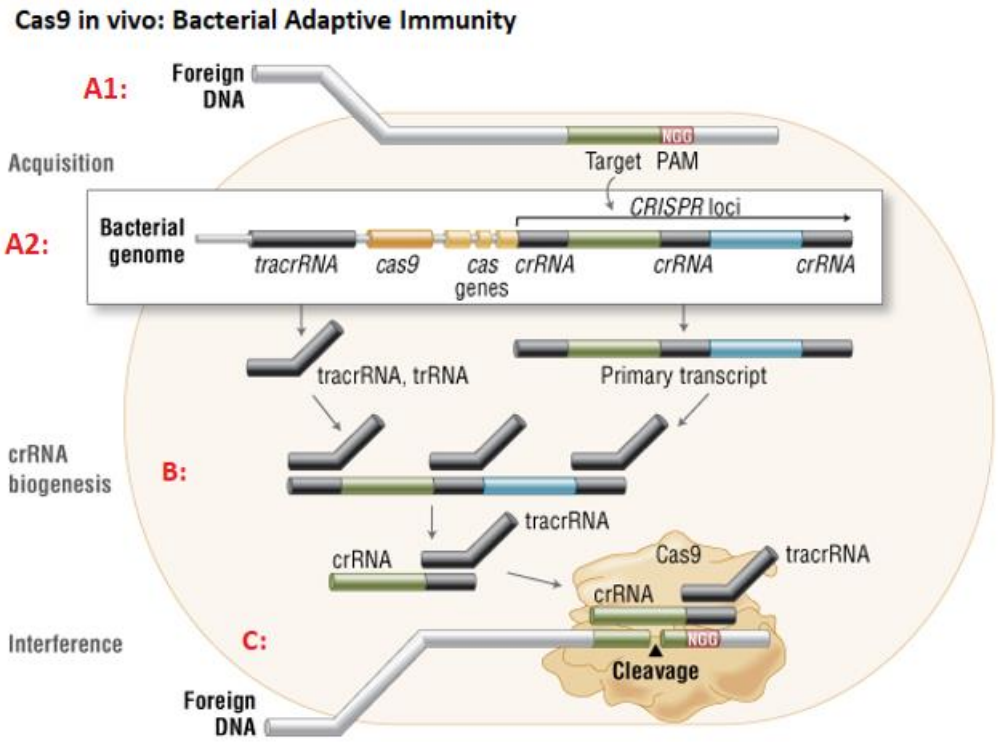



*Figure 2: Pathway of the CRISPR/Cas9 system from adaption of viral DNA to cleavage. A: viral DNA is incorporated into the CRISPR loci and contributes to the recognition of foreign DNA. B: biogenesis of crRNA and tracrRNA. C: The Cas9, guided by the crRNA and recognizing a Protospacer Adjacent Motif (PAM), induces a Double Stranded Break (DSB) in the foreign DNA. From (Reis et al., 2014)*

For the crRNA to be able to associate with Cas, a maturation process is required (Brouns et al., 2008; Carte et al., 2008). This can take many forms, but in 2011 a group of scientists showed maturation of crRNA directed by tracrRNA (*trans*-activating CRISPR RNA) in Streptococcus pyogenes (Fig. 2-C) (Deltcheva et al., 2011). This is made possible by a small structural segment in the crRNA that complements with the tracrRNA (Diep, 2017). tracrRNA is a trans encoded RNA which contains a structural scaffold, or handle, that allows Cas to bind to the tracrRNA (Diep, 2017). The crRNA:tracrRNA complex binds and guides Cas endonucleases to complementary nucleotide sequences.

A final requirement for the Cas to bind to the target area is a Protospacer Adjacent Motif (PAM) (Fig. 3) (Deveau et al., 2008; Mojica et al., 2009). In nature, the PAM sequence keeps the CRISPR/Cas complex from cleaving the complementary spacer sequences within the CRISPR loci (Mojica et al., 2009). Cas contains a PAM sequence which recognizes other PAM sequences in the genome of an organism (Anders et al., 2014). If there is no PAM sequence, the CRISPR/Cas complex will not bind to the DNA even if there is a sequence recognized by the crRNA (Anders et al., 2014). The PAM sequence is usually $^{5'}$NGG$^{3'}$ (Cencic et al., 2014).

If the CRISPR/Cas complex recognizes a PAM sequence and a DNA sequence that is complementary to the crRNA, Cas endonuclease induces cleavage and degradation of the target sequence (Fig. 2-C) (Garneau et al., 2010; Hale et al., 2009).

In 2012, a group of scientists showed that it is possible to engineer the crRNA:tracrRNA complex into a single RNA chimera (guide RNA) (Jinek et al., 2012). In this study, the guide RNA (gRNA) was used to direct Cas9 to induce targeted Double Stranded Breaks (DSB) in DNA (Fig. 3) (Jinek et al., 2012). The specific targeting of nucleotide sequences could be achieved by specifying a 20 nucleotide (nt) sequence within the guide RNA (Ran et al., 2013).

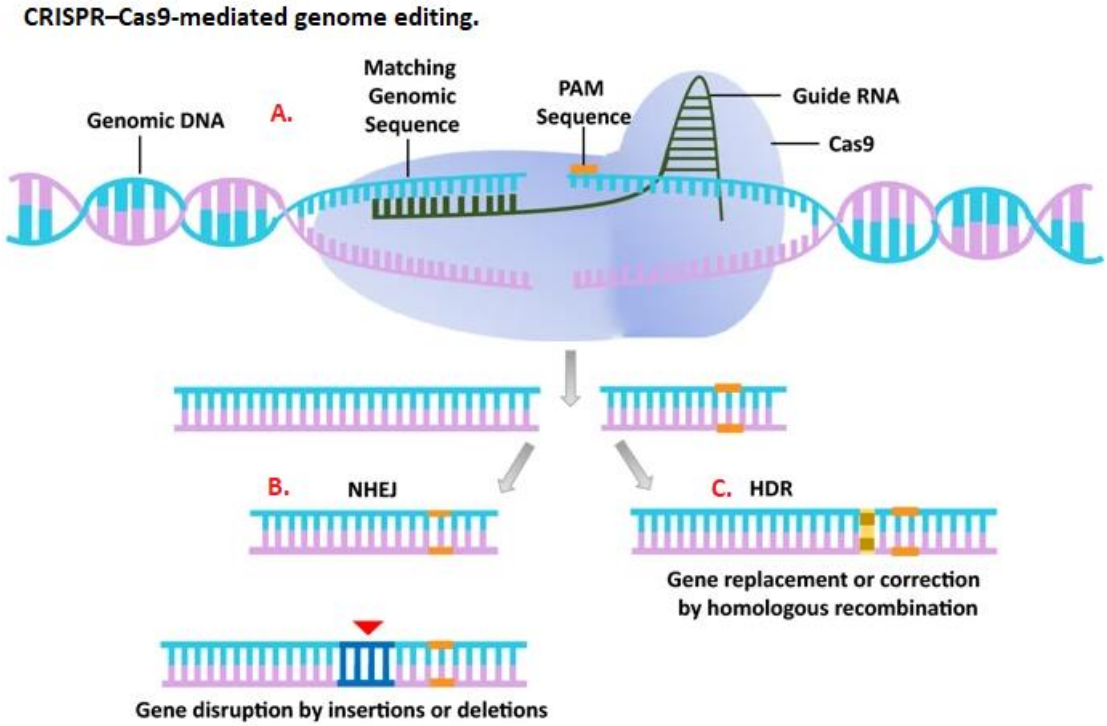



*Figure 3: Shows the CRISPR/Cas9 system and the subsequent repair-mechanisms of DSB. A. Cas9 induces a DSB in a 20 nt sequence recognized by the gRNA. B. Non-Homologous End Joining (NHEJ) repair pathway of DSB. C. Homology Directed Repair (HDR) repair pathway of DSB. Figure from (Ghosh et al., 2019).*

The DSB caused by the CRISPR/Cas9 system, is repaired through two general strategies (Allen et al., 2019); Non-Homologous End Joining (NHEJ) and Homologous Recombination or Homology Directed Repair (HDR) (Fig. 3) (Chiruvella et al., 2013; Liang et al., 1998). During NHEJ, the DSB is joined through direct ligation (Chiruvella et al., 2013). This repair mechanism is error prone, often resulting in the insertion or deletion of small nt segments, which may induce mutations in the repaired region (Ceccaldi et al., 2016). Specific CRISPR/Cas9 induced mutation and subsequent gene knock-out is, therefore,

made possible by utilizing this repair-pathway (Fig. 3) (Barrangou et al., 2015). HDR utilizes homologous DNA sequences as a template to facilitate repair. By supplying donor template DNA, the process can be used to induce mutations or insert foreign DNA (gene knock-in) (Fig. 3) (Gratz et al., 2014). An example of gene knock-in through HDR is the site-specific gene insertion in maize performed by Svitashev et al. (2015). Here, DNA repair templates, as well as gRNAs and the CRISPR/Cas9 complex, were introduced to immature maize embryos by biolistic transformation. Guided by the gRNA, the cas9 endonucleases induced DSB in DNA. These were repaired through HDR using the DNA repair templates, causing a successful insertion of foreign DNA (Svitashev et al., 2015). The use of HDR-facilitated gene insertion may not be as viable in plants as in other organisms. This is because NHEJ is the most common DSB repair mechanism in plants, taking precedence over HDR (Puchta, 2004).

### 1.3 The Flavonoid pathway

When the objective for this thesis was set in spring 2018, few studies concerning the genetic editing of the cultivated and wild strawberry had been undertaken. Since then, several studies have cemented the possibility of using CRISPR to genetically alter strawberries (Martín-Pizarro et al., 2018; Wilson et al., 2019; Xing et al., 2018; J. Zhou et al., 2018).

This thesis aims to show the effects of CRISPR/Cas9 induced mutations in cultivated and wild strawberry. A promising target area is the flavonoid pathway. Anthocyanin is one of the end products of this pathway (Fischer et al., 2014). It is responsible for pigmentation of many fruits, leaves, seeds and flowers (Lin-Wang et al., 2014). The pigmentation ranges from pink, red, violet and blue (Andersen et al., 2010). Anthocyanins are among the most abundant flavonoids in strawberries (Hannum, 2004). Any altered pigmentation caused by the absence of flavonoids will be a very visible effect of the gene editing.

The flavonoid pathway (Fig. 4) is well documented in both cultivated (Almeida et al., 2007; Fischer et al., 2014) and wild strawberry (Lin-Wang et al., 2014). For example, gene silencing of the anthocyanidin reductase (ANR) through intron-spliced hairpin RNAs, has shown the adaptability of the pathway (Fischer et al., 2014). Also, CRISPR/Cas9 has been used to knock-out genes in the flavonoid pathway in the cultivated strawberry (Xing et al., 2018). While the latter of these studies did not observe a change in phenotype, successful integration and gene disruption mediated by CRISPR/Cas9 was verified through Polymerase Chain Reaction (PCR) (Xing et al., 2018).

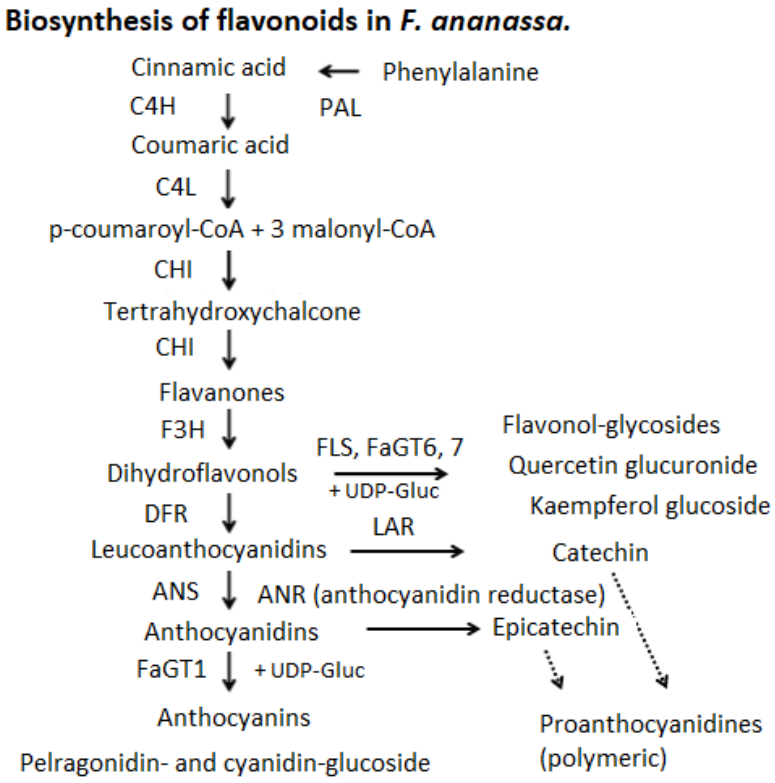



*Figure 4: Only dihydroxylated flavonoids are shown. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid-4-hydroxylase; C4L, 4-coumarate:CoA ligase; CHI, chalcone isomerase; F3H, Flavanone 3-Hydroxylase; DFR dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidinreductase; FaGT1, UDP-glucose:flavonoid-3-O-glucosyltransferasse; FLS, flaconol synthase; LAR, leucoanthocyanidinreductase. Figure from (Fischer et al., 2014).*

An important gene in the flavonoid pathway is Flavanone 3-Hydroxylase (*F3H*) (Fig. 4). *F3H* is an enzyme that catalyzes the hydroxylation of flavanones to dihydroflavonols (Britsch et al., 1992). Down-regulation of *F3H* has shown a correlation between a lack of the 3-hydroxylase activity and a block in the flavonoid pathway (Forkmann et al., 1981). The genetic sequence of the enzyme is also conserved, making the *F3H* a good candidate gene for CRISPR/Cas9 mediated mutations over different cultivars (Britsch et al., 1986; Britsch et al., 1992; Turnbull et al., 2004).

The characteristics of *F3H* makes this an interesting target for disrupting the production of anthocyanins. Therefore, this thesis has an aim to attempt a CRISPR/Cas9 mediated knock-out of this gene.

## 1.4 The regulation of Genetically Modified Organisms in Norway

Genetically Modified Organisms (GMO) production for commercial use is not prohibited in Norway (Genteknologiloven, 1993). However, the current law regulating the use of GMOs is very restrictive, and a case-by-case permit for the release of new GMOs is required (Genteknologiloven, 1993). For this reason, very few genetically modified cultivars are granted commercial permission in Norway.

One of the few genetically modified plants that has been approved for sale is the carnation (*Dianthus caryophyllus*) MoonLite™ (Øverland et al., 2016a). Moonlite™ is modified to have different colored flowers. MoonLite™ is not available for purchase as a whole plant; only the cut flowers are permitted

for sale, distribution and import (Øverland et al., 2016a). Together with MoonLite™, three other shades of purple carnations were also granted permission (Moonvista™, Moonberry™ and Moonaqua™) (Miljødirektoratet, 2019; Øverland et al., 2016b; Øverland et al., 2016c), all produced by Suntory Flowers Limited.

In 2018, The Norwegian Biotechnology Advisory Board proposed a revised method of assessing the risks of commercial use of GMOs (Bioteknologirådet, 2018) to the Norwegian government. The recommended novel way of assessing GMOs would be a tier-based requirement of approval. This would categorize the GMOs based on what genetic change had occurred (Table 1) (Bioteknologirådet, 2018).

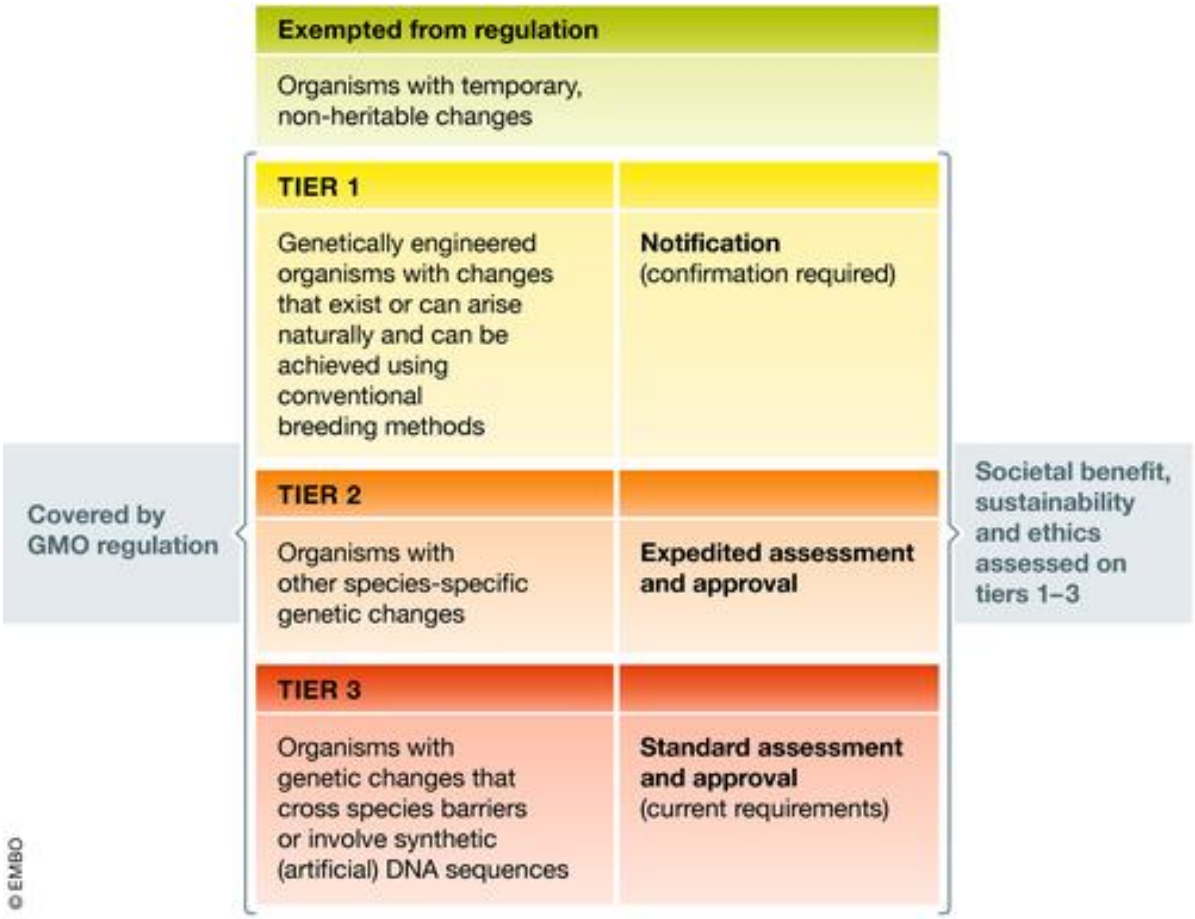
Four main categories were proposed in this tier-based method (Table 1). Level 0 would include genetic changes within an organism that are temporary or non-heritable (Table 1). An example of a method that would fall under this category would be genetically engineered vaccines. This level is exempted from regulations under the Norwegian Gene Technology Law (Genteknologiloven, 1993). If the new proposal is implemented by the Norwegian government, producers will not have to apply for approval if production follows legally established methods. The induction of mutations by conventional methods (chemically or by radiation) would also fall under level 0. This is because these methods have been declared to have a "history of safe use" according to the EU court ruling Case C-528/16 (2018)

Level 1 would pertain to genetic changes that may exist, can occur naturally, or can be achieved through conventional methods (Table 1). Some functions of the CRISPR/Cas9 system, where no foreign DNA is added, could fall under this category. To use organisms that fall under this category, there would be an obligation to notify and obtain confirmation that the notification has been received by the appropriate authorities (Bioteknologirådet, 2018).

Level 2 would govern genetic changes that have happened within a species, or between sexually compatible species (Table 1). This would encompass the deletion of large segments of DNA or the insertion of DNA from the same or related species. Examples of this would be the deletion of disease-related genes in rice (Zhou et al., 2014) and the increasing of late blight resistance by cis-gene stacking in potato (Jo et al., 2014). The producers that handle organisms which could fall under this category would be obligated to provide a simplified consequence analysis/risk assessment. The consequence analysis must contain an assessment of potential environmental and health risks this organism could pose (Bioteknologirådet, 2018).

The third level of this proposal dictates interspecies exchange of DNA and processes that involve synthetic DNA sequences (Table 1) (Bioteknologirådet, 2018). Examples include the 'BT maize' cultivar and 'Golden Rice'. The 'BT maize' gains a built-in defense against the European corn borer (*Ostrinia nubilalis*) by inserting a gene from *Bacillus thuringiensis* that produces Cys proteins (Koch et al., 2015). 'Golden rice' is a rice cultivar that produces β-caroten to prevent Vitamin-A deficiency in areas where rice is the main source of food (Beyer et al., 2002). Level 3 is the most restrictive of the four levels, and it is subject to the most strict regulation (Table 1) (Bioteknologirådet, 2018). This level is in accordance with the current GMO regulation in Norway.

*Table 1: The proposed, revised method for assessing GMOs in Norway. Shows the necessary steps needed to approve GMOs for different tiers pertaining to the type of genetic change that has occurred. Table from Bratlie et al. (2019).*




Levels one through three also require an evaluation of societal benefit, sustainability, and ethical justifiability in the Norwegian society (Table 1). This evaluation may be more severe for the higher tiers (1-3) (Bioteknologirådet, 2018; Bratlie et al., 2019).

Eleven of the 18 members of the Biotechnology Advisory Board voted in favor for this proposal (Bioteknologirådet, 2018). If this proposal is accepted by the Norwegian government, genetic tools like CRISPR/Cas9 could be implemented into Norwegian breeding programs (Christie, 2018).

## 1.5 Goal of this thesis

Studying CRISPR/Cas9 technology is valuable because it provides more tools to strengthen agricultural practices and improve cultivated plants. These tools can help practices adapt to environmental changes and novel diseases that threaten the stability of Norwegian agriculture. Also, the Norwegian Biotechnology Advisory Board's recent proposal to amend the Norwegian Gene Technology Act proposes a "softening" of Norway's regulation of GMOs. If accepted, the use of CRISPR/Cas9 for plant breeding may become a more accepted tool in the future.

This thesis had originally attempted the CRISPR/Cas9 mediated knock-out of the Flavanone 3-Hydroxylase gene in the cultivated and wild strawberry. At the time, the postulated result was the disruption of anthocyanin production and a phenotypic response in mature strawberries. The study attempted to assemble the delivery vector for the CRISPR/Cas9 system, using the seamless assembly method presented by Li et al. (2015). An overlapping PCR with custom primers would generate DNA expression cassettes carrying the gRNAs. These would then be ligated into a plasmid carrying the CRISPR/Cas9 construct (pFGC-pcoCas9). If the construction had been successful, the CRISPR/Cas9 system, with targeted gRNAs, would be introduced through *Agrobacterium tumefaciens* (*Agrobacterium*) mediated transformation, biolistic transformation, and agroinfiltration. Both biolistic and *Agrobacterium* mediated transformation would result in stable transformants. In addition, agroinfiltration would result in transient expression of the CRISPR/Cas9 complex.

However, due to unexpected problems when assembling the destination vector, transformation could not be undertaken. Time constraints caused by the closure of available laboratory facilities, due to the Corona virus disease 2019 (Covid19), also led to the discontinuation of the research process. Therefore, this thesis walks the reader through the steps of troubleshooting plasmid assembly, including both the assembly of DNA expression cassettes and plasmids. The processes that would have been done if the research process were not discontinued is also outlined in this thesis. This thesis also shows how the use of callus could be circumvented using direct regeneration of explants.

# 2 Material and methods

## 2.1 Plant material and MS-media

The plant material used in this experiment came from various sources. The cultivated strawberry 'Jonsok' was supplied by Graminor AS, located in the municipality of Hamar. The wild type 'Hawaii' was supplied by Arti Rai, an engineer in the "Division of biotechnology and plant health" at the Norwegian Institute of Bioeconomy Research (NIBIO) in the Ås municipality. In the first phases of this study the wild type cultivar 'Snöhvit' was used instead of 'Hawaii'. "Snöhvit" was procured from Sagaplant AS in Akkerhaugen, Telemark. Due to difficulty with DNA extraction from this cultivar as well as poor performance in tissue culture, the usage of 'Snöhvit' was discontinued in favor of 'Hawaii'. 'Hawaii' also has the benefit of being fully sequenced (Shulaev et al., 2010). This makes 'Hawaii' potentially easier to work with.

Explants for *in vitro* cultures were obtained from cultivars 'Jonsok' (cultivated strawberry) and 'Hawaii' (wild strawberry). The plant material was kept both *In vivo* and *In vitro*. *In vitro* plants were propagated on a Murashige & Skoog (MS) medium (Table 2), containing MS microelements and vitamins (Appendix I) (Murashige et al., 1962). The MS was obtained from Duchefa Biochemie. The pH of all MS-media was adjusted to 5.7 and distributed to jars in aliquots (≈100 ml). All media was autoclaved for 15 minutes at 121 °C and 1.2 kPa. Conditions for plantlets propagated on MS-media were 20±1 °C (Appendix III) with an 18-h photoperiod and a light intensity of 2,000 lux. The plantlets were sub-cultured to fresh MS-media every three weeks.

*Table 2: Configuration of the MS-media used in this thesis.*

| **1 L MS-medium** | |
|---|---|
| MS | 1x |
| BAP | 0.5 mg/L |
| IBA | 0.2 mg/L |
| Sucrose | 3% |
| Agar | 0.8% |
| $H_2O$ | To 1 L |

*In vivo* plants were sub-cultured from *in vitro* plantlets. Plantlets kept *in vitro* were transferred to a peat-based soil (Tjerbo Gartner jord) in 7.5 cm pots. After being transferred to pots, the small plants were covered with a plastic cover and spray watered every day. Once the plants had outgrown the 7.5 cm pots, they were transferred to 12 cm pots. All *in vivo* plants were kept in a greenhouse at 23±5 °C (Appendix III) in a 16-h photoperiod.

## 2.2 Preliminary test of callus medium

To induce the formation of callus in explants, a Callus Inducing Medium (CIM) containing 2,4-D and BAP was used (Table 3). This is in accordance with Nehra et al. (1990) where BAP and 2,4-D was used to induce callus in the cultivated strawberry.

*Table 3: The base medium used for inducing callus. The concentration of hormones would be varied and can be seen in table 4.*

| **1L Callus inducing medium** | |
|---|---|
| MS | 1x |
| BAP | X |
| 2,4-D | X |
| Sucrose | 3% |
| Agar | 0.8% |
| $H_2O$ | To 1 L |

To test the optimal hormone concentration for callus induction, several hormonal combinations for CIMs were made (Table 4).

*Table 4: The hormonal concentrations of the various callus inducing mediums that were used. Medium 2 proved the most successful.*

| **Medium nr.** | **2,4-D concentration** | **BAP concentration** |
|---|---|---|
| 1. | 0.5 mg/L | 0.5 mg/L |
| 2. | 0.5 mg/L | 0.25 mg/L |
| 3. | 1 mg/L | 1 mg/L |
| 4. | 1.5 mg/L | 1 mg/L |
| 5. | 1.5 mg/L | 1.5 mg/L |

The media was added to 9cm Petri dishes in 20mL aliquots. Two types of explants (leaf disks and petiole fragments) were used, and ten explants were added to each Petri dish. Leaf disks were placed with the adaxial surface away from the media (up-side-down) and the petiole fragments were placed laying side down on the medium. The explants were incubated in total darkness for the duration of the experiment.

## 2.3 Dose response to BASTA

Varying concentrations of glufosinate ammonium was added to CIM2 (Table 3 and 4). In total seven media compositions were made, including the control (Table 5). Each medium was distributed over four, 9 cm Petri dishes. Explants from 'Jonsok' and 'Hawaii' were taken from plants that were cultivated *in vitro*. Both petioles and leaf disks were used, and the different explants were plated out on media. These were incubated in total darkness. After three weeks of incubation, the explants were observed every week for five weeks. Between observations, the explants were kept in total darkness. Medium 3 had the lowest concentration of glufosinate ammonium were no explants could survive. This media composition was used for the rest of this thesis.

*Table 5: The amount of glufosinate ammonium that was added to the callus inducing medium 2 (Table 3 and 4).*

| **Medium nr.** | **Concentration of glufosinate ammonium** |
|---|---|
| 1. | 0.5 mg/L |
| 2. | 1 mg/L |
| 3. | 2 mg/L |
| 4. | 3 mg/L |
| 5. | 4 mg/L |
| 6. | 5 mg/L |
| Control | 0 mg/L |

## 2.4 Preliminary test of regeneration medium

An increasing concentration of IBA and BAP (Table 6) was added to the MS-medium (Table 2) in accordance with Barceló et al. (1998) and El Mansouri et al. (1996). This was added to 9 cm Petri dishes in 20 mL aliquots. Eight explants were placed in each Petri dish, four leaf disks and four petiole fragments. Leaf disks were placed with the adaxial surface away from the medium. The media was further divided into two groups where one would be subjected to an 18-h photoperiod with a light intensity of 2,000 lux at 20±1 °C (Appendix III). The other group would be incubated in darkness for two weeks, before being moved to the same conditions as the previous group. Two parallels were made for each treatment, resulting in a total of 80 Petri dishes each with eight explants.

The explants were moved to fresh media after four weeks and were observed twice over the span of three months. The highest regeneration rate occurred in leaf disks on media F. This medium was used for the rest of the experiment.

*Table 6: Gradient of hormone concentration used in regeneration media.*

| Medium nr. | IBA concentration | BAP concentration |
|---|---|---|
| A | 0.25 mg/L | 2 mg/L |
| B | 0.25 mg/L | 3 mg/L |
| C | 0.25 mg/L | 4 mg/L |
| D | 0.5 mg/L | 2 mg/L |
| E | 0.5 mg/L | 3 mg/L |
| F | 0.5 mg/L | 4 mg/L |
| G | 1 mg/L | 2 mg/L |
| H | 1 mg/L | 3 mg/L |
| I | 1 mg/L | 4 mg/L |
| Control | 0 mg/L | 0 mg/L |

## 2.5 Statistical analysis

A Generalized Linear Model (GLM) was used to see the effects and correlations between different data points in the regeneration media (Table 6) (medium, cultivar, explants, and growth conditions effects on survival). GLM was achieved using "glm2" package in R (https://cran.r-project.org/web/packages/glm2/index.html). Different statistical models implemented in GLM, provides a mathematical basis for the interpretation and examination of parameters and to determine the roles and relative importance of different variables on a process. In this study, GLM was applied to find the significant medium type playing a key role in plant development and its interactions with the cultivar type ('Jonsok' and 'Hawaii') and growth conditions (dark and light). Data collected from plant survival for two cultivars, with several replicates grown in 9 different media compositions, at both light and dark conditions were used for GLM analysis.

## 2.6 DNA isolation

Plant tissue was taken from plants propagated *In vivo.* The tissue was frozen in liquid nitrogen and pulverized using a Retsch MM301 mixer mill. DNA was isolated using QIAgens DNeasy Plant Mini Kit, according to the manufacturer's instructions. The Centrifuge 5471 R from Eppendorf was used to centrifuge samples. This centrifuge was used for all processes requiring a tabletop centrifuge. Successful isolation of DNA was verified using Thermo Scientific's NanoDrop™ One.

## 2.7 Sequencing target area

The sequence of *F3H* from the wild strawberry was obtained from the NCBI website. This sequence was derived from 'Hawaii'. Using the software, Snapgene®, two sets of primers were designed and subsequently ordered from Thermo Fisher Scientific. The primer sets were made to amplify part of exon 1 and 2 of *F3H*. The primer sets were called *F3H*_ex1 and *F3H*_ex2 (Appendix II).

The two primer sets and DNA isolated from 'Jonsok' and 'Hawaii' was used in a PCR master mix (Table 7). This PCR reaction used a OneTaq® DNA polymerase obtained from New England Biolabs® inc. Here,

the isolated DNA from 'Jonsok' and 'Hawaii', served as a template for the PCR reaction. This was done to verify the *F3H* gene in 'Jonsok' and 'Hawaii'.

*Table 7: PCR master mix used for amplification of exon 1 and 2 of F3H.*

| PCR Master Mix | Measurements |
|---|---|
| OneTaq polymerase | 1 unit |
| dNTP | 2 µM |
| Buffer X10 | 1x |
| Template | 10 µL (final concentration >1,000 ng) |
| Reverse primer | 2 µM |
| Forward primer | 2 µM |
| $H_2O$ | To 100 µL |

The annealing temperature of the primers was tested through a temperature gradient, 45-65 °C. The steps of the thermocycling of the PCR routine was as follows:

| | | |
|---|---|---|
| 94 °C: | 30 seconds | |
| 94 °C: | 10 seconds | |
| 45-65 °C: | 30 seconds | 30 cycles |
| 68 °C: | 30 seconds | |
| 68 °C: | 5 minutes | |
| 4 °C: ∞ | | |

Optimal temperature was determined through visualization on a 1% agarose gel (Appendix I). This showed that primer set *F3H*_ex2 was ineffective. Primer set *F3H*_ex1 was therefore used for further experiments. The optimal temperature for annealing, in primer set *F3H*_ex1, was 47.8 °C. This temperature was used when using the *F3H*_ex1 primer set.

The PCR product was purified using the QIAquick® PCR Purification Kit in accordance with the manufacture's specifications. The presence of DNA was verified using Thermo Fisher Scientifics NanoDrop™ One, and Sanger sequenced at GATC, Germany (Appendix IV).

## 2.8 Designing gRNAs and choosing expression vector

Four gRNAs located in exon 1, were designed using the CRISPOR online tool (http://crispor.tefor.net/). The CRISPOR online tool was developed by Haeussler et al. (2016). This tool finds possible gRNAs bordering a PAM sequence, with a defined length, within an uploaded DNA sequence. The webtool also scores on-target efficiency and possible off-target locations across an extensive genomic library, which includes the wild strawberry (Haeussler et al., 2016). All gRNAs had a high on-target activity and a MIT specificity score of 98-100. The MIT specificity score summarizes all off target possibilities into a single number (Haeussler et al., 2016). The higher the number, on a scale of 0-100, the fewer off-target

effects can be expected. All the gRNAs had some potential off-target locations, but none shared the same PAM sequence (Table 8).

*Table 8: The four gRNAs designed using the CRISPOR online tool using the sequenced exon 1 of F3H as a template for the design. PAM sequence shows the PAM sequence pertaining to the respective gRNA. Position shows where the gRNAs binds in exon 1. MIT specificity score pertains to the amount of off-target effects the gRNAs will have.*

| **Name** | **PAM sequence** | **Position** | **MIT specificity score** | **Off targets and off-targets next to PAM sequence** | |
|---|---|---|---|---|---|
| gRNA1_55 | AGG | 55 Forward | 100 | 3 | None with same PAM |
| gRNA3_265 | AGG | 265 Forward | 98 | 8 | None with same PAM |
| gRNA2_172 | GGG | 172 Forward | 100 | 2 | None with same PAM |
| gRNA4_293 | TGG | 293 Forward | 100 | 1 | None with same PAM |

The vector pFGC-pcoCas9 was chosen. This plasmid contains a NOS terminator for plant expression of Cas9 and multiple cloning sites. It also has resistance to BASTA for post-transformational selection and a kanamycin resistance gene for bacterial selection (Fig. 5). The growth strain used for this plasmid was the Subcloning Efficiency™ DH5α™ Competent cells from Thermo Fisher Scientific.

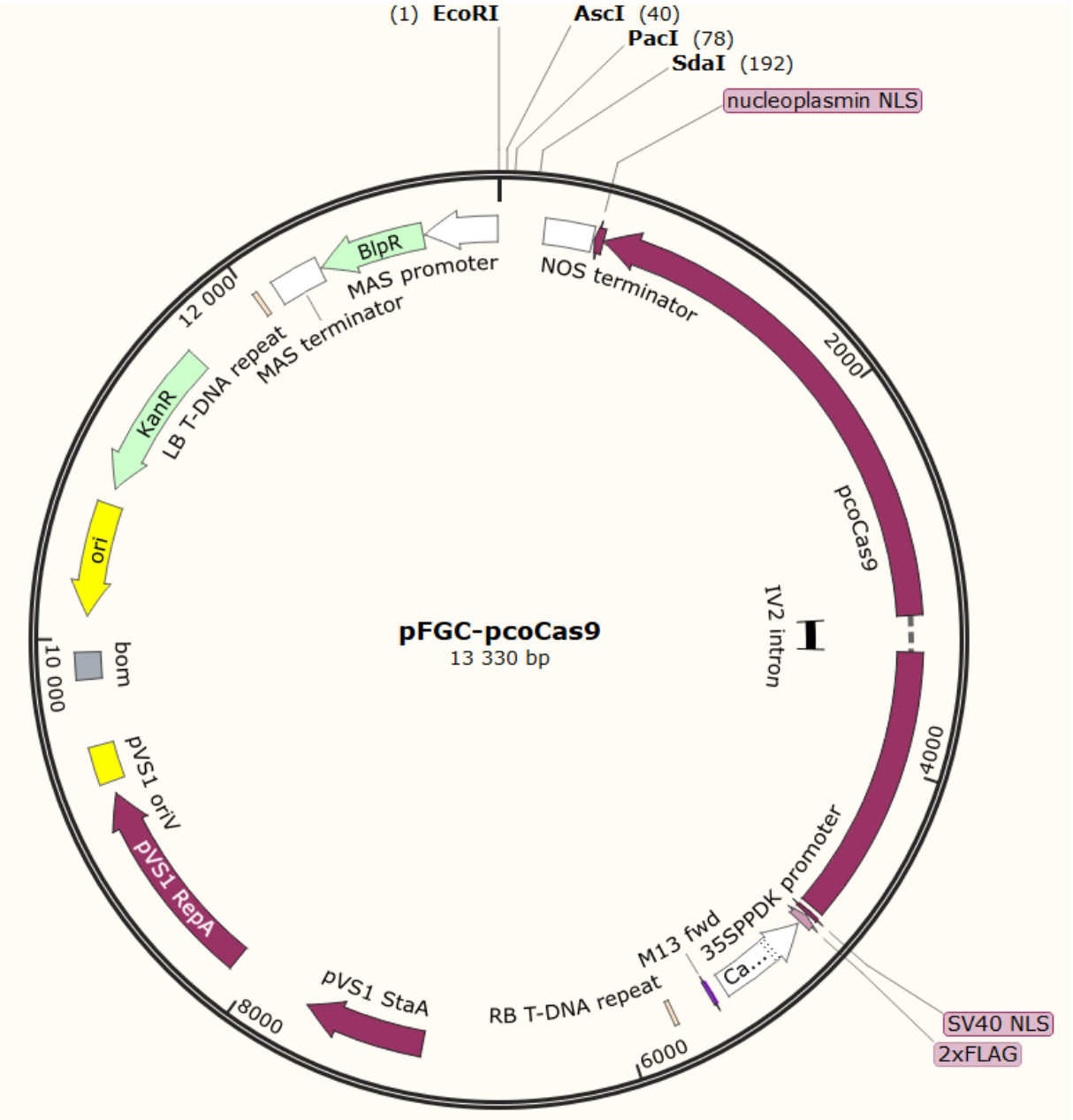



*Figure 5: The binary plasmid pFGC-pcoCas9. The plasmid contains resistance genes against kanamycin and BASTA, plant-codon optimized Cas9 and several restriction sites. All restriction sites, except EcoRI, AscI, PacI and SdaI, have been removed from this figure. This figure is from Jen Sheen (Addgene plasmid # 52256; http://n2t.net/addgene:52256; RRID:Addgene_52256).*

To isolate plasmid from the received glycerol stock, the following steps were taken:

1. 9 cm Petri dishes filled with LB broth (Appendix I) containing 2% agar was pre-warmed at 37 °C for 1 hour.
2. A pipette tip was used to extract and streak the DH5α™ growth strain containing the plasmid onto the plates.
3. The plates were sealed with perfilm from Nescofilm, and incubated overnight at 37 °C.
4. The next morning, colonies were chosen and stabbed from the media with a pipette tip. The pipette tip was added to 5 mL liquid LB broth and incubated in the HT Minitron Incubator Shaker from Infors at 37 °C and 225 rpm overnight.
5. The following day, the sample was pelleted in a tabletop centrifuge at 6,000 rpm.

6. The DNA was extracted from the pelleted cells using the QIAprep® Spin Miniprep Kit in accordance with the manufacturer's instructions.

The extraction of DNA was verified using the NanoDrop™ One, and the DNA was stored at -20 °C.

## 2.9 Designing DNA expression cassettes

Four DNA expression cassettes were designed. These would each carry an AtU6-1 promoter, a gRNA scaffold, one of the four gRNA sequences and two restriction sites (Fig 6).

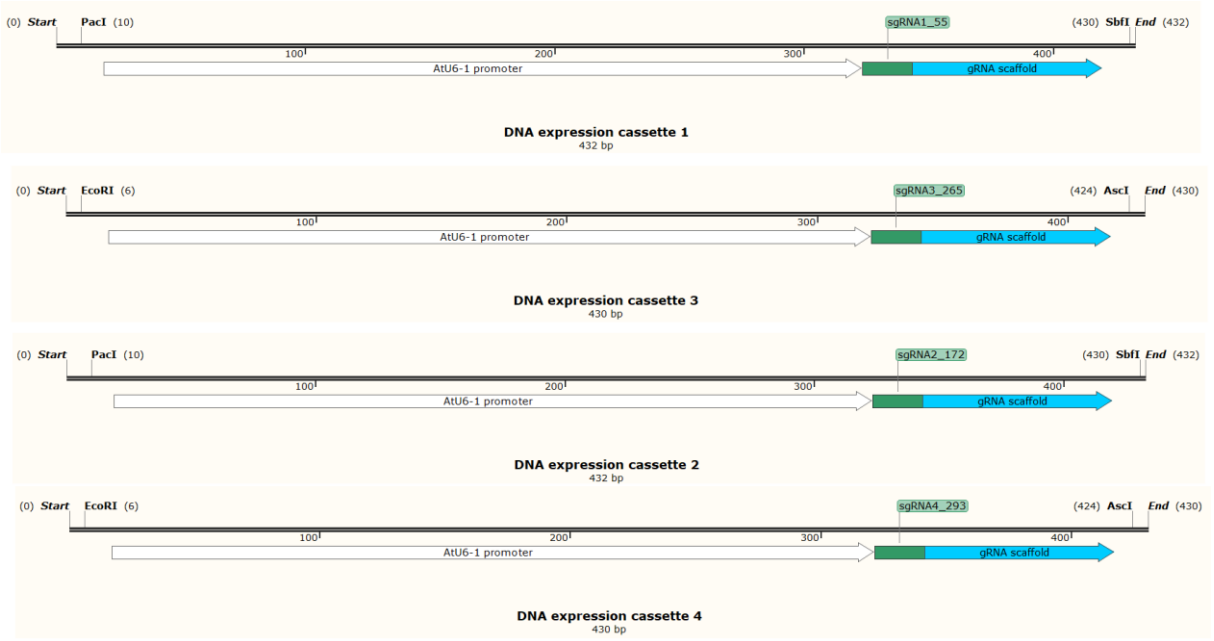



*Figure 6: The four DNA expression cassettes. All contain an AtU6-1 promoter and a gRNA scaffold. In addition to this, Insert 1 carries gRNA_55 and PacI and SdaI (SbfI) restriction sites; Insert 2 carries gRNA_172 and PacI and SdaI (SbfI) restriction sites; Insert 3 carries gRNA_265 and EcoRI and AscI restriction sites and Insert 4 carries gRNA_293 and EcoRI and AscI restriction sites. Figure made with Snapgene®.*

As there were four different gRNA sequences, four cassettes had to be made. These were named Insert 1, 2, 3 and 4. These would be inserted into pFGC-pcoCas9 in pairs. The result would be two plasmids, each containing two DNA expression cassettes each carrying a different gRNA (Fig. 7).

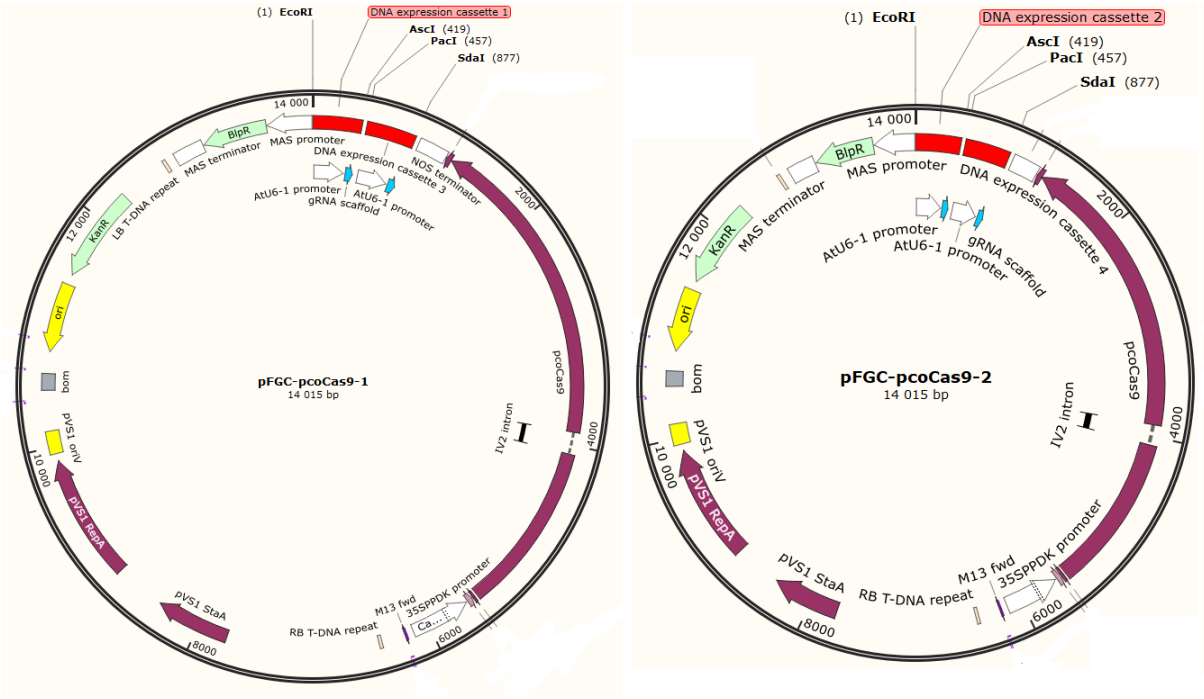



*Figure 7: Expression vectors with the inserts. pFGC-pcoCas9-1 carries Insert 1 and 3 while pFGC-pcoCas9-2 carries Insert 2 and 4. DNA expression cassettes 1 and 2 are indicated over the plasmid while DNA expression cassettes 3 and 4 are indicated inside the plasmid. Figure made with Snapgene®.*

Insert 1 and 2 and Insert 3 and 4 would be ligated into the same spot in their respective plasmids (Fig. 7). The same restriction sites were therefore used. PacI and SdaI (SbfI) or Insert 1 and 2 and EcoRI and AscI for Insert 3 and 4.

## 2.10 Seamless assembly of expression cassette

Four DNA expression cassettes were created using the seamless assembly method presented by Li et al. (2015). This method allows the construction of a DNA sequence through overlapping PCR. During the protocol, two consecutive rounds of PCR are undergone. The first creates intermediate products through PCR (Fig. 8). The second fuses the intermediate products through overlapping PCR (Fig. 10). For this process, a total of 12 primers were generated (Table 9).

*Table 9: the twelve primers used during seamless assembly. The bases are color coded. Red: five additional bases protecting the restriction sites and facilitating restriction digestion. Blue: the restriction sites for the F1 primers, F1_1 has the site for SdaI and F1_2 has AscI. Yellow: the restriction sites for the R2 primers, R2_1 has the site for PacI and R2_2 has EcoRI. Green: The gRNA sequences, all F2 primers have a different gRNA sequence and the R1 primers have the reverse complimentary sequence to the respective F2 gRNA sequences. Black: The sequences that binds to the template during PCR. Note that if the first nucleotide of the gRNA is T, A or C an additional C (Dark Red) has been added to the R1 primers. This is to add a G upstream of $N_1$ to optimize transcription initiation of the Au6-1 promotor during PCR round #1 (Li et al., 2015). Restriction sites associated with primers F1_1, F1_2, R2_1 and R2_2 are shown in column 3.*

| Name | Sequence | Restriction site |
|---|---|---|
| F1_1 | AAGCTCCTGCAGGAGAAATCTCAAAATTCCG | SdaI |
| F1_2 | AAGCTGGCGCGCCAGAAATCTCAAAATTCCG | AscI |
| R1_1 | TAGGGCGCTCATCTTCGTCGCAATCACTACTTCGTCTCT | |
| R1_3 | CCGGCGGCAAAGCGAAGAACAATCACTACTTCGTCTCT | |
| R1_2 | AGTCCTCGCAGGCCTCAACACAATCACTACTTCGTCTCT | |
| R1_4 | CCGGACATGTCGAAACGGAGCAATCACTACTTCGTCTCT | |
| F2_1 | CGACGAAGATGAGCGCCCTAGTTTTAGAGCTAGAAATAGC | |
| F2_3 | GTTCTTCGCTTTGCCGCCGGGTTTTAGAGCTAGAAATAGC | |
| F2_2 | TGTTGAGGCCTGCGAGGACTGTTTTAGAGCTAGAAATAGC | |
| F2_4 | CTCCGTTTCGACATGTCCGGGTTTTAGAGCTAGAAATAGC | |
| R2_1 | AAGCTTTAATTAAAAAAAAGCACCGACTCGGTGC | PacI |
| R2_2 | AAGCTGAATTCAAAAAAGCACCGACTCGGTGC | EcoRI |

The primers can be categorized into three categories — primers carrying a gRNA sequence (F2_1, F2_2, F2_3 and F2_4), primers with a reverse complimentary sequence to the gRNA (R1_1, R1_2, R1_3 and R1_4) and primers with restriction sites as well as a five base nucleotide sequence (F1_1, F1_2, R2_1 and R2_2). These five bases act as a cap to "ensure efficient restriction digestion of PCR products"(Li et al., 2015). These primers would be used to generate four DNA expression cassettes through seamless assembly.

2.10.1 PCR Round #1

In the first round of PCR, two intermediate products (IP1 and IP2) were constructed (Fig 9). IP1 would contain an AtU6-1 promotor, a restriction site, and the reverse complimentary strand of the gRNA (Fig. 8). IP2 would contain the gRNA scaffold, a restriction site, and the gRNA sequence (Fig. 8). These would be joined in PCR round #2, forming the DNA expression cassette. pUC119-gRNA (Li et al., 2013) was used as a template for the PCR reaction.

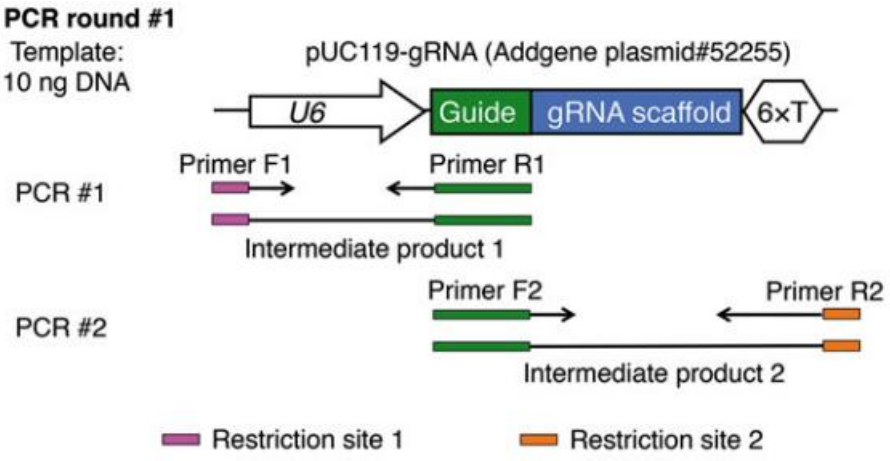



*Figure 8: PCR round #1. Shows the creation of the two intermediate products IP1 and IP2. IP1 containing the AtU6-1 promoter and IP2 containing the gRNA scaffold. Figure from Li et al. (2015).*

The process of constructing the different DNA expression cassettes did not differ for each cassette. Therefore, the general method of DNA expression cassette generation will be presented here.

Two PCR mixtures were prepared (Table 10). All PCR reactions in the seamless assembly were based on instructions for the Q5® High-Fidelity DNA Polymerase from New England Biolabs® inc. The only difference between these mixtures were the primer sets. To make IP1, primers F1 and R1 were added to the PCR mix. To make IP2, primers F2 and R2 were added instead.

*Table 10: The master mix used to create IP1 and IP2. Different primers would be used to create IP1 (F1 and R1) and IP2 (F2 and R2) respectively.*

| Reagents | 50 µL reaction | Final concentration |
|---|---|---|
| 5X Q5 reaction buffer | 10 µL | 1X |
| 10 mM dNTPs | 1 µL | 200 µM |
| 10 µM Forward Primer | 2.5 µL | 0.5 µM |
| 10 µM Reverse Primer | 2.5 µL | 0.5 µM |
| Template DNA | 5 µL | 50 ng |
| Q5 High-Fidelity DNA Polymerase | 0.5 µL | 0.02 U/µL |
| Nuclease-Free Water | To 50 µL | |

The master mixes for both IP1 and IP2 were run in a T100™ Thermal Cycler from Bio-Rad, using these specifications:

98 °C: 30 seconds

98 °C: 10 seconds
54 °C: 30 seconds
72 °C: 30 seconds
} 34 cycles

72 °C: 2 minutes

4 °C: ∞

The F1 and R2 primers would only bind the template partially, creating an overhang containing the restriction site and the cap (Fig. 9). The F2 and R1 primers would bind similarly, creating an overhang containing the complimentary and reverse complimentary gRNA sequence (Fig 9).

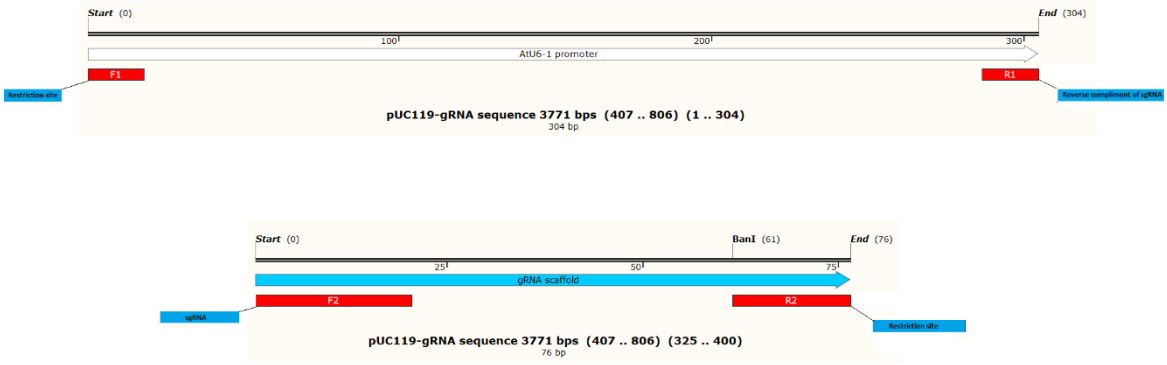



*Figure 9: Shows IP1 and IP2 and how they bind to the pUC199 template. Overhangs created by primers are shown on the 3´ and 5´ ends of both fragments. Figure created using Snapgene™.*

Post thermo cycling, the PCR product was run on a 1% agarose gel for 45 minutes. Bands were visualized using UV light and excised from the gel using a sterilized scalpel. The excised gel was added to a pre-weighed Eppendorf tube and weighed again to find the weight of the excised gel. DNA was extracted from the gel using QIAquick Gel Extraction Kit in accordance to the manufacturer's instructions. To increase the DNA concentration final solution, the DNA was eluted with 15 µL elution buffer instead of the 30 µL suggested in the protocol. The extraction of DNA was verified using Thermo Fisher Scientifics NanoDrop™ One.

2.10.2 PCR round #2

In this reaction the DNA expression cassette was made through an overlapping PCR. This overlapping PCR is a result of the intermediate products binding together. The binding is facilitated by the gRNA and reverse complimentary gRNA. Primers can then extend, joining the two fragments (Li et al., 2015). The intermediate products constructed in PCR round #1 were used as a template and primer set F1-R2 was added to the PCR master mix (Fig. 10).

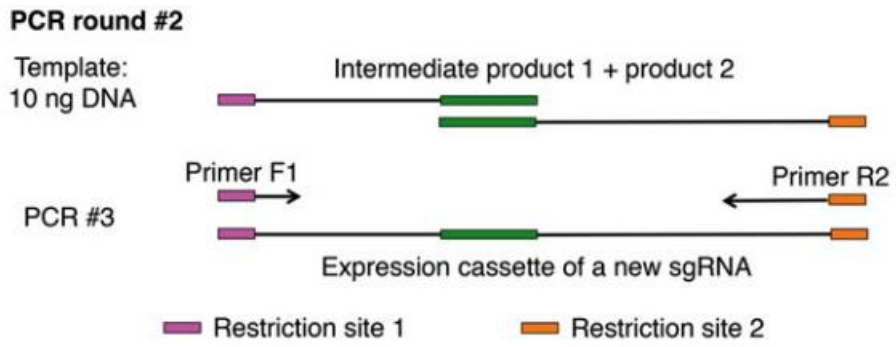



*Figure 10: PCR round #2. Here, IP1 and IP2 are joined through overlapping PCR. The resulting complex is a DNA expression cassette containing a gRNA, an AtU6-1 promoter, gRNA scaffold and restriction sites. Figure from Li et al. (2015).*

The DNA concentration of IP1 and IP2 was measured using the NanoDrop™ One. The concentration was diluted, and 5 ng/ml of IP1 and IP2 was added to the PCR master mix (Table 11).

*Table 11: PCR master mix used to construct DNA expression cassettes. IP1 and IP2 were combined to form the template for this reaction.*

| Reagents | 50 µL reaction | Final concentration |
|---|---|---|
| 5X Q5 reaction buffer | 10 µL | 1X |
| 10 mM dNTPs | 1 µL | 200 µM |
| 10 µM Forward Primer | 2.5 µL | 0.5 µM |
| 10 µM Reverse Primer | 2.5 µL | 0.5 µM |
| Intermediate product 1 | 2.5 µL | 25 ng |
| Intermediate product 2 | 2.5 µL | 25 ng |
| Q5 High-Fidelity DNA Polymerase | 0.5 µL | 0.02 U/µL |
| Nuclease-Free Water | To 50 µL | |

This reaction was run in the T100™ Thermal Cycler, using the same settings as described on page 19.

To increase the distance between the PCR product (430-432 bp) and IP1 (304 bp), the resulting product was run on a 2% agarose gel. The DNA was excised from the gel and extracted as described on page 20. The DNA was also eluted with 15 µL elution buffer and the successful extraction of DNA was verified using Thermo Fisher Scientifics NanoDrop™ One.

## 2.11 Restriction digestion of DNA expression cassettes and pFGC-pcoCas9

The vector and the expression cassettes were digested with the relevant enzymes before ligating the cassettes into pFGC-pcoCas9. This process would linearize and cut out a small part of pFGC-pcoCas9 as well as remove the caps on ends of the expression cassettes. The result was sticky ended products that could be ligated.

### 2.11.1 Digestion using PacI and SdaI (SbfI)

DNA expression cassettes 1 and 2 (Fig. 6) were digested with PacI and SdaI. Alongside this reaction the vector pFGC-pcoCas9 was also digested with the same enzymes. The restriction enzymes were obtained from Thermo Fisher Scientific. These would cut the DNA strand, producing sticky ends (Fig. 11).

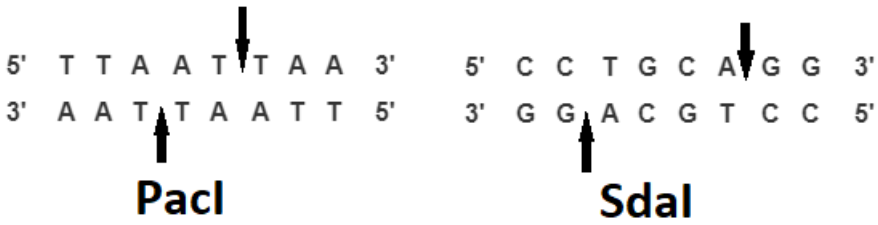



*Figure 11: The restriction sites where PacI and SdaI binds and cuts. The overhang that will be created by PacI and SdaI are indicated by the arrows. Figure adapted from Thermo Fisher Scientifics website.*

pFGC-pcoCas9 had previously been isolated from a DH5α™ growth strain. The plasmid was measured to have a DNA concentration of 557 ng/ µL and only 1 µL was used for the digestion. Restriction digesting using lower concentrations of the plasmid was also tried. The DNA concentration of the extracted DNA expression cassettes was 19 ng/µL for both cassette 1 and 2. When using the NanoDrop™ One, 2 µL of the eluted mixture is used to measure the DNA concentration. When testing

the concentration of cassette 1 the first sample was lost, and a second test had to be done. This meant that for cassette 1 only 11 µL of reaction remained of the initial 15 µL. As a result, the digestion reaction for DNA expression cassette 1 only had 11 µL x 19 ng/µL=209 ng while the reaction for DNA expression cassette 2 had 13 µL x 19 ng/ µL=247 ng. Even though there was less DNA in one of the reactions, this was still within the 0.1-0.5 µg concentration recommended by the manufacturer. All DNA was added to the reaction as seen in table 12. The volume of PacI and SdaI is lowered to 0.5 only 0.5 µL instead of the 1 µL recommended by the manufacturer in accordance with Matsumura (2015).

*Table 12: Shows the reagents and concentration used to perform restriction digestion of DNA expression cassette 1 and 3 as well as pFGC-pcoCas9 using PacI and SdaI.*

| **Reagents** | **Volume: Cassette 1 and 3** | **Volume: pFGC-pcoCas9** |
|---|---|---|
| PacI | 0.5 µL | 0.5 µL |
| SdaI (SbfI) | 0.5 µL | 0.5 µL |
| 10X buffer SdaI (SbfI) | 2 µL | 2 µL |
| DNA | DNAec 1: 11 µL (19ng/ µL) DNAec 2: 13 (19ng/ µL) | 1 µL (557 ng/µL) |
| $H_2O$ | To 20 µL | To 20 µL |

The reaction needed to be incubated at 37 °C. According to the manufactures instructions the incubation period could be varied from 1-h to 16-h. Therefore, three different time intervals were used during restriction digestion. The intervals were 1-h, 3-h and overnight.

After the incubation period, the reaction was run on a 1% agarose gel. This separated the cut-out fragment of DNA, lessening the probability for failing the subsequent ligation. The DNA expression cassettes were again excised from the gel and cleaned as previously described. The plasmid was extracted from the gel using the Monarch® DNA Gel Extraction Kit from New England Biolabs® inc. This kit allows the purification of larger sequences of DNA.

2.11.2 Digestion with EcoRI and AscI

The digestion with restriction enzymes EcoRI and AscI was undergone similarly to PacI and SdaI. The concentration of insert DNA varied but remained within the 0.1-0.5 µg range recommended by the manufacturer. The reaction used for this digestion is shown in table 13.

*Table 13: Shows the reagents and concentration used to perform restriction digestion of DNA expression cassette 2 and 4 as well as pFGC-pcoCas9 using EcoRI and AscI.*

| **Reagents** | **Insert volume: Cassette 2 and 4** | **Volume: pFGC-pcoCas9** |
|---|---|---|
| AscI (SgsI) | 1 µL | 1 µL |
| EcoRI | 1 µL | 1 µL |
| 10X buffer SdaI (SbfI) | 2 µL | 2 µL |
| DNA | DNAec 1: 0.1-0.5 µg<br>DNAec 2: 0.1-0.5 µg | 1 µL (557 ng/µL) |
| $H_2O$ | To 20 µL | To 20 µL |

Restriction digestion with EcoRI and AscI would also produce sticky ends (Fig.12)


*Figure 12: The restriction sites where EcoRI and AscI binds and cuts. The overhang that will be created by EcoRI and AscI are indicated by the arrows. Figure adapted from Thermo Fisher Scientifics website.*

2.12 Ligation of insert into vector

The ligation of the insert into the vector was facilitated by the T4 DNA ligase from Thermo Fisher Scientific. The molar ratio of insert:vector suggested by the manufaturer was 1:1-5:1 and ≈3:1 was used in all reactions. The molar ratio was calculated using the NEBioCalculator™ v.1.10.1 (https://nebiocalculator.neb.com/#!/ligation). Other than this, the reaction was set up according to the manufacturer's instructions, presented in table 14.

*Table 14: Ligation mixture used to ligate DNA expression cassette into pFGC-pcoCas9.*

| **Reagents** | **Amount** |
|---|---|
| Linear vector DNA | 20-100ng |
| Insert DNA | 3:1 molar ratio over vector |
| 10X T4 DNA Ligase Buffer | 2 µL |
| Thermo Scientific T4 DNA Ligase | 1 U |
| Water, nuclease free | To 20 µL |

The reaction was originally incubated for 10 minutes at 22 °C. To increase the number of transformants, the reaction was also incubated for 1 hour.

Lastly, to verify the success of the ligation, 10 µL of the ligation mixture was run on a 1% agarose gel. The bands were visualized with UV light in the ChemiDoc™ XRS+ from BioRad.

Ligation would be undertaken twice to incorporate both expression cassettes.

2.13 Transforming *Escherichia coli* (*E. coli*) with the expression vector

Two methods of transformation were used for transforming *Escherichia coli* (*E. coli*) — heat-shock and electroporation. The DH5α™ growth strain was chosen as this is the growth strain for pFGC-pcoCas9.

2.13.1 Heat shock transformation

The DH5α™ growth strain is chemically competent and could be applied directly following these steps:

1. Subcloning Efficiency ™ DH5α™ from Invitrogen was thawed on ice and separated in aliquots of 50 µL.

2. Samples that were not used were kept at -80 °C in a Thermo Fisher Scientific Ultra-Low Temperature Chest Freezer.
3. Liquid S.O.C. medium was pre-warmed at 37 °C for 1 hour.
4. 5 µL of ligation mixture was added directly to the DH5α™ cells and flicked gently to mix. The samples were kept on ice for 30 minutes.
5. The samples were put in a 42 °C water bath for exactly 20 seconds before being returned to ice for 5 minutes.
6. 950 µL pre-warmed S.O.C. medium was added to the sample and the mixture was incubated at 37 °C at 225 rpm for 1 hour.
7. Selection media containing 50 µg/mL kanamycin, was pre-warmed to 37 °C.
8. The samples were plated on pre-warmed selection media, inverted, and incubated at 37 °C overnight.

The following morning, colonies were picked and added to a liquid LB media containing kanamycin. This was incubated overnight at 37 °C and 275 rpm in a HT Minitron Incubator Shaker. The resulting sample was pelleted at 8,000 rpm and the plasmid was extracted from the bacteria using the QIAprep® Spin Miniprep Kit in accordance with the manufacturer's instructions. In addition to the destination vector, puC19 was used as a control for the transformation. The transformation using this plasmid was undertaken in the same way, except for the selection medium. For selection of puC19, LB medium containing 100 µg/mL ampicillin was used.

2.13.2 Electroporation

To undergo electrotransformation, the DH5α™ strain had to be altered to be electrocompetent. This was done through the following steps:

1. An overnight culture of DH5α™ was grown in liquid LB medium.
2. 100 mL of fresh LB medium was prepared in a 500 mL flask.
3. This was inoculated with the overnight, stationary-phase culture to an OD of ≈0.05. 100x dilution.
4. The cells were grown for approximately 3 hours until they reached the mid-exponential phase, $OD_{600}$ of 0.4-0.6.
5. Cells were kept chilled from this point and all centrifugation was undergone at 4 °C.
6. The cells were transferred to 2x50 mL Falcon tubes.
7. The cells were pelleted by centrifugation for 5 minutes at 6,000 rpm. They were promptly removed, and the supernatant was poured off.
8. The cells were washed by adding 40 mL of chilled 10% glycerol, then resuspended by vortexing the sample vigorously. The sample was pelleted by centrifugation for 3.5 minutes at 6,000 rpm and the supernatant was poured off. This was repeated for four wash cycles with 10% glycerol.
9. The pellet was resuspended in 500 µL 10% glycerol, making a 100x concentration of the initial culture.
10. The sample was divided into aliquots of 50 µL.

Samples that were not immediately used for electroporation, were stored at -80 °C.

Before electroporation, the ligation mixture was heated to 65 °C for 10 minutes using a heating block to inactivate the T4 ligase. The DNA was also purified using the QIAquick® PCR Purification Kit in accordance with the manufacture's specifications. The electroporation was undergone following these steps:

1. 17 mm x 100 mm round bottom tubes (e.g. VWR #60818-667) were prepared at room temperature. The S.O.C. recovery medium was placed in a 37 °C water bath. The selective plates were pre-warmed at 37 °C for 1 hour.
2. Electroporation cuvettes (1 mm) and microcentrifuge tubes were placed on ice.
3. As a positive control for transformation, the control pUC19 was diluted by 1:5 to a final concentration of 10 pg/µL using sterile water.
4. NEB 5-alpha Electrocompetent cells were thawed on ice (about 10 minutes) and cells were mixed by flicking gently. 25 µL of the cells were transferred to a chilled microcentrifuge tube. 1 µL of the DNA solution was added.
5. The cell/DNA mix was carefully transferred into a chilled cuvette, without introducing bubbles, ensuring that the cells deposited across the bottom of the cuvette. Electroporation was done using the following conditions for BTX ECM 630 and Bio-Rad GenePulser electroporators: 1,7 kV, 200 Omega and 25 µF. The typical time constant is 4.8 to 5.1 milliseconds.
6. 975 µL of 37 °C S.O.C. was immediately added to the cuvette and gently mixed up and down twice. The mixture was transferred to the 17 mm x 100 mm round-bottom culture tube.
7. The mixture was vigorously shaken (250 rpm) at 37 °C for 1 hour.
8. The cells were diluted and then 100-200 µL cells were spread onto pre-warmed selective plates containing 50 µg/mL kanamycin.
9. Plates were incubated overnight at 37 °C.

puC19 was used as a control during electroporation. The method was the same as with the expression vector but puC19 was plated out on selection plates containing 100 µg/mL ampicillin.

### 2.13.3 Verification of transformation

Post transformation, DNA would be isolated from colonies. The presence of the expression vector would be visualized on a 1% agarose gel and verified through sanger sequencing at GATC, Germany.

## 2.14 Note on the text

As will be seen in the result and discussion chapter, the transformation of *E. coli* was unsuccessful. Due to the impact of Covid19, our lab had to close. This made it impossible to continue research. The steps that would have taken place after a successful transformation of *E. coli* were still planned and will be described here.

## 2.15 Testing constructs through transient transformation

The protocol for transient transformation was adapted from Cui et al. (2017):

1. Inoculate 2 mL of low-salt LB (Appendix I) with *Agrobacterium* strain. Grow cells at 20 °C overnight, shaken at 225 rpm in a HT Minitron Incubator Shaker.
2. Use the 2 mL culture to inoculate 50 mL of the same medium in a 250 mL flask. Incubate until cells reach an $OD_{600}$ between 0.5 and 1.0. Chill the culture on ice.
3. Prepare a resuspension liquid containing 3 mM $Na_2HPO_4$, 50 mM 4-morpholineethanesulfonic acid (MES) (pH 5.6), 0.5% glucose, and 100 µM acetosyringone (AS).
4. Pellet cells by centrifugation at 4 °C for 10 minutes at 10,000 g in an SS-34 rotor. Discard the supernatant and resuspend cells in 5 mL resuspension liquid. Repeat centrifugation and discard the supernatant.

5. Resuspend in 35 mL resuspension liquid.
6. Remove fully extended leaves from *in vitro* 'Jonsok' and 'Hawaii'.
7. Submerge leaves in 35 mL of bacterial suspension.
8. Apply a vacuum of 0.085 MPa for 30 minutes before slowly releasing the vacuum.
9. Remove left-over liquid with sterile filter paper.
10. Place leaves on MS-medium and keep in normal conditions (20±1 °C, 18-h photoperiod, light intensity of 2,000 lux) for 3 days.

After the three days, DNA can be isolated from the leaf and examined by Sanger sequencing of the target area.

### 2.16 Transformation of *Agrobacterium tumefaciens*

The freeze-thaw transformation of *Agrobacterium* would be undertaken in accordance with Wise et al. (2006). This protocol was slightly modified in accordance with Miller (2019):

#### 2.16.1 Preparation of freeze-thaw competent cells

1. Inoculate 2 mL of low-salt LB (Appendix I) with *Agrobacterium* strain. Grow cells at 20 °C overnight, shaken at 225 rpm in a HT Minitron Incubator Shaker.
2. Use the 2 mL culture to inoculate 50 mL of the same medium in a 250 mL flask. Incubate until cells reach an $OD_{600}$ between 0.5 and 1.0. Chill the culture on ice.
3. Pellet cells by centrifugation at 4 °C for 10 minutes at 10,000g in an SS-34 rotor. Discard the supernatant and resuspend cells in 5 mL chilled 20 mM $CaCl_2$. Repeat centrifugation and discard the supernatant.
4. Resuspend cells in 1 mL chilled 20 mM $CaCl_2$ and transfer to chilled Eppendorf tubes in aliquots of 150 µL. Keep on ice for immediate transformation or freeze at -80 °C.

#### 2.16.2 Freeze-thaw Transformation of *Agrobacterium*

1. Add ~1 µg plasmid DNA to a vial of competent cells. Incubate on ice for 30 minutes.
2. Freeze the cell/DNA mix in liquid nitrogen for 5 minutes. Submerge and hold the tubes using tweezers.
3. Thaw the frozen cell/DNA mixture for 5-10 minutes at room temperature. Transfer the contents of the tube to 2 mL liquid low-salt LB medium and incubate at 28 °C with shaking at 225 rpm for 2-4 hours in a HT Minitron Incubator Shaker.
4. Pellet the cells in a microcentrifuge at 13,000 rpm for 2 minutes. Resuspend the cells in 200 µL low-salt LB medium containing 10 mg/L rifampicin, 25 mg/L gentamicin and 50 mg/L kanamycin.
5. Plate 100-300 µL of the cell suspension on agar plates with low-salt LB with 10 mg/L rifampicin, 25 mg/L gentamicin and 50 mg/L kanamycin. Invert plates and incubate at 28 °C overnight.

#### 2.16.3 *Agrobacterium* mediated transformation of plant material

Plant material would be transformed through the following steps:

1. Select single colonies of transformed *Agrobacterium* from selection media. Grow the *Agrobacterium* in 5 mL of low-salt LB medium, containing 10 mg/L rifampicin, 25 mg/L gentamicin and 50 mg/L kanamycin in a shaking incubator at 28 °C for ~7 hours.
2. In 250 mL flasks, use 750 µL of the culture to inoculate 50 mL low-salt LB medium containing 10 mg/L rifampicin, 25 mg/L gentamicin and 50 mg/L kanamycin.

3. Incubate at 28 °C overnight until an $OD_{600}$ of 0.5-1.0 is reached.
4. Transfer the cells to 50 mL tubes and pellet the cells by centrifugation at 2,700 g at 18 °C for 20 minutes.
5. Pour off the supernatant and resuspend cells in 20 mL MS-2 medium (Appendix I).
6. Centrifuge the cells at 2,850 rpm at 18 °C for 5 minutes, pour off supernatant and resuspend in 8 mL MS-2 medium.
7. Distribute the cells in 5 cm Petri dishes in aliquots of 4 mL.
8. Place explants from 'Jonsok' and 'Hawaii' in the cell suspension and shake for 5 minutes.
9. Dry explants on sterile filter paper and place in 10 cm Petri dishes containing regeneration medium F with 0.8% agar.

## 2.17 Gold particle bombardment of leaf disks

Plasmid DNA would be isolated from single colonies of *E. coli*. The protocol used was adapted from Hvoslef-Eide et al. (2019).

### 2.17.1 Sterilization of gold particles, 60 mg/mL

1. Add 1 mL 100% EtOH to 60 mg Au-Particles in a 2 mL Eppendorf tube and vortex.
2. Centrifuge the Eppendorf tube at 10,000 rpm for 1 minute and pour off the supernatant.
3. Resuspend the pellet in 1 mL sterile water and store at -20 °C.

### 2.17.2 Preparation of plasmid DNA, 1 µg/µL

1. Use the amount and concentration of plasmid DNA to calculate required amount. Add to an Eppendorf tube.
2. Add 20 µL sodium acetate (3 M, pH 5.2).
3. Add 500 µL absolute EtOH, chilled to -20 °C, and vortex.
4. Centrifuge at 10,000 rpm for 5 minutes. Carefully pour off supernatant.
5. Wash with 1 mL absolute EtOH and dry in a flow hood for 20-30 minutes.
6. Add sterile MilliQ water to a DNA concentration of 1 µg/µL.
7. Chill in fridge for 20-30 minutes and vortex.
8. Store at -20 °C.

### 2.17.3 Covering gold particles with DNA

1. Pipette the following into a 1.5 mL Eppendorf tube:
    - 100 mL gold particle solution (1 µM, 60 mg/mL)
    - 25 µL DNA (1 µg/µL)
    - 100 µL $CaCl_2$ (2.5 M)
    - 40 µL spermidine (0.1 M)
2. Vortex for 3 minutes and leave on ice for 5 minutes.
3. Pipette off the supernatant.
4. Wash with 100 µL 100% EtOH and leave on ice for 5 minutes.
5. Pipette off supernatant.
6. Resuspend in 120 µL EtOH and chill on ice until needed.

### 2.17.4 Shooting the gene gun

The gene gun that would be used for this process is the Biolistic ™ Particle Delivery System Model PDS-1000 (DuPont). The procedure is as follows:

1. Sterilize the stop plate, macro carrier and rupture disk (1,150 psi) in 70% EtOH. Dry in a flow hood. Wash the inside of the gun chamber with 70% EtOH.
2. Open the helium gas flask and turn on the vacuum pump.
3. Dip the rupture disk in isopropanol and place it into the holder. Place the holder and rupture disk into the gun and tighten with a special tool.
4. Put a macro carrier plate into the macro carrier holder. Add 10 µL resuspended gold particles covered with DNA to the middle of the macro carrier. Air dry in flow hood.
5. Place a stop plate and the macro carrier into the holder and place the holder into the gun in the upper position.
6. Place a 9 cm Petri dish with the leaf disk explant on MS-medium inside the gun in position 2 from below.
7. Remove the lid and shut the door.
8. Adjust the vacuum in the chamber to ~28 mmHg.
9. Shoot by holding the button until the rupture disk is broken.
10. Remove the Petri dish, replace the lid and seal it with parafilm.

### 2.17.5 Post transformation

After the transformation, explants would be transferred to regeneration medium F (Table 6) and incubated at 20±1 °C temperature and an 18-h photoperiod with a light intensity of 2,000 lux. After two weeks the plants would be moved to a selection medium containing 2 mg/L glufosinate ammonium.

### 2.18 Verification of transformation

If plants regenerated on a regeneration medium containing glufosinate ammonium, the transformation may have been successful. DNA would then be isolated using QIAgens DNeasy Plant Mini Kit. The potential mutation would be verified through Sanger sequencing and gel analysis. The plants would also be grown and allowed to bear fruit. If the plants bore fruit a visual analysis could also be undertaken.

# 3 Results and discussion

The production of gene edited plants is a complex process. The transformation of plants is a prerequisite for this process. However, other methods, e.g. regeneration of explants and selection of transformants, are also vital to produce gene edited plants. In addition, the ability to troubleshoot when things do not go as planned and find possible faults in a method is an important part of all research.

### 3.1 Tissue culture experiments

In the following subchapters, results detailing all experiments using tissue culture are presented.

### 3.1.1 Regeneration medium

The direct regeneration of explants confers clear advantages over the regeneration from callus as there is less risk of somaclonal variation. Somaclonal variation is defined as the variation which may arise during an *in vitro* culture (Larkin et al., 1981). Several authors have confirmed that direct regeneration can result in a more genetically stable end-product (Arene et al., 1993; Phillips et al., 1994). Direct regeneration of strawberry has proven successful in several studies (Barceló et al., 1998; El Mansouri et al., 1996; Mohamed et al., 2007; Nehra et al., 1989).

However, regeneration protocols tend to be genotype specific. To test the application of direct regeneration in the cultivated strawberry 'Jonsok' and the wild type 'Hawaii', we started with the studies of Barceló et al. (1998) and El Mansouri et al. (1996). These studies used the same hormones, BAP and IBA, but Barceló et al. (1998) used the cultivated strawberry while El Mansouri et al. (1996) used the wild strawberry. The optimal hormone concentration for successful regeneration differed (0.5 mg/L IBA (auxin) and 2.0 mg/L BAP (cytokinin) for the cultivated strawberry, 0.25 mg/L IBA and 4 mg/L BAP for the wild strawberry). This could suggest that the optimal hormone concentration for successful regeneration is dependent on the cultivar. Therefore, it was necessary to find the optimal hormone concentration for both 'Jonsok' and 'Hawaii'. The use of light was also an interesting aspect, as Barceló et al. (1998) recorded the best results when the explants were incubated in total darkness for two weeks before being transferred to light conditions.

Both studies used leaf disks as explants (Barceló et al., 1998; El Mansouri et al., 1996). Petiole fragments have also been used successfully in direct regeneration (Husaini et al., 2011; Zebrowska et al., 2002). It would be interesting to see whether similar media could be used to perform a direct regeneration of different explants. Therefore, petiole fragments were also used as explants.

The explants were observed at two time-points and evaluated on a scale of 0-10 (Table 15).

*Table 15: The scale used to visually evaluate strawberry-explants propagated on regeneration media. These grades have been the basis for the statistical analysis.*

| **Grade** | **Appearance** |
|---|---|
| 0 | Dead |
| 1 | Very sickly |
| 2 | Sickly |
| 3 | Some signs of degradation. |
| 4 | No growth |
| 5 | Healthy explants |
| 6 | Healthy plants showing signs of minor growth |
| 7 | Forming new structures |
| 8 | Formed structures |
| 9 | New shoots |
| 10 | Can be transferred to MS-media |

The evaluation was based on the appearance and perceived vitality of the explant (Fig. 13).

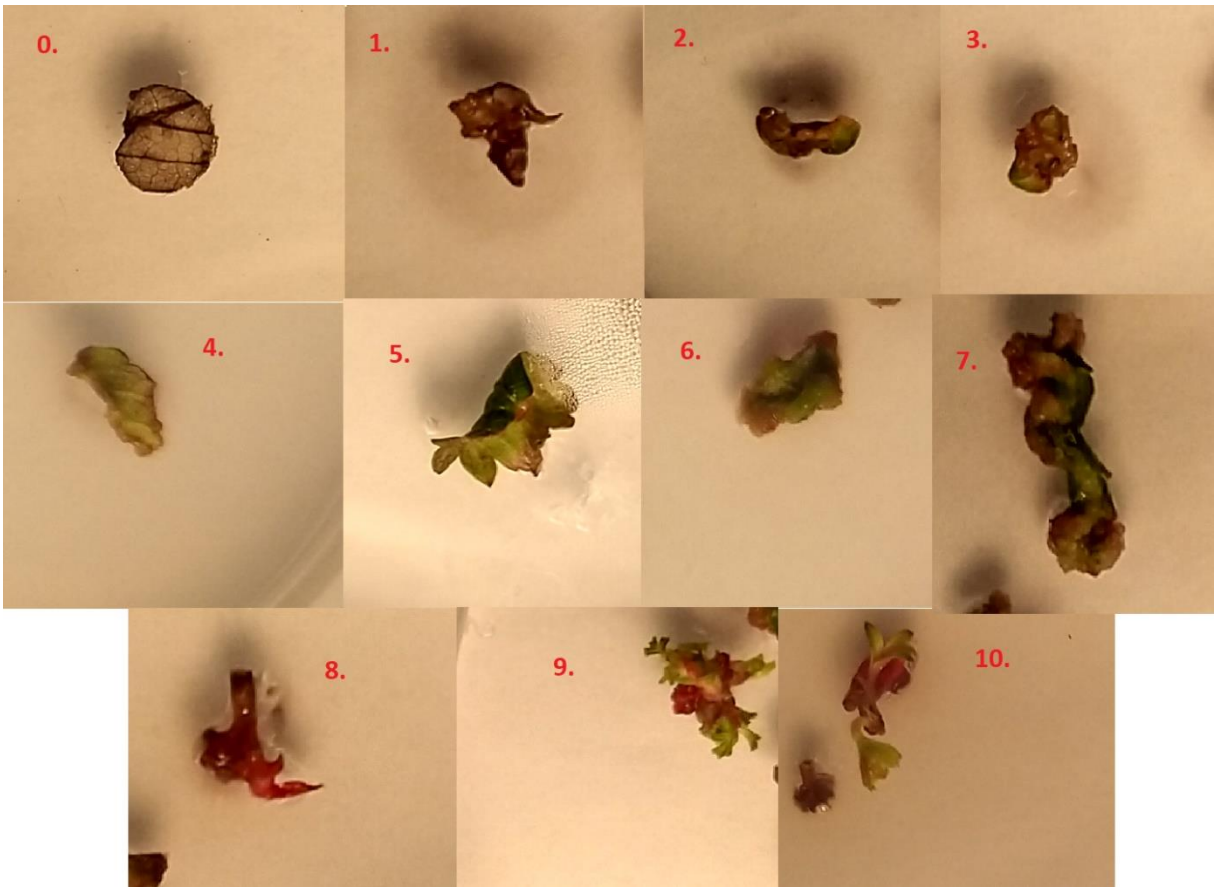



*Figure 13: Explants grown on the regeneration medium. The numbers refer to the different points in the scale presented in table 15. Explants shown here are taken from both cultivated and wild strawberry.*

The effects of hormone concentration, cultivar, explant, and growth conditions were analyzed using Generalized Linear Model (GML) and linear regression analysis (Fig. 14).

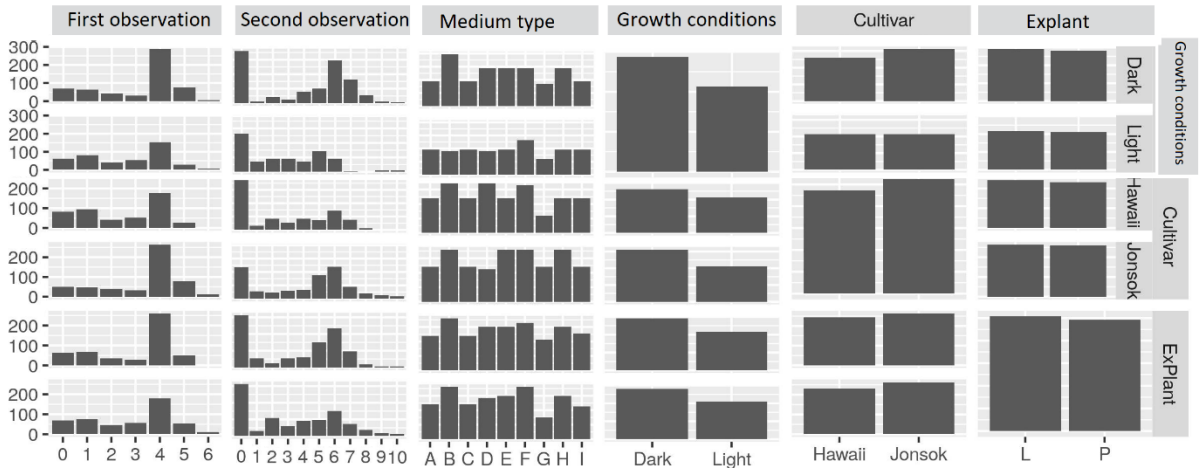



*Figure 14: Results of the linear regression analysis. Column 1 and 2 shows explant survival at the two different observation times. The third column shows the performance of the different medium types. Column 4 shows the effect of light conditions. Column 5 shows the effect of cultivars on the survivability of explants. Column 6 shows the effect of explant type (L=leaf disk, P=petiole fragment). The rows show the correlation between the different data points used in the analysis. Figure created with the statistical program R.*

### 3.1.1.1 Results of the linear regression analysis

The explants were first observed after 44 days. At this point little growth was observed, and most explants were graded in category 4 (Table 15) (Fig. 14). The first column in Figure 14, pertaining to the first observation, has a scale of 0-6. This is because no explant was graded >6 at this point. The second observation was done 33 days after the first (column 2, Figure 14). A greater spread of values (0-10) and more plant death were registered during this observation. This is expected as explants will respond more positively or negatively to the treatment over time. There is a strong correlation between the first and second observation (0.88) (Appendix V), based on the GLM analysis.

The effect of variables on medium type was mostly consistent (column 3, Figure 14). Here medium B seemed to have the best effect on the explants except in column 3 row 1 pertaining to effect of light conditions. However, these results are not significant (Appendix V). Medium G performed worst in all scenarios (Fig. 14). Growth conditions in column 4 shows that incubation in total darkness had a significant effect on the explants. 'Jonsok' performed better than 'Hawaii' in all instances (Fig. 14). In general, it seems that explant type had some minor differences, but they are not statistically significant (Column 6, Figure 14) (Appendix V).

### 3.1.1.2 Effect of media

Most of the media used had a significant effect on the survivability of the explants. Visualizing the data shows that medium B, overall, had the most positive impact on the explants. However, this result is not significant on a confidence interval (CI)>95 (Appendix V). Medium F also had a positive impact over most variables and the result is significant at a 99.99 CI (Appendix V). This medium contains 0.5 mg/L IBA and 4 mg/L BAP. This is the optimum concentration for regeneration of the wild strawberry found by El Mansouri et al. (1996). The worst performing medium was medium G. These results are significant at a 99.9 CI (Appendix V).

### 3.1.1.3 Effect of auxin (IBA) and cytokinin (BAP)

Medium G, H and I exhibits poor performance when compared with the other media. This could suggest that a high concentration of auxin (IBA) is coupled with poorer regeneration of explants. This is especially true for medium G. This medium contained the highest auxin concentration of 1 mg/L and

the lowest cytokinin concentration of 2 mg/L. This could indicate that there is a negative interaction between high auxin concentrations and low concentrations of cytokinin. Medium A also showed poor performance. This medium has the same cytokinin concentration as medium G. This could indicate that a low concentration of cytokinin is detrimental to explant regeneration.

The media which performed best contained 3-4 mg/L cytokinin and 0.25-0.5 mg/L auxin. This is in accordance with the optimal hormone concentrations found in the studies by Barceló et al. (1998) and El Mansouri et al. (1996).

#### 3.1.1.4 Effect of cultivar

The effect of choice of cultivar was significant at a 99 CI (Appendix V). This indicates that 'Jonsok' performed better than 'Hawaii' overall for all conditions during regeneration. This is contradictory to the findings of El Mansouri et al. (1996) where their wild strawberry genotypes showed a 98% regeneration rate at the optimum hormone concentration. The regeneration rate for medium B was 34.4% for 'Jonsok' and 25% for 'Hawaii' while medium F showed the highest regeneration rate of 50% for 'Jonsok' and 31% for 'Hawaii'. These results are in accordance with Barceló et al. (1998) where the cultivated strawberry had a 47% regeneration rate at the optimum hormone concentration. This could indicate that genotypes may play a significant role in the results of El Mansouri et al. (1996) and Nehra et al. (1989) since they had a higher success rate with their genotypes on their regeneration media.

#### 3.1.1.5 Effect of light quality

The effect of light quality was significant at a 99.9 CI (Appendix V). This shows that incubating explants in total darkness for two weeks before moving to light conditions had a positive effect on survivability. This is in accordance with the studies of Barceló et al. (1998).

#### 3.1.1.6 Effect of explant

Effects of explants were not significant to survivability. This suggests that the choice of explant is not imperative in our strawberries.

#### 3.1.1.7 Conclusions on media.

Based on the statistical analysis, it seems the best treatment for both 'Jonsok' and 'Hawaii' is the following: leaf disk or petiole fragments incubated in darkness for two weeks before being moved to light conditions. The medium used should be medium F as it had a significant positive performance under all conditions.

In addition to this the statistical analysis indicates that cytokinin concentrations <3.0 mg/L and auxin concentrations >0.5 mg/L is detrimental to explant regeneration for our strawberries.

### 3.1.2 Dose response to BASTA

If the transformation of explants were successful, the pFGC-pcoCas9 would confer a resistance to the herbicide BASTA (Fig. 5). BASTA is a non-selective herbicide, where the active ingredient is glufosinate ammonium (Bayer Crop Science Australia, 2016). A selection process could therefore be undertaken by growing explants on a growth medium containing glufosinate ammonium. Transformed explants should have a higher tolerance to glufosinate ammonium than plants that were not transformed. However, high concentrations of glufosinate ammonium is detrimental to plant growth, even if the explant has a higher tolerance (Ganasan et al., 2010). Therefore, it was necessary to find the lowest concentration of glufosinate ammonium that would kill all explants that were not transformed with pFGC-pcoCas9 but let the transformants survive the treatment.

This was established through observation of explants on different media containing an increasing concentration of glufosinate ammonium. After the observation period of 49 days, all explants died in medium 3-6 indicating a threshold glufosinate ammonium tolerance of 2 mg/L for 'Jonsok' and 'Hawaii'. (Fig. 15).

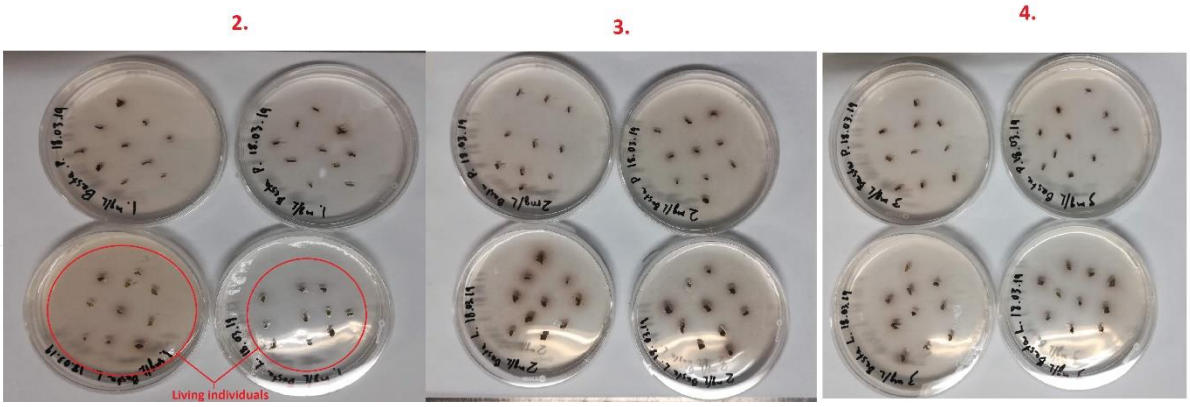



*Figure 15: Three of the seven BASTA selection media. Medium 2 still had living individuals after five weeks. A higher concentration than what was used in medium 2 resulted in the death of explants. This can be seen in medium 3 and 4.*

During the preliminary tests of the dose response to BASTA, the explants were grown on callus inducing media (Table 3 and 4). This is because the testing of the callus inducing medium was concluded before the preliminary tests of the regeneration medium began. If the transformation of plant material had been undertaken 2 mg/L glufosinate ammonium would have been added to regeneration medium F.

### 3.1.3 Plant response to MS-media

The cultivated strawberry 'Jonsok' and the wild type 'Hawaii', both responded well to the MS-media used in this thesis. Little plant death but from infections on the media was observed.

This was not the case for the wild strawberry 'Snöhvit'. Over the course of six months of subculturing 'Snöhvit' on MS-media (Table 2), plantlets showed increasing signs of deterioration. Plants lost color, turning light green, had low vigor and formed few new structures during growth periods. This, in addition to issues when isolating DNA, was the reason for the switch from the wild type 'Snöhvit' to the wild type 'Hawaii'.

### 3.1.4 Callus induction media

Only callus inducing medium 2 produced viable callus. This medium was used to test the dose response to BASTA.

### 3.1.5 Callus regeneration medium

To regenerate plants from callus, the testing of a callus regeneration medium was planned. The medium would contain BAP and 2,4-D in accordance to Biswas et al. (2007) and Jones et al. (1988). A gradient would also be established to determine the optimal hormone concentration for regeneration of 'Jonsok' and 'Hawaii' from already established callus.

Successful attempts at direct regeneration made the testing of a callus regeneration medium obsolete and testing was not done.

## 3.2 DNA isolation

DNA was isolated from both cultivars several times. The final concentrations were between 7.50-20.72 µg/µL for 'Jonsok' and between 5.23-18.52 µg/µL for 'Hawaii'. Samples with a concentration >10 µg/µL and an absorption level between 1.80-2.00 were selected for further use. These were used as templates for PCR amplification of the target area. Initial studies with the wild type cultivar 'Snöhvit' yielded 1.46-8.47 µg/µL. In addition to this DNA isolated from 'Snöhvit' generally had an 260/280 absorbance (ABS) level of <1.80. A suboptimal purity is an indicator that the sample carries contaminants from the DNA extraction (Lorenz, 2012). Such contaminants are common inhibitors of PCR which may have contributed to the unsuccessful PCR amplification of the target area (Lorenz, 2012).

## 3.3 Verification of target area

Parts of exon 1 and 2 of *F3H* was amplified through a PCR reaction. The amplification of exon 2 was unsuccessful. This could suggest that the primers, designed using sequences obtained from NCBI, were not complimentary to exon 2 of 'Jonsok' and 'Hawaii'. Amplification of Exon 1 was successful, showing high similarity to sequences obtained from NCBI (Fig. 16). This similarity suggests that exon 1 of *F3H* is highly conserved.

Taq polymerases has an error rate greater than that of high-fidelity polymerases (New England Biolabs® inc., 2020a). This could result in the single base differences seen in Figure 16.

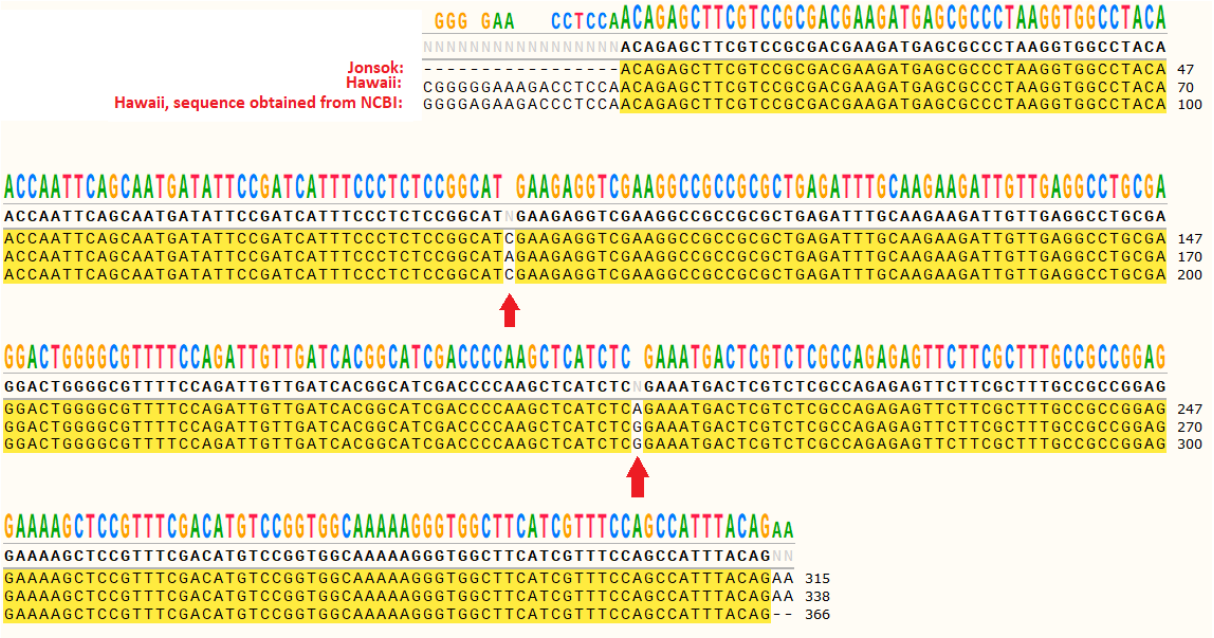



*Figure 16: Shows the alignment of sequenced material from 'Jonsok' and 'Hawaii' as well as sequences obtained from NCBI. The single base misalignments are indicated by arrows. Figure was made using SnapGene®.*

Indel events in a genetic sequence are likely to induce a "frameshift mutation" (Crick, 1962). If such an event occurs early in the genetic sequence, there is a higher probability of producing a non-functional or truncated protein (Klug et al., 2014). Therefore, it was decided not to pursue the sequencing of exon 2 of the *F3H* gene, and design gRNAs that would recognize sequences in exon 1.

## 3.4 Seamless assembly, digestion, ligation, and transformation

The successful integration of the destination vector into *E. coli* was not achieved in this thesis. Despite an extensive troubleshooting of the processes leading up to transformation (Fig. 17.A-C) and the transformation (Fig. 17.D), no conclusive reason for the unsuccessful transformation could be identified. This next section will cover the troubleshooting process of the seamless assembly of the DNA expression cassettes, restriction digestion of DNA expression cassettes and plasmid, ligation of the DNA expression cassettes and the plasmid and transformation of *E. coli*.

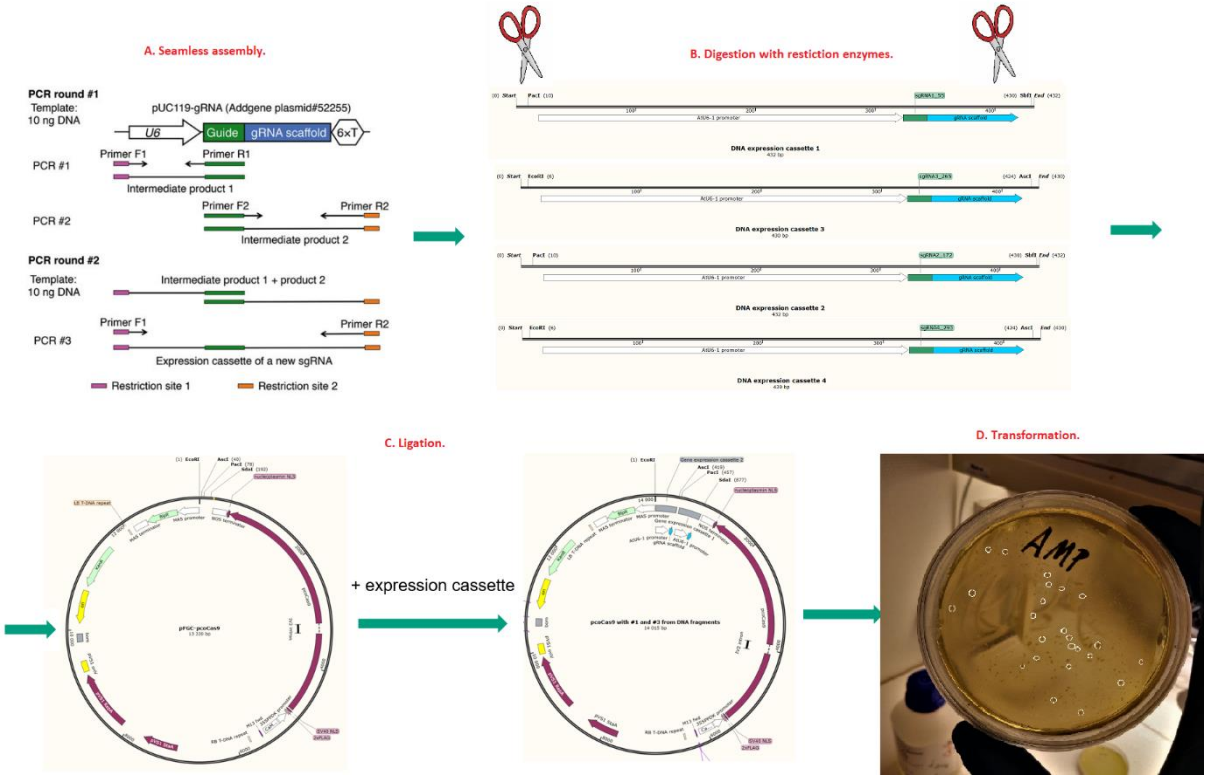



*Figure 17: Shows the general steps in the path from seamless assembly to transformation. A. shows the two steps taken in seamless assembly. B. shows the subsequent digestion of the DNA expression cassettes as well as pFGC-pcoCas9. C. shows the ligation of expression cassette and plasmid, creating an expression vector. D. shows a post transformational colony. Figures obtained from Li et al. (2015) or made with SnapGene®.*

### 3.4.1 Troubleshooting seamless assembly

In the two steps of the seamless assembly, four DNA expression cassettes were created. Each cassette contained a gRNA, a DNA scaffold, an AtU6-1 promotor, and capped restriction sites at both the 3' and 5' end (Fig. 6). The integrity of the DNA cassettes is important for down-stream applications when performing restriction digestion, ligation, and transformation of *E. coli*. However, unsuccessful attempts at transformation suggests that this integrity could have been damaged — especially that of the restriction sites in the DNA cassettes.

The restriction sites are very important as these are required to perform restriction digestion. If these are inhibited, they may not be cleaved by the proper restriction enzymes. This makes subsequent ligation impossible.

Between the PCR reactions of seamless assembly, the DNA had to be separated on a gel, excised, and purified. This process could create some challenges. Exposure to UV could potentially damage the integrity of the DNA (Gründemann et al., 1996). The technical difficulty in excising the DNA fragments

could lower the purity of the final product. Also, during clean-up of DNA excised from the agarose gel, possible unsuccessful disassociation of high-fidelity polymerase could hinder restriction digestion (Matsumura, 2015). These details are important to keep in mind moving into the next phase of troubleshooting.

To excise DNA from a gel DNA must be visualized during UV exposure. When exposed to UV, it is then possible to excise the DNA fragment from the gel and, subsequently, purify the DNA. With that said, UV exposure can damage the DNA (Gründemann et al., 1996) so exposure should be minimal. Initially this was disregarded but minimal UV exposure was implemented in late attempts at troubleshooting. In addition to minimizing the UV exposure, it is possible to protect the DNA by adding cytidine or guanosine to the electrophoresis buffer (Gründemann et al., 1996). This was not attempted due to time constraints but could be implemented in future studies where UV exposure could pose a problem.

The excision of DNA from the gel also poses some technical difficulties. This is especially true after round two of the seamless assembly (Fig. 17-A), where the template and the DNA expression cassette are very similar in size (Fig. 18).

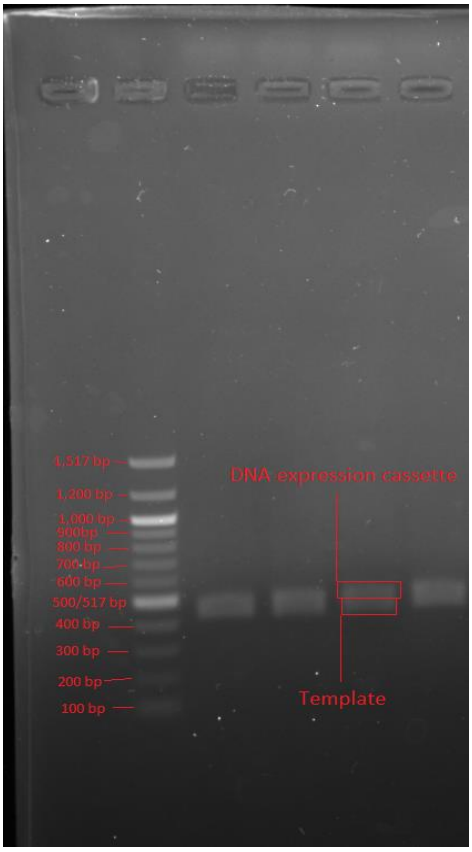



*Figure 18: Shows the PCR product of seamless assembly round #2. The assembled DNA cassette is 430-432 bp while the template is 304 bp.*

The excision of the DNA expression cassette is done manually with a scalpel. This manual extraction can be challenging because of the small difference in size (Fig. 18). The DNA expression cassette and the template are only 100 bp apart in size. An inexact excision can end up isolating both the DNA expression cassette as well as the template. To counteract this, it was attempted to increase the agarose content of the gel two-fold to 2% agarose. The gel could then be subjected to electrical currents for longer periods without risking running the DNA fragment out of the gel. The postulated result was that a larger distance between the DNA expression cassette and the template would make

excision easier. The result was just a slight increase in band distance, which made excision somewhat easier.

A high-fidelity polymerase is required to make DNA expression cassettes for cloning. This will minimize the incorporation of mismatched nucleotides during PCR (Pezza et al., 2013). It will also increase the chance of correct activity of the DNA expression cassette when transforming plants. While high-fidelity polymerases are necessary for exact replication, there are some problems that can be caused by such polymerases.

DNA mechanisms that confer high processivity to polymerases are often specific to a single polymerase (Wang et al., 2004). Low processivity can result in primers not extending, causing "ragged" ends (Matsumura, 2015). The restriction sites in the DNA expression cassette are located towards the ends of the sequence. If these ends are not fully extended the restriction sites will not be recognized by restriction enzymes (Matsumura, 2015). These ends are difficult to spot on an agarose gel, and so this issue may go undetected.

High-fidelity polymerases binds to the template very tightly (Kong et al., 1993). The binding polymerases can "occlude the ends of the DNA from restriction enzymes or fill in overhangs created by them" (Matsumura, 2015). This can be solved by denaturing the polymerase during DNA purification. According to Matsumura (2015), the guanidine hydrochloride concentrations used to denature polymerases in commercial kits are not sufficient to denature high-fidelity polymerases. To counteract this, two kits were used to purify DNA that had been excised from an agarose gel. These were QIAquick Gel Extraction Kit from QIAGEN and Monarch® DNA Gel Extraction Kit from New England Biolabs® inc. However, no detectable change occurred as transformation of *E. coli* was unsuccessful.

It is difficult to determine if these factors inhibited restriction digestion. It could be one of the above reasons, a combination of problems or something that was not detected. But if the protocol changes that were implemented had an effect the issue could be with restriction digestion of the plasmid or the DNA expression cassettes.

### 3.4.2 Troubleshooting restriction digestion

The restriction digestion of both the DNA expression cassette and the plasmid is necessary to create sticky ends required for ligation (Mertz et al., 1972). Some challenges associated with this process are substrate inhibition, star-activity, and non-functional restriction enzymes (Matsumura, 2015).

Substrate inhibition, where sequences similar to the target area functions as enzyme inhibitors, can occur if the concentration of DNA is too high during the restriction digestion (Matsumura, 2015). Other substances can also function in this way, resulting in what is known as 'star activity' (Robinson et al., 1993). To reduce these events, different concentrations and incubation times were used. The concentrations specified by the manufacturer were used (Table 12 and 13) and the incubation period was varied from 1 hour to overnight. In addition to this, the concentration of restriction enzymes as well as DNA was lowered by half and incubated from 1 h to overnight.

It is hard to determine whether this had any effect as all transformations were unsuccessful regardless of incubation time.

Restriction enzymes displaying irregular activity were also encountered (Fig. 19).

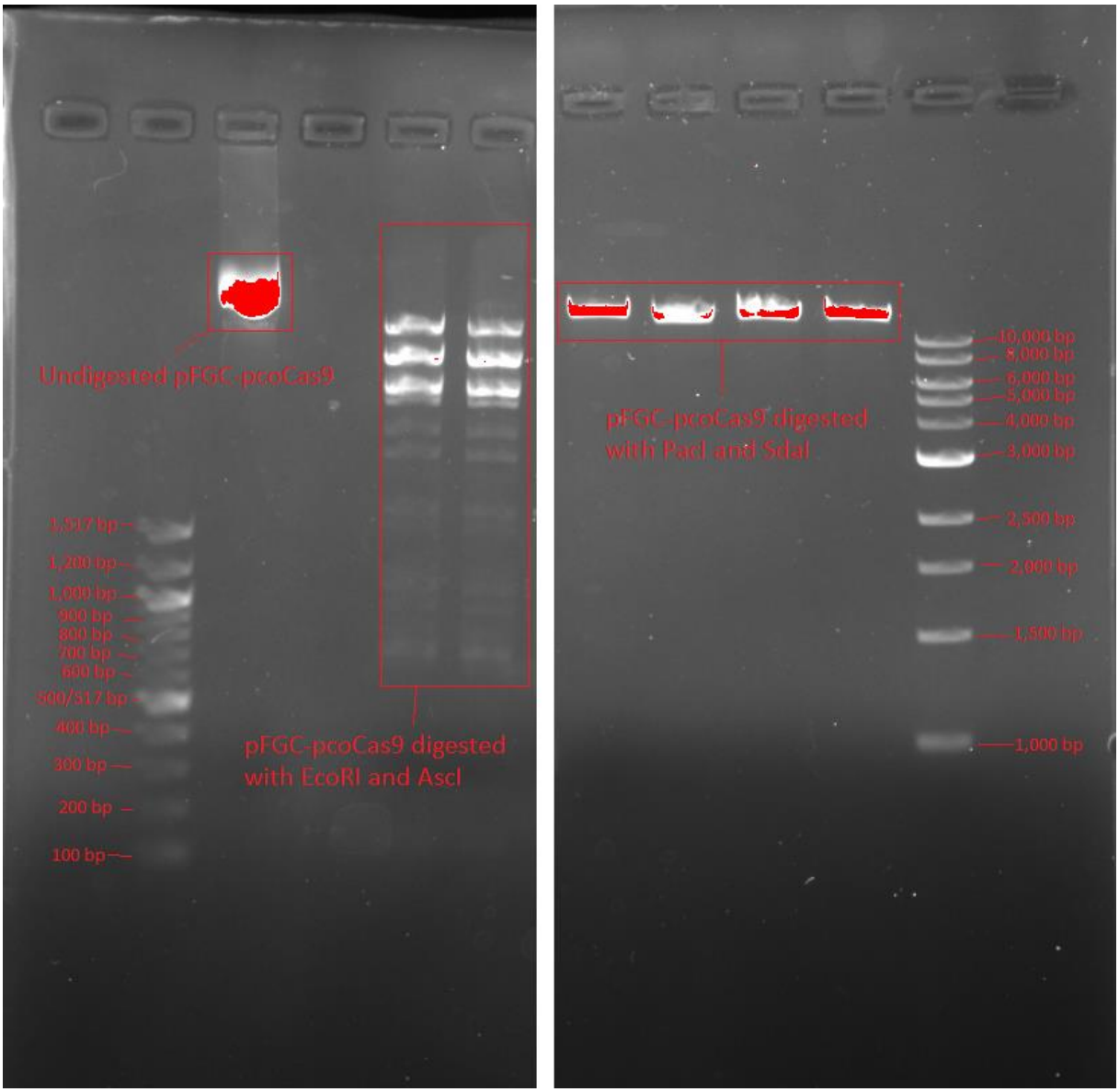



*Figure 19: Shows undigested pFGC-pcoCas9 (left), pFGC-pcoCas9 digested with EcoRI and AscI (left) and pFGC-pcoCas9 digested with PacI and SdaI (right). The gel on the right is warped as a result of the gel setting at an angle.*

The AscI and EcoRI restriction enzymes had been left at room temperature overnight by mistake before attempting the digestion that yielded the results in Figure 19. This could be the reason for the result seen in this figure.

If restriction enzymes display activity such as can be seen in Figure 19, ligation where the product is a functional plasmid becomes virtually impossible.

Plasmid digestion, using PacI and SdaI, seems to have been successful. Here bands of appropriate size could be observed on an agarose gel. When compared with undigested plasmid it is also possible to hypothesize that the digestion resulted in the linearization of pFGC-pcoCas9 (Fig. 19). This is because uncut DNA will travel through an agarose gel differently than linearized DNA (Oppenheim, 1981). If the restriction digestion was successful, it may be detected after ligation.

### 3.4.3 Troubleshooting ligation

Ligation of DNA expression cassettes and linearized plasmid are needed to create a functional circular expression vector. Ligases are an essential tool for cloning as they "catalyze the formation of a phosphodiester linkage between DNA chains" (Engler et al., 1982).

In this thesis, T4 ligase from Thermo Fisher Scientific was used. The process — including incubation time, inactivation of T4 ligase and purification of the ligation mix — was done according to the manufacturer's specifications. However, subsequent transformation was not achieved.

To see whether ligation was successful despite the unsuccessful transformation, the ligation mixture was separated on an agarose gel (Fig. 20).

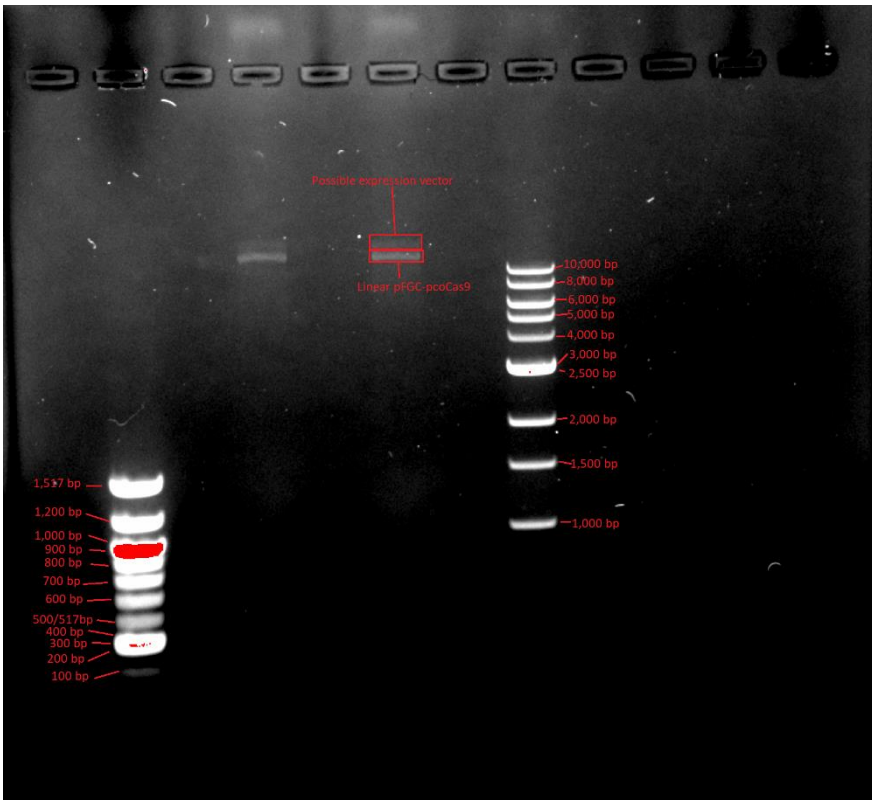



*Figure 20: Agarose gel containing ligation mixture. Two bands >1 kb can be observed. This could suggest that the ligation was wholly or partially successful.*

Here two bands could be observed. When compared with digested plasmid (Fig. 19), it seems that ligation was achieved. These two fragments are ~400 bp apart which matches the 13636 bp size of the constructed expression vector containing a single DNA expression cassette. This was excised from the gel and used for subsequent transformation. As the transformation was unsuccessful, it can be hypothesized that the ligation may have been only partly successful. This could result in a linearized plasmid where the DNA expression cassette only bound itself to one of the sticky ends of the plasmid. If this were the case, the linearized plasmid would not be able to function post-transformation.

A factor that could have contributed to the failure of ligation could be the quality of the reagents that were used. The T4 ligase had been used in past experiments and, thusly, been subjected to multiple freeze-thaw cycles. Repeated freeze-thaw cycles are not recommended when working with T4 ligase. This is because the T4 ligase uses ATP as a cofactor which is degraded by freeze-thaw cycles (Ponte, 2020). This degradation may result in a faulty ligation.

After ligation, the next step is transformation.

### 3.4.4 Troubleshooting transformation of *E. coli*

Many attempts at transformation with newly purchased strands of competent cells were attempted throughout the lab work for this thesis.

The strain that was used was Subcloning Efficiency™ DH5α™ Competent cells from Thermo Fisher Scientific. These are ideal for subcloning genes into plasmid vectors (Invitrogen, 2006). These cells are chemically competent, and the main method of transformation was through heat-shock. As a control, the plasmid pUC19 Control DNA was used to transform DH5α™ alongside pFGC-pcoCas9. This control was positive, which suggest that the DH5α™ competent cells behaved as intended.

However, the size of the 13636 bp expression vector could have had a detrimental effect on the transformation. New England Biolabs® inc. (2020b) suggests that if the plasmid is >10kb, transformation through electroporation should be attempted. Electroporation is also a more efficient mode of transformation than chemical transformation (Neumann et al., 1982). Transformation through electroporation did not yield transformants carrying pFGC-pcoCas9 (Fig. 21). The control, carrying pCU19, did yield transformants (Fig. 21).

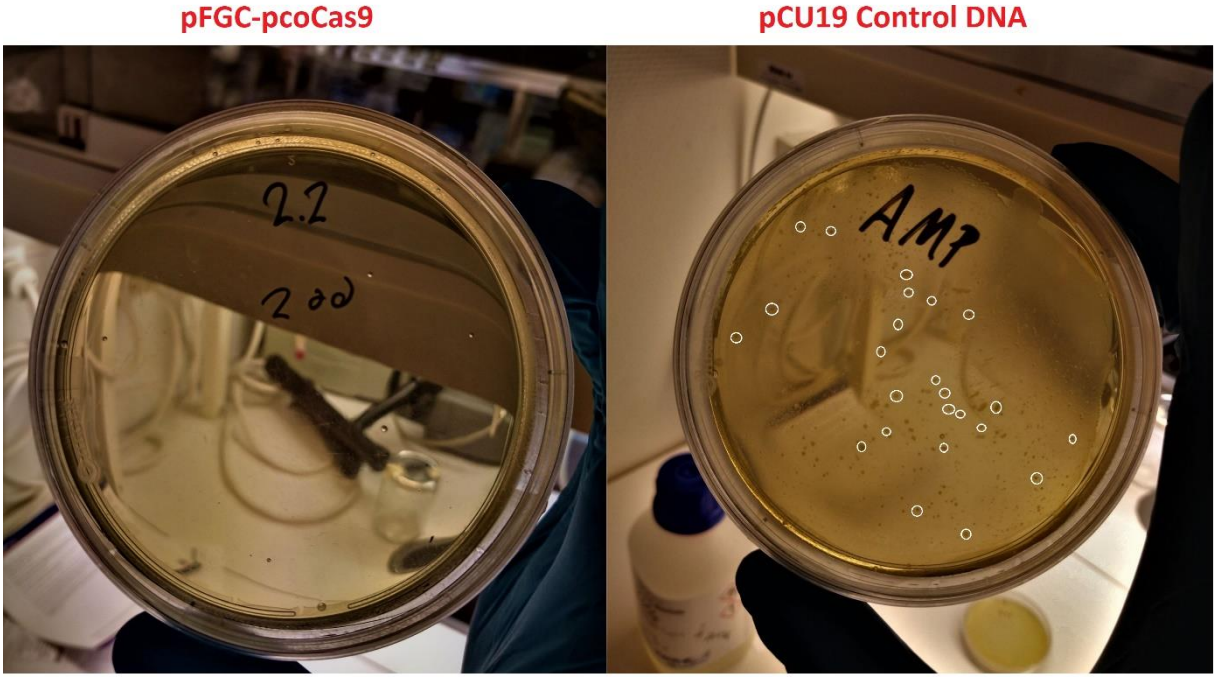



*Figure 21: Possible transformants were plated on selection media post-transformation. Kanamycin was used for pFGC-pcoCas9 and ampicillin for pUC19. Both were transformed through electroporation and treated the same. In the petri-dish containing pCU19, it is possible to observe multiple colonies (circled). In the petri-dish containing pFGC-pcoCas9, no colonies were observed. The small round spots in the petri-dish containing pFGC-pcoCas9 are air bubbles formed when cooling the media after pouring.*

This suggests that the reason for unsuccessful transformation does not lie with the transformation protocol.

### 3.4.5 False positives

False positives were encountered after transformation. False positives are events that wrongly indicate the success of a process. These colonies were similar to the colonies that can be expected when using *E. coli* (Fig. 22).

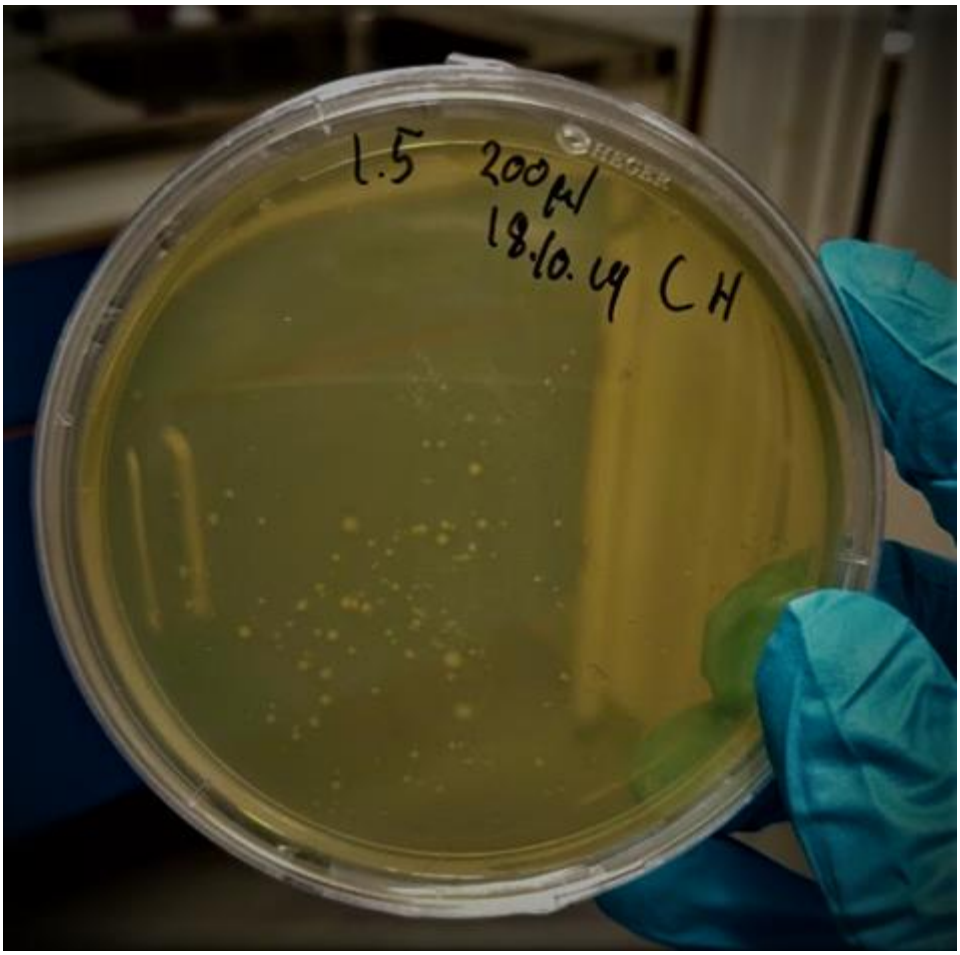



*Figure 22: This petri-dish contained colonies like those formed by E. coli. The colonies appeared after overnight incubation at 37 °C.*

These colonies did not contain the desired expression vector. This was discovered by isolating DNA from the colonies and running the DNA on an agarose gel. This showed a slight band at ~1,500 bp (Fig. 23).

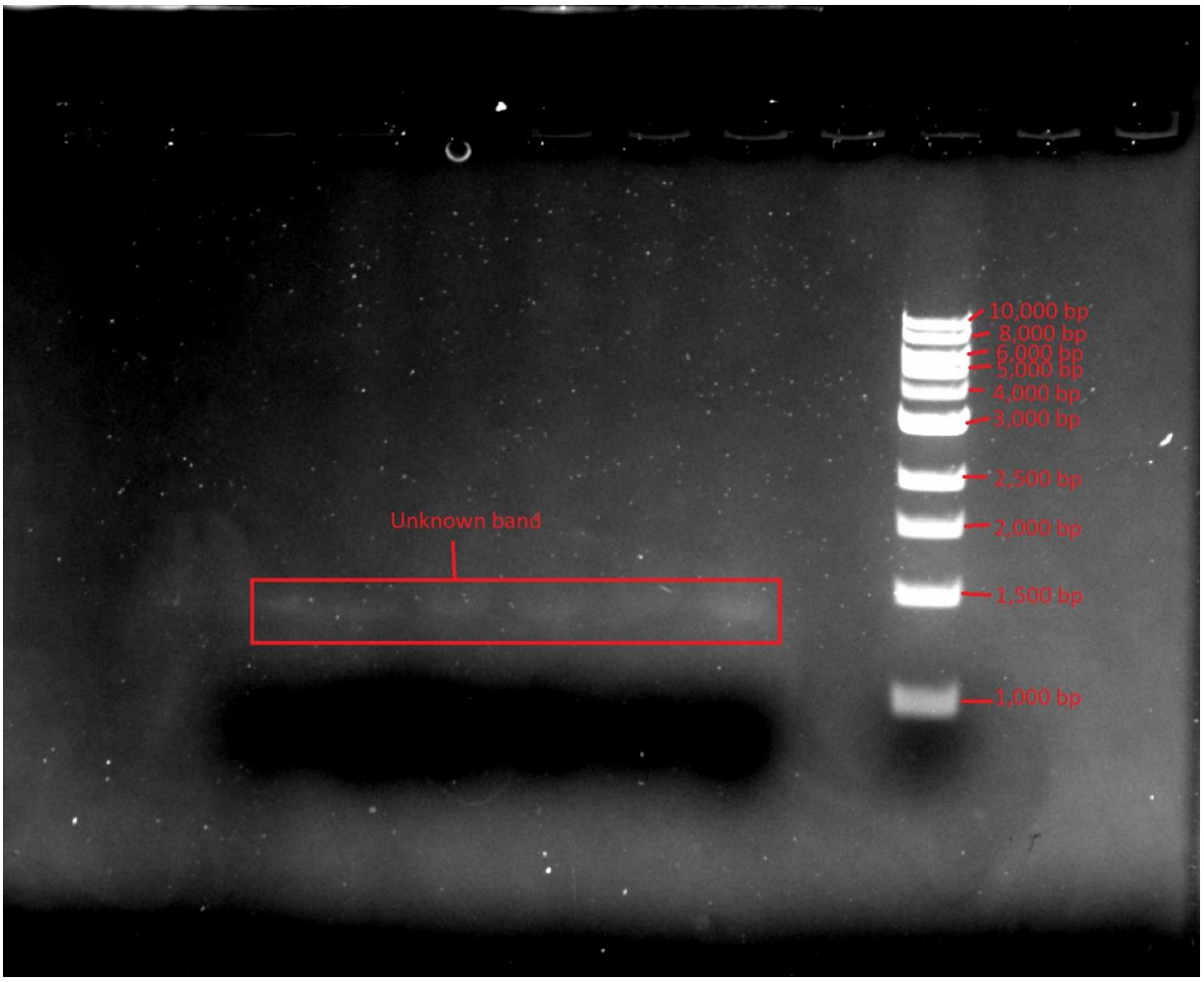



*Figure 23: Shows DNA isolated from the false positive colonies of E. coli. These bands are ~1,500 bp and are extremely faint. The gel was over-exposed to show these bands.*

The reason for this issue could have been contamination from kanamycin-resistant bacteria or a result of low concentrations of kanamycin in the selection media. The integrity of kanamycin is liable at pH 5.0 (Peteranderl et al., 1990). This should not have been a problem as the pH of the media was adjusted to pH 7.0 before autoclaving.

3.4.6 Troubleshooting, summary, and discussion

It is difficult to pinpoint the exact reason the transformation was unsuccessful. Many of the issues are difficult to detect, as small changes can be detrimental to the final product. This is especially true regarding the restriction sites carried by the DNA expression cassettes.

The linearization of the pFGC-pcoCas9 using PacI and SdaI suggests that the restriction enzymes are functional. The presence of two bands during gel separation after round #2 (Fig. 18) of seamless assembly indicates that the overlapping PCR joined IP 1 and 2. These facts indicate that the assembly of the DNA expression cassette was successful and that PacI and SdaI performed as expected when used on pFGC-pcoCas9. However, if the restriction sites on the ends of the DNA expression cassette were compromised, these would not be digested. In that case, the DNA expression cassette would not have the complementary sticky ends necessary to successfully ligate into pFGC-pcoCas9, resulting in an unsuccessful transformation.

Even if the two bands observed after ligation (Fig. 20) suggest at least partial ligation, the possible degradation of ATP in the T4 ligase poses a serious issue. This experiment was undertaken several times, which resulted in the ligase being subjected to multiple freeze-thaw cycles. When this issue was discovered, time constraints did not make room for attempts with fresh reagents.

Reagents for the other steps were routinely replaced.

If similar cloning attempts using seamless assembly should be undertaken, some protocol changes should be done. The addition of cytidine or guanosine to the running buffer may eliminate DNA damage sustained during UV exposure. Using kits that employ a higher concentration of guanidine hydrochloride may also aid in denaturing high-fidelity polymerases.

The construction of plasmids is a common process in modern biotechnology. The fact that this was not achieved during this thesis could indicate that the seamless assembly method is not as stable as other methods. There are other methods of plasmid construction that, potentially, could provide more consistent results.

'Golden gate cloning' utilizes T IIS restriction enzymes that cleave outside the restriction sites (Engler et al., 2008). This would mean that the integrity of the cleavage sites would not be of such large import as with the seamless assembly method. A longer cap at the 3' and 5' ends could be utilized, as the integrity of this cap is not as important. The use of 'Golden gate cloning' could therefore mitigate challenges associated with the restriction sites, e.g. ragged ends and bound polymerases.

As previously stated, it is difficult to find the exact reason why this process failed. The most likely explanation is that multiple factors resulted in the negative result. The vulnerability of the digestion sites and the degradation of ATP in the T4 ligase could have a compound effect on the process. It is also possible that other, undetected factors also played a role. If future attempts at plasmid construction utilize protocols like the one used in this thesis, the possible pitfalls described here should be kept in mind.

## 3.5 Planned workflow

Due to the Covid-19 virus and the shut-down of the university's facilities, the troubleshooting of the seamless assembly protocol was interrupted before it was possible to resolve the issue. If the transformation of *E. coli* had been successful, the assembled expression vector would have been isolated from the E. *coli.* After this, *Agrobacterium* would have been transformed with the expression vector. Subsequently, three modes of plant transformation would have been attempted. The final step that would be undertaken is the verification of possible transformation. The planned transformations, as well as the verification of transformation, are described in the following four subsections.

### 3.5.1 Transient transformation

To test the expression vector, transient transformation of 'Jonsok' and 'Hawaii' would have been attempted. The main method for achieving transient expression in strawberry is through inoculation of the fruit (Agius et al., 2004; Guidarelli et al., 2015; Hoffmann et al., 2006). The strawberry plants that were cultivated in the greenhouse did not carry fruit. Therefore, a protocol using leaf explants for transient expression was chosen (Cui et al., 2017).

### 3.5.2 Stable transformation of explants

Two modes of stable transformation were also planned — *Agrobacterium* mediated transformation and particle bombardment. Particle bombardment is especially interesting, as it makes it possible to create transgene-free gene edited plants through the transient expression of CRISPR/Cas9 (Zhang et al., 2016). Bombardment of other tissues than callus can also be achieved (Christou, 1992; Gal-On et al., 1995; Klein et al., 1988; Snyder et al., 1999). This makes it possible to use leaf disks as explants during particle bombardment.

Explants transformed in this way could be directly regenerated using the regeneration medium described in this thesis. This would offer an opportunity to bypass callus culture, avoiding potential genetic change that may occur in this stage (Phillips et al., 1994).

### 3.5.3 Verification of transformation

The verification of transformational events in transformed plants would be undertaken in four steps: 1. selection using a selection medium, 2. DNA isolation and visualization on agarose gel, 3. Sanger sequencing of target area, and 4. visual evaluation of fruit color.

Pertaining to step 1, after two weeks of growing transformed explants on the regeneration media, the explants would be transferred to fresh regeneration media now containing 2 mg/L glufosinate ammonium. This would contribute to the identification of transformed plants as pFGC-pcoCas9 confers BASTA resistance.

Related to step 2, when surviving plants developed new structures, it would be possible to take samples from the explants without risking plant death. DNA could be isolated from these samples, which would be used to amplify the target area using PCR. The PCR product would be visualized on an agarose gel to detect large indels caused through CRISPR/Cas9 mediated mutation. Pertaining to step 3, the PCR product would also be Sanger sequenced, making it possible to pinpoint the presence of point mutations in the target area.

The final step would be visual evaluation of fruit color. Given the octoploid nature of the cultivated strawberry it would be interesting to observe alongside the diploid wild strawberry. A postulated result is the possibility of different shades of red/white in the cultivated strawberry. This could be possible if a varied number of *F3H* copies were knocked out by the CRISPR/Cas9 complex. In contrast, there is a probability that the wild strawberry would exhibit total knock-out of the *F3H* gene resulting in white berries.

If there is partial knock-out of allelic genes in the octoploid the cultivated strawberry, it could be interesting to examine how many copies were knocked out by the CRISPR/Cas9 complex. This could be achieved through high throughput sequencing. It is possible to estimate ploidy levels by examining allelic frequencies of nuclear genes using a target-capture method (Viruel et al., 2019). This would make it possible to examine the versatility of the CRISPR/Cas9 system when transforming polyploid organisms.

# 4 Conclusions

The CRISPR/Cas9 mediated transformation of the cultivated and wild strawberry were not successful in this experiment. This was due to unexpected challenges during assembly of the expression vector. During the experiment, an extensive troubleshooting process of expression vector assembly was undertaken. This process highlights several pitfalls that can be encountered during plasmid assembly.

Even if this experiment did not yield transformed strawberries, there is little doubt that it is possible to transform both the cultivated and wild strawberry using the CRISPR/Cas9 system (Martín-Pizarro et al., 2018; Wilson et al., 2019; Xing et al., 2018; J. Zhou et al., 2018). These studies provide a proof of concept for the use of CRISPR/Cas9 in the production of new strawberry cultivars.

This thesis shows that it is possible to circumvent the use of callus when regenerating strawberries. Moreover, a direct regeneration could be an important asset in future research where strawberries are transformed using genetic tools such as the CRISPR/Cas9 system. The regeneration medium could be implemented to a regeneration protocol that would provide a more genetically stable end-product.

Future research should explore other methods of plasmid assembly, to help determine how to achieve the desired results. If other methods, like 'golden gate cloning', were successful, a comparison between this method and the seamless assembly could be done. Furthermore, a comparative method could potentially help develop a more stable protocol for plasmid assembly for CRISPR/Cas9 mediated transformation.

The use of biolistic transformation and subsequent direct regeneration of explants would also be an interesting approach to the CRISPR/Cas9 mediated transformation of strawberry. This could be used to develop gene edited, transgene-free cultivars. Also, the development of gene edited, transgene-free cultivars would be especially important if Norway implements the proposal of the Norwegian Biotechnology Advisory Board to introduce a tier-based evaluation system of GMOs.

## Literature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# Appendix I: Medium compositions

I.I Murashige & Skoog Medium Including Vitamins

*Table 16: Shows the vitamins, micro and macro elements in the MS obtained from Duchefa Biochemie. Total concentration of Micro and Macro elements including vitamins is 4405.19 mg/L.*

| **Micro elements** | **mg/L** | **µM** |
|---|---|---|
| CoCl2.6H2O | 0.025 | 0.11 |
| CuSO4.5H2O | 0.025 | 0.10 |
| FeNaEDTA | 36.70 | 100.00 |
| H3BO3 | 6.20 | 100.27 |
| KI | 0.83 | 5.00 |
| MnSO4.H2O | 16.90 | 100.00 |
| Na2MoO4.2H2O | 0.25 | 1.03 |
| ZnSO4.7H2O | 8.60 | 29.91 |
| **Macro elements** | **mg/L** | **mM** |
| CaCl2 | 332.02 | 2.99 |
| KH2PO4 | 170.00 | 1.25 |
| KNO3 | 1,900.00 | 18.79 |
| MgSO4 | 180.54 | 1.50 |
| NH4NO3 | 1,650.00 | 20.61 |
| **Vitamins** | **mg/L** | **µM** |
| Glycine | 2.00 | 26.64 |
| myo-Inositol | 100.00 | 554.94 |
| Nicotinic acid | 0.50 | 4.06 |
| Pyridoxine HCl | 0.50 | 2.43 |
| Thiamine HCl | 0.10 | 0.30 |

I.II Lysogeny broth (LB) 1 L

*Table 17: Lysogeny broth used for growing E. coli.*

| **Reagents** | **Amount** |
|---|---|
| Tryptone | 10 g |
| NaCl | 10 g |
| Yeast extract | 5 g |
| $H_2O$ | To 1 L |
| pH 7.0 | |

I.III Low-salt lysogeny broth (Low-salt LB) 1 L

*Table 18: Low-salt lysogeny broth, used for growing Agrobacterium.*

| **Reagent** | **Amount** |
|---|---|
| Tryptone | 10 g |
| NaCl | 5 g |
| Yeast extract | 5 g |
| $H_2O$ | To 1 L |
| pH 7.0 | |

I.IV S.O.C. medium

*Table 19: S.O.C. medium made for incubation of E. coli.*

| Reagents | Amount |
|---|---|
| Yeast extract | 5 g |
| Tryptone | 20 g |
| NaCl | 0.584 g |
| KCl | 0.186.4 g |
| $MgCl_2$ | 0.952 g |
| $MgSO_4$ | 1.2 g |
| Glucose* | 1.8 g |
| $H_2O$ | To 1 L |
| *Glucose must be added after autoclaving. | |

I.V MS-2 medium

*Table 20: MS medium with 2% sucrose.*

| Reagents | Amount |
|---|---|
| MS | 1x |
| BAP | 0.5 mg/L |
| IBA | 0.2 mg/L |
| Sucrose | 2% |
| Agar | 0.8% |
| $H_2O$ | To 1 L |

I.VI Agarose gel

*Table 21: Agarose gel used to separate and visualize DNA. Gel Loading Dye, Purple (6X) from New England Biolabs® inc. was used to stain the DNA samples. The amount of agarose was varied for some DNA samples.*

| Reagents | Amount |
|---|---|
| Agarose | 1% |
| TBE | 1x |
| GelRed® | 2.5 µL per 50 mL of 1x TBE |

# Appendix II: Primers

*Table 22: Primers used to sequence exon 1 and 2 of F3H.*

| Name | Sequence |
|---|---|
| *F3H*_ex1F | ATGGCCCCTACTCCTACTACTCT |
| *F3H*_ex1R | CTGTAAATGGCTGGAAACGATGAAGC |
| *F3H*_ex2F | GGAGAGGCGGTGCAGGA |
| *F3H*_ex2R | ATGTCCATGGTCTCCAAGATTCACC |

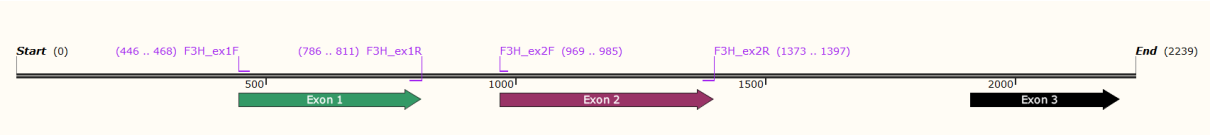


*Figure 24: Shows the complete coding sequence (CBS) for F3H in the wild strawberry. Primers used to sequence exon 1 and 2 of F3H is marked in purple.*

# Appendix III: Green house and growth chamber conditions

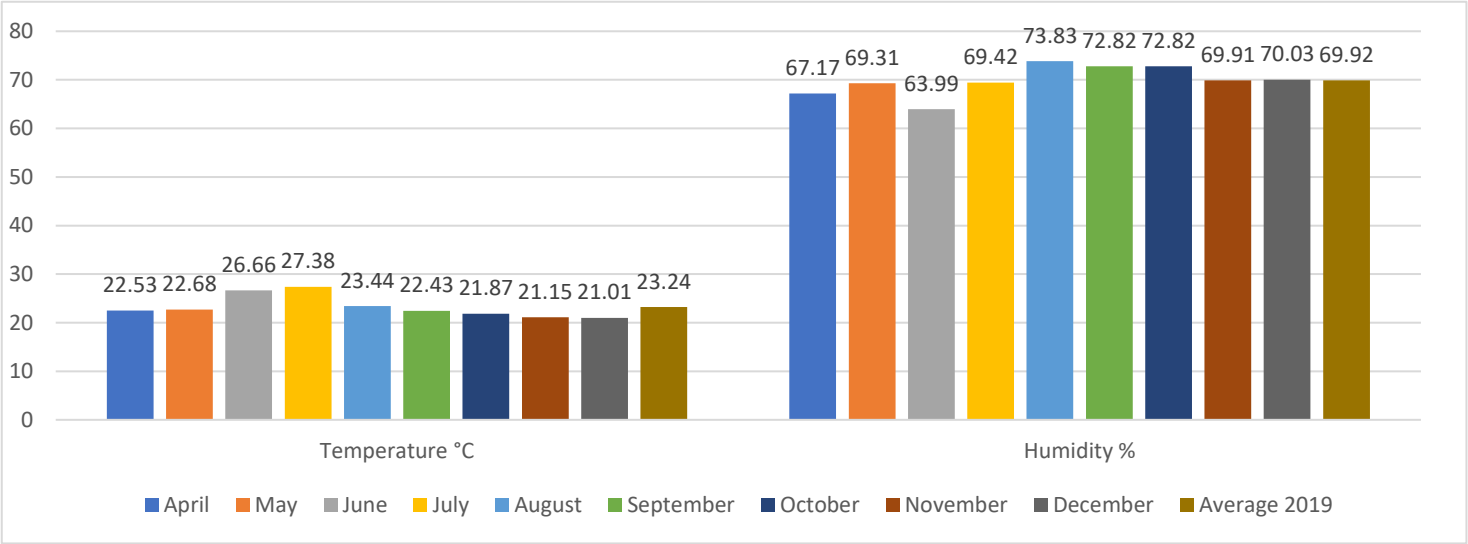


*Figure 25: The average temperature and humidity in the greenhouse from April 2019 to December 2019. Measurements were done every 15 minutes for a total of 96 measurements per day.*

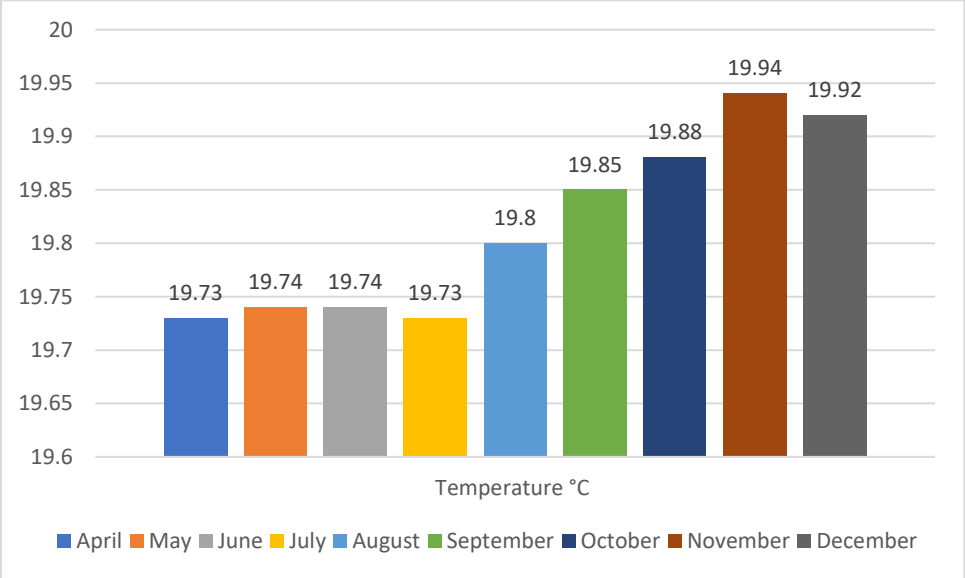


*Figure 26: The average temperature in the growth chamber from April to December 2019. Measurements were done every 15 minutes for a total of 96 measurements per day.*

# Appendix IV: Aligned sequences

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 601 bits(325) | 2e-171 | 330/332(99%) | 2/332(0%) | Plus/Plus |

```
Query  7    CGGGGGA-AAGA-CCTCCAACAGAGCTTCGTCCGCGACGAAGATGAGCGCCCTAAGGTGG  64
            ||||||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  CGGGGGAGAAGACCCTCCAACAGAGCTTCGTCCGCGACGAAGATGAGCGCCCTAAGGTGG  303

Query  65   CCTACAACCAATTCAGCAATGATATTCCGATCATTTCCCTCTCCGGCATAGAAGAGGTCG  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  CCTACAACCAATTCAGCAATGATATTCCGATCATTTCCCTCTCCGGCATAGAAGAGGTCG  363

Query  125  AAGGCCGCCGCGCTGAGATTTGCAAGAAGATTGTTGAGGCCTGCGAGGACTGGGGCGTTT  184
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364  AAGGCCGCCGCGCTGAGATTTGCAAGAAGATTGTTGAGGCCTGCGAGGACTGGGGCGTTT  423

Query  185  TCCAGATTGTTGATCACGGCATCGACCCCAAGCTCATCTCGGAAATGACTCGTCTCGCCA  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  TCCAGATTGTTGATCACGGCATCGACCCCAAGCTCATCTCGGAAATGACTCGTCTCGCCA  483

Query  245  GAGAGTTCTTCGCTTTGCCGCCGGAGGAAAAGCTCCGTTTCGACATGTCCGGTGGCAAAA  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484  GAGAGTTCTTCGCTTTGCCGCCGGAGGAAAAGCTCCGTTTCGACATGTCCGGTGGCAAAA  543

Query  305  AGGGTGGCTTCATCGTTTCCAGCCATTTACAG  336
            ||||||||||||||||||||||||||||||||
Sbjct  544  AGGGTGGCTTCATCGTTTCCAGCCATTTACAG  575
```

*Figure 27: Sequence obtained from 'Hawaii' through amplification of exon 1 of F3H. The amplification was done using primer F3H_1. The sequence was blasted and aligned with Fragaria vesca subsp. americana clone, through NCBI database.*

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 584 bits(316) | 2e-166 | 318/319(99%) | 0/319(0%) | Plus/Minus |

```
Query  1    CCTCCAACAGAGCTTCGTCCGCGACGAAGATGAGCGCCCTAAGGTGGCCTACAACCAATT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  952  CCTCCAACAGAGCTTCGTCCGCGACGAAGATGAGCGCCCTAAGGTGGCCTACAACCAATT  893

Query  61   CAGCAATGATATTCCGATCATTTCCCTCTCCGGCATCGAAGAGGTCGAAGGCCGCCGCGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  892  CAGCAATGATATTCCGATCATTTCCCTCTCCGGCATCGAAGAGGTCGAAGGCCGCCGCGC  833

Query  121  TGAGATTTGCAAGAAGATTGTTGAGGCCTGCGAGGACTGGGGCGTTTTCCAGATTGTTGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  832  TGAGATTTGCAAGAAGATTGTTGAGGCCTGCGAGGACTGGGGCGTTTTCCAGATTGTTGA  773

Query  181  TCACGGCATCGACCCCAAGCTCATCTCAGAAATGACTCGTCTCGCCAGAGAGTTCTTCGC  240
            |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  772  TCACGGCATCGACCCCAAGCTCATCTCGGAAATGACTCGTCTCGCCAGAGAGTTCTTCGC  713

Query  241  TTTGCCGCCGGAGGAAAAGCTCCGTTTCGACATGTCCGGTGGCAAAAAGGGTGGCTTCAT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  712  TTTGCCGCCGGAGGAAAAGCTCCGTTTCGACATGTCCGGTGGCAAAAAGGGTGGCTTCAT  653

Query  301  CGTTTCCAGCCATTTACAG  319
            ||||||||||||||||||
Sbjct  652  CGTTTCCAGCCATTTACAG  634
```

*Figure 28: Sequence obtained from 'Jonsok' through amplification of exon 1 of F3H. The amplification was done using primer F3H_1. The sequence was blasted and aligned with Fragaria vesca subsp. americana clone, through NCBI database.*

# Appendix V: Statistical analysis

V.I Statistical analysis of regeneration media

*Table 23: Linear regression analysis of observed values from regeneration media. The * indicates the level of significance.*

| | | | | | |
|---|---|---|---|---|---|
| (Intercept) | 1.36109 | 0.09501 | 14.325 | < 2e-16 | *** |
| Survival2 | 0.53821 | 0.01036 | 51.956 | < 2e-16 | *** |
| Medium_typeB | -0.14401 | 0.10154 | -1.418 | 0.156423 | |
| Medium_typeC | -0.58887 | 0.10922 | -5.392 | 8.74e-08 | *** |
| Medium_typeD | -0.59512 | 0.10480 | -5.678 | 1.79e-08 | *** |
| Medium_typeE | -0.35409 | 0.10444 | -3.390 | 0.000726 | *** |
| Medium_typeF | -0.85461 | 0.10063 | -8.492 | < 2e-16 | *** |
| Medium_typeG | -0.55286 | 0.11941 | -4.630 | 4.15e-06 | *** |
| Medium_typeH | -0.14520 | 0.11165 | -1.301 | 0.193730 | |
| Medium_typeI | -0.27647 | 0.10919 | -2.532 | 0.011494 | * |
| CultivarJonsok | 0.10161 | 0.05046 | 2.013 | 0.044341 | * |
| Growth_ConditionLight | 0.13987 | 0.05066 | 2.761 | 0.005867 | ** |
| --- | | | | | |
| Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1 | | | | | |
| | | | | | |
| Residual standard error: 0.7564 on 987 degrees of freedom | | | | | |
| Multiple R-squared: 0.7989, Adjusted R-squared: 0.7966 | | | | | |
| F-statistic: 356.4 on 11 and 987 DF, p-value: < 2.2e-16 | | | | | |

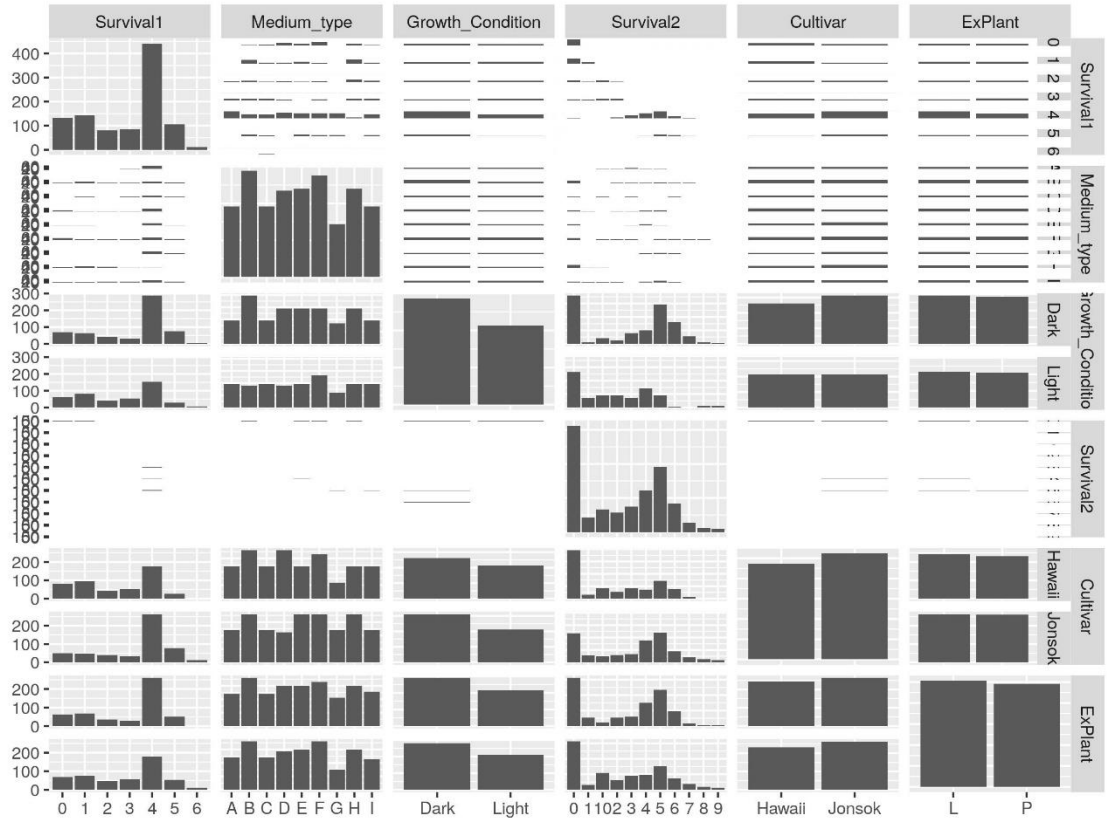


*Figure 29: All results of linear regression analysis of the data gathered from regeneration media. Figure made with the statistical program, R.*

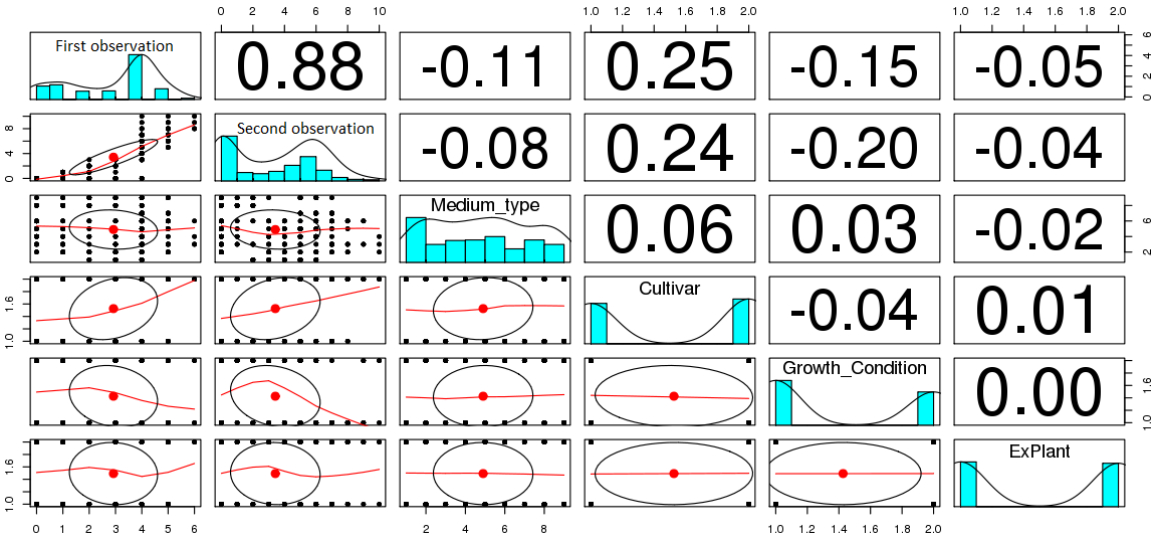


*Figure 30: Correlation between the different variables from regeneration media experiment. Figure made with the statistical program, R.*

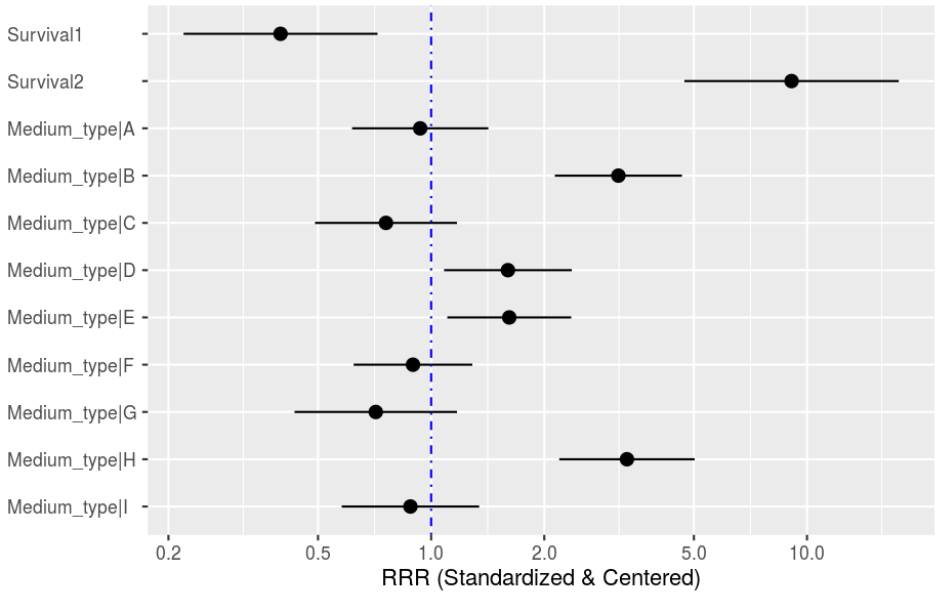


*Figure 31: The Relative Risk Reduction (RRR) of Jonsok. Figure made with the statistical program, R.*

*Table 24: Generalized linear model analysis of explant effect. The " indicates the level of significance.*

| *Standardized coefficients selected* | |
|---|---|
| *label,"OR","coefficient","std.error","z.value","p.value","sig_star","dummy","mean","sd","min","max","importance"* | |
| *(Intercept), 0,0.0113869581746169, 0.226546495320261, 0.0502632281224192,0.959912626419268,"", 0,1,0,1,1,0* | |
| *Survival1, 0.859086718579471, -0.151885409148617 ,0.28280481349737, -0.537067977274829, 0.591220659539484,"", 0,2.92192192192192, 1.67736012349098, 0,6, 1.16402684196251* | |
| *Survival2, 0.932696290538642, -0.0696756503788724, 0.301206553244608, -0.231321827590813, 0.817064790526486, "",0, 3.41741741741742, 2.84096446565725, 0,10, 1.07216037004124* | |
| *Medium_type\|B, 0.936103543940315, -0.0660291847550554, 0.268936840619222, -0.245519299635648, 0.806054366451016, "",1, 0.144144144144144, 0.351411758042648, 0,1, 1.06825789355601* | |
| *Medium_type\|C, 0.983801052085836, -0.0163315852111864, 0.293188122134207, -0.0557034340010221 ,0.955578063815073, "",1, 0.0960960960960961, 0.294870602330942, 0,1, 1.01646567451805* | |
| *Medium_type\|D, 0.903896310750042, -0.101040625666307, 0.281910051104933, -0.3584144136411, 0.720033206657396,"", 1,0.117117117117117, 0.32172085090823, 0,1, 1.10632158590205* | |
| *Medium_type\|E, 0.940344067736993, -0.0615094411831061, 0.278015818252503, -0.221244393825251, 0.824902144335041, "", 1, 0.12012012012012, 0.325264784361106, 0,1, 1.06344053661823* | |
| *Medium_type\|F, 1.03336762319478, 0.0328230060093098, 0.275923921648154, 0.11895672478577, 0.905309640879026, "",1, 0.138138138138138, 0.345217739277635, 0,1 ,1.03336762319478* | |
| *Medium_type\|G, 0.732547009067281, -0.311227763949053, 0.321731920094183, -0.96735121544031, 0.333368488887455, "", 1, 0.0720720720720721, 0.258736739198811, 0,1, 1.36510010637168* | |
| *Medium_type\|H, 0.855106339305083, -0.156529444322877, 0.295688670525371, -0.529372478305509, 0.596547086152419,"", 1,0.12012012012012, 0.325264784361106, 0,1, 1.16944519533403* | |
| *Medium_type\|I, 0.867300718002075, -0.14236951343311, 0.290025662112087, -0.490885918150541, 0.623507137363342,"", 1, 0.0960960960960961, 0.294870602330942, 0,1, 1.15300261978753* | |
| *Cultivar\|Jonsok,1. 1094980155503, 0.103907674790719, 0.134150551146525, 0.774560178118288, 0.438599595829724,"", 1,0.525525525525526, 0.499598134194354, 0,1, 1.1094980155503* | |
| *Growth_Condition\|Light, 0.964241209354183, -0.0364137984982986, 0.134810513387204, -0.270110969711321, 0.78707488307407,"", 1,0.426426426426426, 0.494805018522501,0,1, 1.03708490188857* | |

*Table 25: Generalized linear model analysis of the effect of the cultivated strawberry cultivar 'Jonsok'. The * indicates the level of significance.*

| *Standardized coefficients selected* | |
|---|---|
| *level,"label","RRR","coefficient","std.error","z.value","p.value","sig_star","dummy","mean","sd","min","max","importance"* | |
| *Jonsok,"Survival1", 1.86201547135125,0.621659487816731, 0.299109438283613, 2.07836800932801, 0.037675476852221,"* ", 0,1,0,1,1, 1.86201547135125* | |
| *Jonsok,"Survival2", 2.19186035278689, 0.784750659386135, 0.311115661172428, 2.52237594349584, 0.0116565057403278,"* ",0,3.41741741741742, 0.5,2.81596367543497, 4.57592937033966, 2.19186035278689* | |
| *Jonsok,"Medium_type\|A", 0.77787543478291, -0.25118887715618, 0.210362632587488, -1.19407555451519, 0.232448414617925," ",1, 0.0960960960960961, 0.294870602330942,0,1, 1.28555287297263* | |
| *Jonsok,"Medium_type\|B", 1.1303590847897, 0.122535356394915, 0.179200025991884, 0.683790952131141, 0.494107180775262," ",1, 0.144144144144144, 0.351411758042648,0,1, 1.1303590847897* | |
| *Jonsok,"Medium_type\|C", 0.805041313581678, -0.216861681660987, 0.218729788516777, -0.991459293823407, 0.321461359601623," ",1, 0.0960960960960961, 0.294870602330942,0,1, 1.24217227504877* | |
| *Jonsok,"Medium_type\|D", 0.619696378345959, -0.478525633236479, 0.201266648274374, -2.37757043871538, 0.0174271141675591,"* ",1, 0.117117117117117, 0.32172085090823,0,1, 1.61369347142082* | |
| *Jonsok,"Medium_type\|E", 1.59250304493002, 0.465307020506319, 0.197878561874171, 2.35147767448504, 0.0186990117084787,"* ",1, 0.12012012012012, 0.325264784361106,0,1, 1.59250304493002* | |
| *Jonsok,"Medium_type\|F", 1.08842236996747, 0.0847292807913274, 0.187504315009479, 0.451879098286587, 0.651356082877418," ",1, 0.138138138138138, 0.345217739277635,0,1, 1.08842236996747* | |
| *Jonsok,"Medium_type\|G", 1.0934357426776, 0.0893247964246298, 0.261312906400992, 0.341830786909386, 0.732478239098992," ",1, 0.0720720720720721, 0.258736739198811, 0,1, 1.0934357426776* | |
| *Jonsok,"Medium_type\|H", 2.92081277201231, 1.07186192412852, 0.206118945598676, 5.20021059206995, 0.000000199062851622621,"***", 1,0.12012012012012, 0.325264784361106,0,1, 2.92081277201231* | |
| *Jonsok,"Medium_type\|I", 0.836378451349909, -0.178674075329716, 0.213176527758869, -0.838150790840445, 0.401946020303568," ",1, 0.0960960960960961, 0.294870602330942,0,1, 1.19563099501907* | |

*Table 25: Generalized linear model analysis of the effect of the cultivated strawberry cultivar 'Jonsok'. The * indicates the level of significance.*

| *Standardized coefficients selected* |
|---|
| *level, label, RRR, coefficient, std.error, z.value, p.value, sig_star, dummy, mean, sd, min, max, importance* |
| *Dark,Survival1, 0.397456689, -0.922669309, 0.303125386, -3.043853634, 0.002335687,** ,0,1,0,1,1, 2.515997408* |
| *Dark,Survival2, 9.097346335, 2.20798276,0.335110792, 6.588814244,4.43353E-11,***, 0,3.417417417, 0.5, 2.815963675, 4.57592937, 9.097346335* |
| *Dark,Medium_type\|A, 0.935061867, -0.067142584, 0.212917763, -0.31534515, 0.752499608, ,1, 0.096096096, 0.294870602, 0,1, 1.069447953* |
| *Dark,Medium_type\|B, 3.148857971, 1.147039838, 0.198582236, 5.776145248, 7.64314E-09,***,1, 0.144144144, 0.351411758, 0,1, 3.148857971* |
| *Dark,Medium_type\|C, 0.758266465, -0.276720418, 0.221789201, -1.247673094, 0.212150799, ,1,0.096096096, 0.294870602, 0,1, 1.318797608* |
| *Dark,Medium_type\|D, 1.600528069, 0.470333618, 0.199547033, 2.35700632, 0.018422933,* ,1, 0.117117117, 0.321720851, 0,1, 1.600528069* |
| *Dark,Medium_type\|E, 1.613625728, 0.478483652, 0.193672251, 2.470584452, 0.013489246,* ,1, 0.12012012, 0.325264784, 0,1, 1.613625728* |
| *Dark,Medium_type\|F, 0.89465933, -0.11131227, 0.185458098, -0.600201722, 0.548371806, ,1, 0.138138138, 0.345217739, 0,1, 1.11774389* |
| *Dark,Medium_type\|G, 0.712236352, -0.339345467, 0.253918618, -1.336433975, 0.181407468, ,1, 0.072072072, 0.258736739, 0,1, 1.404028308* |
| *Dark,Medium_type\|H, 3.318492771, 1.199510695, 0.211599234, 5.66878561, 1.43813E-08,***, 1, 0.12012012, 0.325264784,0, 1, 3.318492771* |
| *Dark,Medium_type\|I, 0.880739071, -0.126993871, 0.214739604 ,-0.59138542, 0.554262207, ,1, 0.096096096, 0.294870602, 0,1, 1.135410058* |


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