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Gene Editing of Red Raspberry (Rubus ideaus L.) with CRISPR/Cas9 Knocking out *F3'H*

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Plant Science

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Abstract

Breeding new improved crop varieties is a lengthy process that can take many years. With climate change and more rapidly changing environmental conditions, it becomes even more important to develop new varieties quickly. Gene editing presents a useful tool to achieve this. The relatively new gene editing method CRISPR/Cas9 is simple, cost-effective and above all more precise than most other methods. Due to its novelty there is no protocol for CRISPR/Cas9 for many plants. The aim of this thesis is to develop such a protocol for raspberry which is an economically important crop, especially in the northern parts of Europe. As a proof of concept, the gene *F3'H*, an important gene for synthesis of the main anthocyanin cyanidin in the berries of raspberry, was knocked out.

Several different delivery methods for CRISPR/Cas9 have been used in this thesis. Two types of *Agrobacterium*-mediated transformation have been tested: transformation of in-vitro explants to regenerate entire transformed pants and agroinfiltration of greenhouse-grown plants as a quick method to test the designed CRISPR/Cas9 constructs. Biolistic transformation by gold particle bombardment of callus was also tested. The focus however was on obtaining transformed plants by *Agrobacterium*-mediated transformation, by far most the common transformation method for plants.

Plants were regenerated only after the *Agrobacterium*-mediated transformation. These plants were likely transformed, but the plants were too small to verify this through a PCR screening when the experiment had to end due to the submission date for this thesis. It turned out that agroinfiltration, as it is used for tobacco, is not a suitable transformation method for raspberry. Neither were any plants regenerated from the bombarded callus. However, it could be verified that there were changes in the genome of some callus cells within the target region of the CRISPR/Cas9 constructs.

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Introduction

Raspberry production: Importance in the EU

Red raspberry (*Rubus idaeus L.*) belongs to the genus *Rubus* and to the family of the *Rosaceae* like many other fruit crops such as apple (*Malus pumila*), cherry (*Prunus avium*) or peach (*Prunus persica*) (Watson and Dallwitz, 1992). It is mainly cultivated in the northern parts of Europe, but also in North America, Russia, Korea, Chile and Australia (Jennings et al., 1991, Strik, 2007).

Raspberry production in Europe has more than quadrupled in the last 50 years (FAO, 2018). The increase in raspberry production is a result of an increased demand for both processed and fresh fruit (Darnell et al., 2006). The raspberry presumably owes its rising popularity to its reputation as a so called 'superfood' (Breyer, 2013, Ebeling, 2016, Barns, 2014). This reputation as 'superfood' seems to be somewhat justified, since raspberry has been proven to have a number of health beneficial components: It has a high content of dietary fibre (6.5g/100g fresh weight) and is a source for various nutrients: vitamin C, magnesium, potassium, vitamin K, calcium and iron (USDA, 2018). Apart from vitamin C raspberries also contain other antioxidants: anthocyanins and ellagitannins, which makes them one of the fruit with the highest antioxidant activity (Beekwilder et al., 2005). Anthocyanins can decrease oxidative damage to cellular DNA, proteins and lipids through reactive oxygen species (Heinonen et al., 1998). Besides that, anthocyanins have been shown to contribute to healthy vision, urinary tract health, and dermal health. They also possess potential health benefits such as cardiovascular and neuroprotective potential, antidiabetic and anticancerogenic properties (Zafra-Stone et al., 2007, Roy et al., 2002, Bagchi et al., 2004).

Even though the production in Europe has grown so much, the demand is much higher especially for fresh raspberries (CBI Ministry of Foreign affairs Netherlands, 2018). Europe is also losing market share to international competitors, that is why there is a need for better varieties with improved health benefits and whose cultivation is better for the environment (European Comission, 2014). This could make raspberries from Europe more competitive in comparison to cheaper raspberries from other parts of the world. The gene editing tool CRISPR/Cas9 could be a useful technique to produce such varieties much faster than by conventional breeding and help meet the increasing demand.

CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats (CRISPR) together with CRISPR associated proteins (Cas) play an important role in adaptive immunity against phage in bacteria and archaea (Pourcel et al., 2005, Makarova et al., 2006, Barrangou et al., 2007). The CRISPR/Cas system silences invading nucleic acids by recognising them with complementary guide RNA sequences and then cleaving them (Hale et al., 2009). Cas9 which is derived from *Streptococcus pyogenes* can cleave DNA guided by two RNAs, the trans activating CRISPR RNA (tracrRNA) and the CRISPR RNA (crRNA). The crRNA is composed of a sequence complementary to the target (spacer) and a structural part (repeat) that complements the tracrRNA (Diep, 2017). The trcrRNA consists of a longer constant stretch of bases that provide a "stem-loop" structure Cas9 can bind to (Simeonov).The site-specific cleavage occurs at locations determined both by the crRNA as well as the protospacer and a short motif protospacer adjacent motif (PAM), which usually has the sequence 5'-NGG-3'. The protospacer and the PAM are

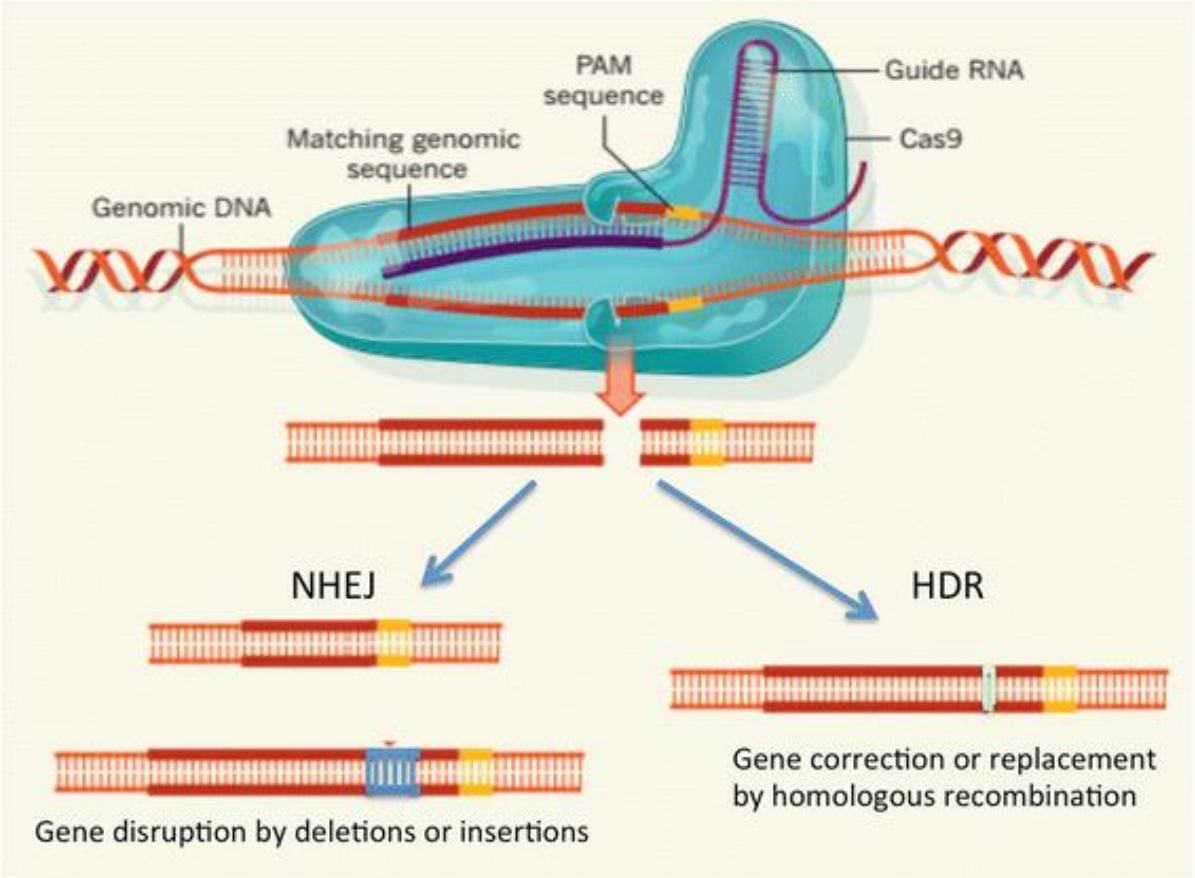


Figure 1: CRISPR-Cas9 targeting system. The guide RNA recognises the 20-nt target DNA sequence immediately preceding the NGG DNA motif (protospacer-associated motif or PAM). This results in Cas9 causing a double-strand break (DSB) 3 bp upstream of the PAM sequence. These DSBs are then either repaired by nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ can lead to gene disruption by either causing deletions or insertions. HDR results in gene correction with the sister chromatid as a template or in gene knock-in when an oligonucleotide sequence for insertion is provided. From Tu et al. (2015)

both located on the invading viral DNA. The tracrRNA and the crRNA can be combined into one single guide RNA (sgRNA) to program CRISPR/Cas9 to cleave specific DNA sites for genome editing (Jinek et al., 2012). This sgRNA contains a 20-nt sequence that matches the target site, for which the only requirement is to be directly upstream of a PAM sequence (Hsu et al., 2014). When the target is recognised, Cas9 induces site-specific double-stranded breaks (DSBs), which can be repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Cong et al., 2013). NHEJ is used to knock out genes, since its pathway is error prone and therefore creates small insertions or deletions that cause genes to become unfunctional (Barrangou et al., 2015). The HDR pathway, on the other hand, uses as DNA template, usually the sister chromatid, to repair DSBs (Liu et al., 2016). This repair mechanism is more precise and also suited for the insertion of DNA sequences at a desired site (Barrangou et al., 2015) (Figure 1).

Anthocyanin pathway

Since the CRISPR/Cas9 technology is so new – it was first used in mammalian cells in 2012 (Jinek et al., 2012) and then shortly after in plants (Pennisi, 2013, Shan et al., 2013, Feng et al., 2013, Li et al., 2013, Nekrasov et al., 2013, Xie and Yang, 2013) - there are no established protocols for most plants. The aim of this master thesis is to create such a protocol for raspberry by knocking out parts of the anthocyanin synthesis pathway. Anthocyanins, pigments in plants that are responsible for red, purple, blue and even black colouring in flowers, fruits and other plant parts, are especially suited for genetic studies in plants, because their synthesis results in such a wide range of colours (Davies, 2004). Mendel's work on the flower colour of peas already made use of the colourful anthocyanins and the easy detectability of visible mutations in anthocyanin genes. Mutations in anthocyanin genes are not only well visible, but they generally also do not have a deleterious effect on plant growth and development (Holton and Cornish, 1995). Another reason anthocyanins are a very interesting target for modification is their health benefits as mentioned previously, and the good knowledge of the anthocyanin synthesis pathway (Shirley, 1996).

Anthocyanins belong to the flavonoids, a diverse group of secondary metabolites, which occur in many plant species (Aizza and Dornelas, 2011). The flavonoid biosynthetic pathway is one of the best studied pathways in plants (Shirley, 1996) and almost all of the genes regulating anthocyanin synthesis are fully characterised (Liu et al., 2018). In addition, the successful modification of the anthocyanin pathway has been demonstrated many times (Tanaka and Ohmiya,

2008). Parts of the anthocyanin pathway were suppressed by RNA interference with *F3'5'H* in cyclamen (Boase et al., 2010). With the same method, the whole anthocyanin synthesis was down-regulated in *Torenia hybrida* (Fukusaki et al., 2004) and tobacco (*Nicotiana tabacum L.*) (Nishihara et al., 2005) and pelargonidin synthesis was downregulated in carnation (*Dianthus caryophyllus*) (Zuker et al., 2002). Knocking out a gene with CRISPR/Cas9 represents a more direct approach than RNA interference since the gene itself is made non-functional. This has

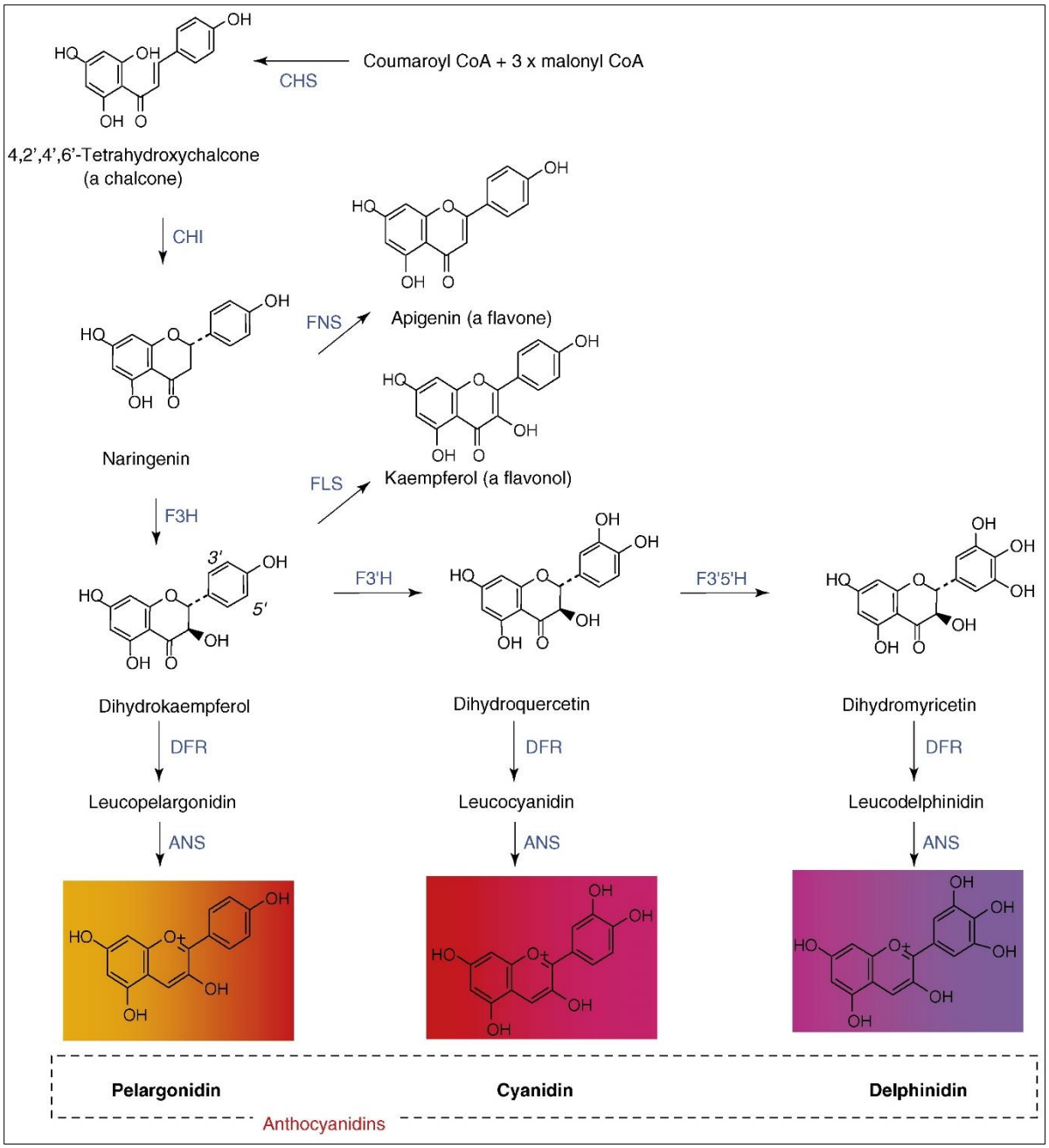


Figure 2: Synthesis pathway for the 3 major anthocyanins in plants: CHS:: chalcone synthase; CHI: chalcone isomerase; F3H: flavone 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; F3'5'H: flavonoid-3',5'-hydroxylase; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; FLS: flavonol synthase; FNS: flavanone synthase. Modified from Tanaka and Ohmiya (2008)

been successfully demonstrated in carrot (*Daucus carota subsp. sativus*) callus by knocking out the *F3'H* gene and thereby inhibiting all anthocyanin synthesis (Klimek-Chodacka et al., 2018).

Figure 2 shows the main anthocyanin synthesis pathway which is shared among higher plants. The target gene for this experiment is *F3'H* which codes for flavonoid 3'-hydoxylase also called flavonoid 3'-monoxygease. This is the enzyme that converts dihydrokaempferol to dihydroquercetin, a crucial step for synthesising cyanidin. Knocking out *F3'H,* and with it the cyanidin synthesis, should produce a visible colour change in the berries of raspberry since the main anthocyanin the fruits of raspberry contain is cyanidin. The only other anthocyanin that can be found in raspberry is pelargonidin, but not in all varieties and then only in traces (Jennings and Carmichael, 1980). Other pigments than anthocyanins found in raspberries are carotenoids (Carvalho et al., 2013, Bradish et al., 2015), which are yellowish to reddish coloured (Wibowo et al., 2015), so it would be expected that knocking out the cyanidin synthesis would result in yellow to orange berries due to the carotenoids and possibly pelargonidin present in the berries.

Aim of the Thesis

The aim of this thesis is to use the gene editing tool CRISPR/Cas9 to knock out *F3'H*, a gene part of the anthocyanin pathway, in raspberry. This is to be done as a proof of concept that CRISPR/Cas9 can be successfully applied in raspberry. In order to achieve this, different delivery methods for CRISPR/Cas9 will be used with the main focus on *Agrobacterium*-mediated transformation.

Material and Methods

There are several different delivery methods that can be used to introduce foreign DNA into a plant's genome, such a CRISPR/Cas9 complex. Some of the most common ones include transformation with *Agrobacterium tumefaciens*, protoplast transformation via electroporation, biolistic-mediated transformation with a particle gun, electrophoresis (Rakoczy-Trojanowska, 2002) and transformation with a viral vector (Ali et al., 2015). In this experiment the focus lies on *Agrobacterium*-mediated delivery of CRISPR/Cas9, because this is the method has mostly been used to transform raspberry before and there are established protocols for it (Mathews et

al., 1995, Mezzetti et al., 2004, Mezzetti et al., 2002). The second transformation method used is gold particle bombardment, because transient expression of CRISPR/Cas9 and with it transgene-free gene edited plants have been achieved before in wheat (*Triticum aestivum L.*) (Yi et al., 2016). Transgene-free genome edited plants only display a very small change in their genome, with no foreign DNA added, and do not carry a herbicide or antibiotic resistance gene common in traditional genetically modified organisms. Such plants are more likely to be accepted by the public and enter the market.

Plant Material

In vitro cultures of two Norwegian raspberry cultivars 'Veten' and 'Ninni' were obtained from Sagaplant AS, Akkerhaugen, Norway. The plants were sub-cultured every 3-5 weeks on full MS medium (Murashige and Skoog, 1962) with 3% sucrose, 0.8% agar, 0.1 mg/L indole-3-butyric acid (IBA), 0.5 mg/L benzyl adenine (BAP), 36 mg/L Fe-EDDHA and a pH of 5.7. To test if additional iron in the medium is beneficial for the plants, some plants were grown on the very same medium, but without the extra Fe-EDDHA added.

DNA isolation

To obtain DNA, shoots from the in vitro cultures were grown in soil in a greenhouse. Leaf samples from those plants were frozen in liquid nitrogen and homogenised with a Retsch MM301 mixer mill. The DNA was then isolated with a QIAGEN DNeasy Plant Mini Kit according to the manufacturer's instructions.

Sequencing *F3'H*

A sequence for *F3'H* in red raspberry was obtained from Jahn Davik at the Norwegian Institute of Bioeconomy Research (NIBIO), Norway. Four primer pairs were designed to amplify part of *F3'H*, the gene of interest, in order to verify it within the genome of 'Ninni' and 'Veten'. To verify that the primers work and to find their optimal annealing temperature, a PCR with a gradient of the annealing temperature ranging from 46.9 to 65.2 °C was conducted with OneTaq polymerase. This was verified by visualising the PCR product on a 1% agarose gel. Only one

primer pair, RuF3'H_1sc, worked well and was selected for further work. The temperature gradient showed that 60 ℃ was the optimal annealing temperature for this primer pair, that is why 60 ℃ was used as annealing temperature for RuF3'H_1sc in later PCRs.

The PCR product was purified from a gel with the Monarch® DNA Gel Extraction Kit and sent to GATC, Germany for Sanger sequencing.

Single guide RNA design and vector selection

To predict the activity of potential single guide RNAs (sgRNAs) the sgRNA Scorer 2.0, a software designed to predict the activity and specificity of sgRNAs for Cas9 from *S. pyogenes* and Cas9 from *S. thermophilus I,* was used. The tool can identify possible sgRNAs for an input sequence with a defined spacer length and PAM sequence and gives them a score according to their predicted activity (Chari et al., 2017). The scores for the potential gRNAs within the scaffold sequence obtained from Jahn Davik ranged from ~ -2 to ~ 2. Four sgRNAs were selected, all with a score over 1. The first sgRNA selected was the one with the highest score 2.01 which also lies within the region of the sequenced PCR product. The second sgRNA lies 214 bp upstream of the first one in hope to be able to cause the section between them to be deleted. The last two sgRNAs selected are very close to each other with their cut sites only being 23 bp apart as recommended by Li et al. (2015).

It was not possible to identify possible off-target sequences for the selected gRNAs, because the sequence for the whole raspberry genome was not available. The sequences obtained from Jahn Davik only covered *F3'H*.

The binary plasmid pFGC-pcoCas9 (Addgene plasmid # 52256) designed for *Agrobacterium*-mediated stable and transient expression was selected because it contains multiple cloning sites and is optimised for plants. pUC119-gRNA (Addgene plasmid # 52255) was used as the PCR template to assemble the expression cassettes for the gRNAs (Li et al., 2015). Both plasmids were a gift from Jen Sheen's lab at Harvard University, Cambridge, Massachusetts.

Plasmid isolation and clean up

The *Escherichia coli* stab cultures containing each of the two plasmids were streaked onto plates with LB medium containing an antibiotic, 100 mg/L ampicillin for the strain containing pUC119-gRNA and 50 mg/L kanamycin for the strain containing pFGC-pcoCas9. The plates

were incubated at 37 °C overnight. Single colonies were transferred to tubes, each containing 5 mL of liquid LB medium supplemented with the same concentration of the corresponding antibiotic as the LB plates. The tubes were incubated overnight at 37 °C in a shaking incubator (230 rpm).

Assembly of the expression cassette and the expression vector

The expression cassettes for the four guide RNAs were assembled via overlapping PCR in two steps, as in Li et al. (2015). The assembled expression cassettes are composed of an *Arabidopsis* U6-1 promoter, the gRNA and a gRNA scaffold flanked by two restriction sites, either SbfI and PacI or AscI and EcoRI, to insert it into the vector plasmid pFGC-pcoCas9 later. The following primers were designed (Table 1) according to Figure 4:

Table 1: Vectors designed for PCR assembly of the expression cassettes: The colours and names correspond with Figure 4.

Name	Sequence
F1_1	AAGCTCCTGCAGGAGAAATCTCAAAATTCCG
F1_2	AAGCTGGCGCGCCAGAAATCTCAAAATTCCG
R1_1	CCACGTTGGGCTTTTCGCCGCCCAATCACTACTTCGTCTCT
R1_2	TTTCCTTGACGACGGCCTGGAGGCAATCACTACTTCGTCTCT
R1_3	CCTCCTTTCCCGTCACCGAACACAATCACTACTTCGTCTCT
R1_4	CCTCCGCCCTACCATCACCCGCCAATCACTACTTCGTCTCT
F2_1	GGGCGGCGAAAAGCCCAACGTGGGTTTTAGAGCTAGAAATAGC
F2_2	CCTCCAGGCCGTCGTCAAGGAAAGTTTTAGAGCTAGAAATAGC
F2_3	GTGTTCGGTGACGGGAAAGGAGGGTTTTAGAGCTAGAAATAGC
F2_4	GGCGGGTGATGGTAGGGCGGAGGGTTTTAGAGCTAGAAATAGC
R2_1	AAGCTTTAATTAAAAAAAGCACCGACTCGGTGC
R2_2	AAGCTGAATTCAAAAAAGCACCGACTCGGTGC

For F1 and R2 there are two different primers for the different restriction sites. The vector pFGC-coCas9 has two sites flanked by restriction enzymes (Figure 3) that can be replaced with a Cas9 expression cassette, one is flanked by SbfI and PacI and the other one by AscI and EcoRI. To insert two gRNAs into one vector, gRNA1 together with gRNA2 and gRNA3 together with gRNA4, the two expression cassettes that are to be inserted into the same vector have to be flanked by two different restriction sites. Therefore, there has to be two versions of F1 and R2. F1_1 and R2_1 were used to assemble the expression cassette for gRNA1 and gRNA3 and F1_2 and R2_2 for gRNA2 and gRNA4. For the primers F2 and R1 there are four versions since they include the sequence for each gRNA or the reverse complement respectively.

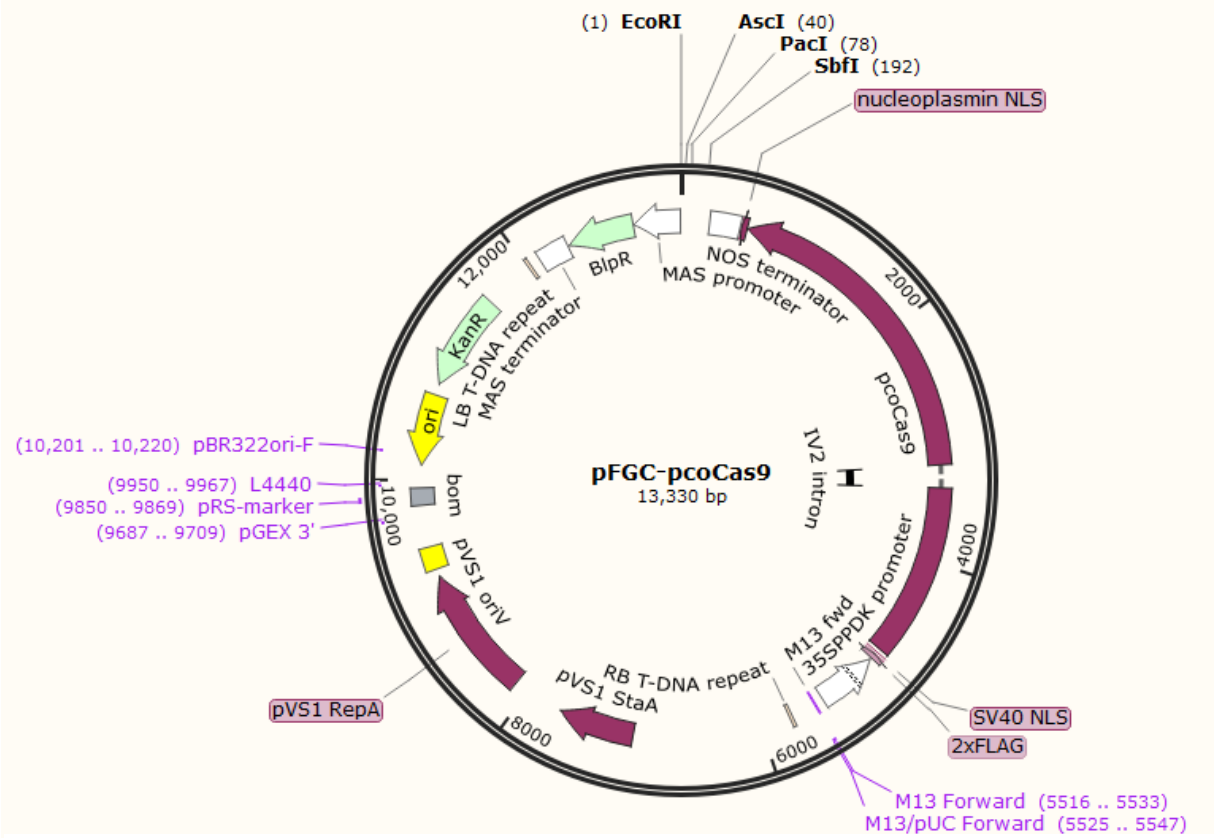
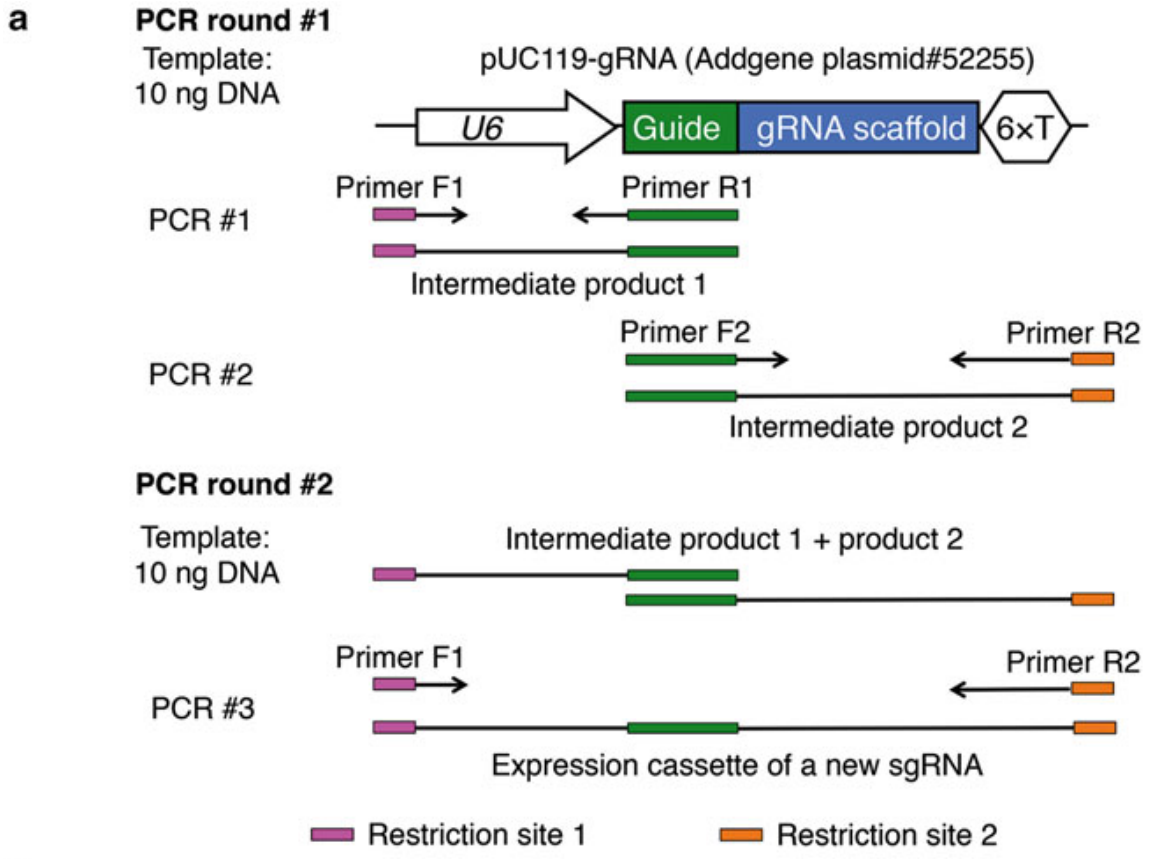


Figure 3: pFGC-pcoCas9 (Addgene)



b

Primer name	Primer sequence (5' to 3') for a gRNA targeting to N_{20}NGG
F1	XXX + restriction site 1 + AGAAATCTCAAAATTCCG
R1	Reverse complement of N_{20} + AATCACTACTTCGTCTCT 3' (if N_1 = G)
	Reverse complement of N_{20} + CAATCACTACTTCGTCTCT 3' (if N_1 = C, A, T)
F2	N_{20} + GTTTTAGAGCTAGAAATAGC
R2	XXX + restriction site 2 + AAAAAAGCACCGACTCGGTGC

XXX: Additional nucleotides to ensure efficient restriction digestion of PCR products

Figure 4 from Li et al. (2015): a. Seamless assembly of the expression cassettes via overlapping PCR. b. primer design instruction. The colours and names are consistent with those shown in (a). In the design of primer R, an additional "C" (marked in red) is added if the gRNA doesn't start with a "G" to generate a "G" upstream of N 1 during PCR #1 to optimize transcription initiation by the Arabidopsis U6 - 1 promoter.

The expression cassettes were assembled in two rounds of PCR as shown in Figure 4. In the first round DNA of the plasmid pUC119-gRNA was amplified with primer F1 and R1 as well as primer F2 and R2 in two separate reactions. Two different products resulted from this, one containing the first restriction site alongside the U6-1 promoter and the gRNA sequence, the other containing the second restriction site, the gRNA scaffold and the gRNA sequence. These

two overlapping products were combined and amplified with the primers F1 and R2 to create a fused product, which is the finished expression cassette.

The PCR was conducted with Q5® High-Fidelity DNA Polymerase under the conditions shown in Figure 5.

98 °C	30 secs	
98 °C	10 secs	10-30 cycles
55 °C	30 secs	
72 °C	30 secs	
72 °C	5 mins	

Figure 5: PCR conditions for the assembly of the expression cassettes

To assemble the expression vector, the plasmid pFG-pcoCas9 and expression cassette 1 and 3 (containing gRNA 1 and 3) were restriction digested with SbfI and PacI from Thermo Fisher for 2-3 hours at 37 °C (Table 3).

Table 2: First round of restriction digestion

Component	Volume: pFGC-pcoCas9	Volume: expression cassette 1 and 3
PacI (10 U/µL)	2 µL	2 µL
SdaI (SbfI) (10 U/µL)	2 µL	2 µL
10x Buffer SdaI (SbfI)	4 µL	4 µL
DNA	2 µL (~ 200 ng/µL)	19 µL (~ 30 ng/µL)
Nuclease-free water	30 µL	13 µL

The digested vectors were run on a 1% agarose gel and purified with a GeneJET Gel Extraction Kit from Thermo Fisher. To increase the relatively low DNA yield of the over 13 kb big linearised plasmid, the elution step was conducted with only 20 instead of 50 µL, which was preheated to 65 °C and run through the spin column twice. The digested expression cassettes were purified with a QIAquick PCR Purification Kit from QIAGEN. The linearised vector was then ligated with either expression cassette 1 or 3 with T4 DNA ligase from Thermo Fisher in the reaction shown in Table 4 and incubated at room temperature for 10 minutes:

Table 3: Ligation reaction after Lee et al. (2019): Conditions under which all ligation reactions were performed. The volumes of the digested plasmid (X) and the expression cassettes (Y) were adjusted according to obtained DNA concentrations. The reaction was incubated at room temperature for 10 minutes.

Component	Volume
10x T4 DNA ligase buffer	1 µL
digested pFCG-pcoCas9 (~50 ng)	X µL
digested expression cassette 1 or 3 (~10 ng)	Y µL
T4 DNA ligase (5 U/µL)	1 µL
nuclease-free water	add to 10 µL

The ligated plasmid was then transformed into e. coli as follows:

1. One 50 µL vial of One Shot® cells for each ligation was thawed on ice.
2. 5 µL of the ligation mix was added into each vial of competent cells and mixed by gentle tapping.
3. The vials were incubated on ice for 30 mins,
4. Then incubated in a 42 °C water bath for exactly 30 secs and transferred to ice again.
5. 250 µL of prewarmed S. O. C. medium was added to each vial.
6. The vials were then incubated at 37 °C in a shaking incubator (225 rpm) for 1 hour.
7. 150 µL of the content of each transformation vial were spread on LB agar plates containing 50 mg/L kanamycin. The plates were sealed with parafilm, inverted and incubated at 37 °C overnight.

Single colonies were picked from the plates after the overnight incubation and grown overnight in a shaking incubator at 30 °C and 225 rpm in 13 mL cap tubes containing 5 mL liquid LB supplemented with 50 mg/L of kanamycin. From these liquid cultures the plasmids, containing one expression cassette each, were extracted with a QIAprep Spin Miniprep kit. The insertion of the expression cassettes was confirmed by Sanger sequencing through GATC, Germany. The newly obtained plasmids, as well as expression cassette 2 and 4, were digested with EcoRI and AscI at 37 °C for 2 hours (Table 5).

Table 4: Second restriction digestion of pFGC-pcoCas9 with EcoRI and AscI

Component	Volume: pFGC-pcoCas9 gRNA 1/gRNA 3	Volume: expression cassette 2 and 4
EcoRI (10 U/µL)	2 µL	2 µL
SgsI (AscI) (10 U/µL)	2 µL	2 µL
10x Buffer Tango	8 µL	8 µL
DNA	7 µL (~ 80 ng/µL)	19 µL (~ 30 ng/µL)
Nuclease-free water	21 µL	9 µL

The digested pFGC-pcoCas9 gRNA 1 was then ligated with expression cassette 2 and pFGC-pcoCas9 gRNA 3 with expression cassette 4 as described in Table 4 and transformed into *e. coli* as already described previously. The insertion of the expression cassettes was confirmed by Sanger sequencing once more.

Transformation of *Agrobacterium*

To obtain competent *Agrobacterium* cells and to introduce the two desired plasmids into them by the freeze-thaw method a slightly altered protocol of the one described by Wise et al. (2006) was used:

Making competent cells

1. Glycerol stock of the *Agrobacterium tumefaciens* strain GV3101 was used to inoculate 2 mL of low-salt liquid LB medium, which was then incubated overnight at 20 °C and shaken at 225 rpm.

2. Since the cells grew very vigorously overnight and a visible pellet formed only 25µL of the overnight culture was used to inoculate 50 mL of low-salt LB in a 250-mL flask. The culture was then incubated overnight once more.

3. The OD_{600} of the culture was measured with a NanoDrop One. Although with a value of 1.7 the O_{D600} of the culture was much higher than the desired 0.5 – 1.0, the culture was chilled on ice to proceed in the making of competent cells.

4. The culture was then divided into two centrifuge tubes and the cells pelleted at 10,000 G at 4 °C for 10 min in an SS-34 rotor. The supernatant was discarded, and the cells

resuspended in 5 mL chilled 20 mM $CaCl_2$ and centrifuged again. The supernatant was discarded once more.

5. The cells were then resuspended in 1 mL chilled 20 mM $CaCl_2$ and aliquoted into chilled Eppendorf tubes in portions of 150 µL. Four tubes were kept on ice for immediate transformation and the rest frozen at -80 ℃.

Transformation of *Agrobacterium* through freeze-thaw method

1. Approximately 1 µg of plasmid DNA was added to each vial of competent cells. 10 µL of pFGC-pcoCas9 gRNA 1+2 with a concentration of ~100 ng/µL was added to two tubes with competent cells. To the two other tubes 20 µL of pFGC-pcoCas9 gRNA 3+4, with a concentration of ~50 ng/µL, was added. The tubes were incubated on ice for 30 minutes.
2. The tubes containing the cells and the DNA were lowered into liquid nitrogen for 5 mins and submerged in it with tweezers.
3. The tubes were thawed at room temperature for approximately 10 mins. The content of the tubes was then transferred into 13 mL cap tubes containing 2 mL of liquid low-salt LB medium and incubated at 28 ℃ and shaken at 225 rpm for 2 ½ hours.
4. The cells were pelleted by spinning the tubes in a microcentrifuge at 13,000 rpm for 2 minutes. The cells were resuspended in 200 µL of low-salt LB with 10 mg/L rifampicin, 25 mg/L gentamicin and 50 mg/L kanamycin.
5. 200 µL of the cell suspension were plated on agar plates with low-salt LB with 10 mg/L rifampicin, 25 mg/L gentamicin and 50 mg/L kanamycin. The plates were inverted and incubated at 28 ℃ for 3 days.

Agrobacterium-mediated transformation of red raspberry ‘Ninni’ and ‘Veten’

Three transformation events were conducted in total, all under slightly varying conditions. Different centrifugation speeds were used to harvest the bacterial cells from the overnight cultures. The OD_{600} of the cultures used in the transformation events also varied (Table 6), since the

OD_{600} of the starter cultures varied as well as the incubation time of the cultures. The number of transformed explants also varied slightly (Table 5).

Transformation event nr. 1, 12.2.2019

Single colonies of the transformed *Agrobacterium*, containing either pFGC-pcoCas9 gRNA 1+2 or pFGC-pcoCas9 gRNA 3+4, were picked the day before and grown in 5 mL liquid low-salt LB, supplemented with 50 mg/L kanamycin, 25 mg/L gentamicin and 10 mg/L rifampicin, for approximately 7 hrs in a shaking incubator at 28 ℃. This culture was used to inoculate an overnight culture. Four 250 mL flasks, containing 50 mL low-salt LB with 50 mg/L kanamycin, 25 mg/L gentamicin and 10 mg/L rifampicin, were inoculated with 750 µL of culture, two with the culture harbouring pFGC-pcoCas9 gRNA 1+2 and two with the culture harbouring pFGC-pcoCas9 gRNA 3+4. The rest of the culture used for inoculation was kept and also incubated overnight.

After measuring the $OD_{600,}$ which was slightly higher than the desired 0.5 – 1.0, the overnight cultures were transferred to 50 mL falcon tubes and centrifuged at 800 G and 10 ℃ for 10 minutes. The supernatant was discarded, and the cells resuspended in 20 mL MS3 medium. The cultures were centrifuged once more under the same conditions and resuspended in 8 mL MS3.

The *Agrobacterium* suspension was transferred into high-walled 5 cm Petri dishes in 4 mL aliquots. Leaf discs, as well as petiole segments, were cut from in vitro cultivated explants of ‘Ninni’ and ‘Veten’ and shaken in the *Agrobacterium* suspension for at least 5 mins. The explants were dried on sterile filter paper and transferred onto 10 cm Petri dishes containing MS3 with 0.8% agar.

Transformation event nr. 2, 13.2.2019

The rest of the 5 mL cultures which were kept overnight were used once more to inoculate four 250 mL flasks containing low-salt LB and the three antibiotics to maintain the strains. Since the starter cultures had already reached a very high $OD_{600,}$ only 50 µL of each was used to inoculate 50 mL of medium. These new cultures were incubated at 28 ℃ in a shaking incubator.

The next morning the OD_{600} was measured to be between 0.5 and 1.0 this time. 200 µL of these cultures were used to inoculate 5 mL of low-salt LB supplemented with the three antibiotics to make new starter cultures for another transformation the next day.

The overnight cultures were transferred into clean centrifuge tubes and since the tubes could not hold the full volume of 50 mL contained in one flask, the content of each flask was split up into two centrifuge tubes. The cultures were centrifuged at 4 ℃ and 5,000 G for 10 mins and resuspended in 20 mL of MS3 medium. The cultures were centrifuged again at 4 ℃ and 5,000 G and resuspended in 5 mL of MS3 medium. These 5 mL of *Agrobacterium* suspension were filled into high-walled 5 cm Petri dishes. Leaf discs, as well as petiole segments, were cut from in vitro cultivated explants of 'Ninni' and 'Veten' and shaken in the *Agrobacterium* suspension for at least 5 mins. The explants were dried on sterile filter paper and transferred onto 10 cm Petri dishes containing MS3 with 0.8% agar.

Transformation event nr. 3, 14.2.2019

The starter cultures made from the previous overnight cultures were incubated at 28 ℃ in a shaking incubator for approximately 6 hrs. 500 µL of these cultures were used to inoculate 50 mL of low-salt LB supplemented with the three antibiotics in 250 mL flasks, whereas two flasks for each plasmid were used again. These cultures were incubated overnight at 28 ℃ in a shaking incubator.

The next morning the OD_{600} of the cultures was measured and even though it was well over 1.0 a transformation with those cultures was proceeded with in the same way as the day before.

The explants from all three transformation events were incubated in the dark at 23 ℃ for 3 days and then transferred to a regeneration medium which was full MS medium with 3% sugar, 36 mg/L Fe-EDDHA, 0.1 mg/L thidiazuron (TDZ), 0.1 mg/L IBA, 500mg/L cefotaxime and 0.8% agar. After two weeks the plants were transferred onto new regeneration medium, now containing 3 mg/L glufosinate in addition to select for transformed plants.

Table 5: Transformation events, number of transformed explants and OD_{600} of the used cultures

Date	**Nr. of leaf discs**	**Nr. of petiole segments**	**Total nr. of explants**	**OD_{600}**
12.2.2019	160	300	460	0.85-1.39
13.2.2019	170	300	470	0.58-0.94
14.2.2019	160	300	460	1.53-1.85
		Total:	**1 390**	

Testing the dose response to glufosinate

Since pFGC-pcoCas9 confers resistance to glufosinate, the transformed plants were to be selected by adding glufosinate to the regeneration medium. To find the right dose which kills non-transformed plants but leaves the transformed ones to grow, several concentrations of glufosinate were tested. Petri dishes with regeneration medium (MS3 + 0.1 mg/L TDZ, 0.1 mg/L IBA, 3% sugar, 36 mg/L Fe-EDDHA, 0.8% agar) with five different concentrations of glufosinate (0 mg/L, 0.5 mg/L, 1 mg/L, 3 mg/L and 5 mg/L) were filled with leaf discs or whole leaves from in vitro cultures. Two Petri dishes of each concentration were filled with ten leaf discs of 'Veten' and two Petri dishes of each concentration were filled with ten whole leaves of 'Ninni', since the leaves of 'Ninni' were too small to cut leaf discs out of them.

Callus induction

Petioles, segmented petioles and leaf discs were put on a medium with the same composition as the growth medium apart from the hormone concentration. Three different hormone concentrations were tested: medium 1 containing 2.5 mg/L of each 2,4-D and BAP, medium 2 containing 1 mg/L 2,4-D and 0.5 mg/L BAP and medium 3 containing 1 mg/L of each 2,4-D and BAP. The plates were then incubated in the dark at 23 °C. After 8 weeks the explants were transferred to new callus medium, only to medium 2 this time since that yielded the healthiest callus.

Gold particle bombardment of callus

Plasmid DNA of pFGC-pcoCas9 gRNA 1+2 and 3+4 was obtained from single colonies of *E. coli* harbouring those plasmids with a QIAGEN Plasmid Maxi Kit.

For the shooting, a modified protocol after Hvoslef-Eide et al. (2018) was used:

Making gold particle solution (1 µm, 60 mg/mL):

1. 60 mg gold particles were mixed with 1 mL 100% EtOH in a 2 mL Eppendorf tube and vortexed.
2. The Eppendorf tube was spun at 10,000 rpm for 1 min and the supernatant poured off.
3. The gold pellet was resuspended in 1 mL sterile water and stored at -20 °C.

Preparing plasmid DNA (1µg/µL)

1. 20 µL sodium acetate (3 M, pH 5.2) was added to 25 µg plasmid DNA (volume according to DNA concentration, 100 – 400 µL in this case).
2. 500 µL 100% EtOH (from -20 ℃) were added and the tube vortexed.
3. The tube was spun at 10,000 rpm for 5 min and the supernatant pipetted off carefully.
4. The pellet was washed with 1 mL 100% EtOH and left to air dry in a flow hood for 20-30 minutes.
5. 25 µL of sterile MilliQ water was added onto the pellet and the tube left in the fridge for 15-20 minutes.
6. The tube was vortexed and stored at -20 ℃.

Covering gold particles with DNA

1. The following was pipetted into a 1.5 mL Eppendorf tube:

100 µL gold particle solution	(1 µm, 60 mg/mL)
25 µL DNA	(1µg/µL)
100 µL $CaCl_2$	(2.5 M)
40 µL spermidine	(0.1 M)

2. The tube was finger vortexed for 3 min and left on ice for 5 min after.
3. The supernatant was pipetted of and the pellet washed with 100 µL 100% EtOH.
4. The tube was left on ice for 5 min.
5. The supernatant was pipetted off and the pellet resuspended in 120 µL 100% EtOH.
6. The tube was then left on ice until it was needed.

Shooting with the gene gun

The Biolistic ™ Particle Delivery System Model PDS-1000 (DuPont) was used as follows:

1. The stop plate, macro carrier and rupture disc (1150 psi) were sterilised in 70% EtOH and left to air dry in a flow hood. The inside of the gun chamber was also washed with 70% EtOH.
2. The helium gas flask was opened, and the vacuum pump was turned on.
3. The rupture disc was dipped in isopropanol and placed into the holder which was placed into the gun and tightened with a special tool.
4. The DNA covered gold particles were resuspended by dipping the tube into an ultra-sound bath.
5. 10 µL of the gold particle solution was spread on the middle of the macro carrier, which was then air-dried in a flow hood.
6. A stop plate and the macro carrier were put in the holder and the holder then placed inside the gun in the upper position.
7. A 9 cm Petri dish with callus from 'Veten' on raspberry growth medium was placed inside the gun in position 2 from below, the lid removed, and the door shut.
8. The vacuum in the chamber was adjusted to just above 25 mmHg.
9. The callus was shot by holding the button until the rupture disc broke.
10. The Petri dish was removed from the gun, the lid placed back on it and it was sealed with parafilm.

After the shooting, the callus was kept in the dark at 23 ℃ for 4 days. It was then transferred to regeneration medium and placed in a lighted growth room (28µmol/s) at 20 ℃.

Testing the gRNAs with agroinfiltration

The aim was to test the guide RNAs through transient expression via agroinfiltration in green house-grown 'Veten' plants derived from the in vitro cultures. A protocol according to Huhdanmäki et al. (2018) was used:

1. Single colonies of *Agrobacterium* harbouring the desired plasmid were picked and grown (at 28 ℃ and 225 rpm) overnight in 5 mL of medium (low-salt LB in this case).

2. The cultures were transferred to 15 mL Falcon tubes and spun for 10 min at 4,000 G at room temperature.
3. The supernatant was poured off and the tubes spun again for 10 sec.
4. The remaining supernatant was removed with a pipette.
5. The pellet was resuspended in 2 mL Mg-MES (200 µm acetosyringone, 10 mM $MgCl_2$, 10 mM MES, pH 6.0)
6. 200 µL of this *Agrobacterium* suspension were diluted to 3 mL in Mg-MES.
7. The OD_{600} was measured and adjusted to 0.5.
8. The bacteria were incubated at room temperature for 3 hours.

Approximately 12-week old 'Veten' plants were infiltrated with this *Agrobacterium* solution: The leaves were punctured with a clean scalpel. A 2 mL syringe without a needle filled with the *Agrobacterium* solution was placed with its opening over the puncture in the leaf. By pressing the finger on the other side of the leaf and slowly pressing down the plunger down the air space in the leaf was filled with the solution. This was only merely successful, and the liquid did not spread as far as it does in tobacco for example. The infiltrated areas were marked by circling them with a marker pen. After a week the infiltrated leaf parts were analysed with the REDExtract-N-Amp™ Tissue PCR Kit by Sigma-Aldrich.

Results and Discussion

Medium with Fe-EDDHA versus medium without additional iron source

The extra Fe salt has been added because it has previously been shown to prevent chlorosis in tissue culture of raspberries (Zawadzka and Orlikowska, 2006). Plants grown on medium without Fe-EDDHA showed slight chlorosis after about three weeks of cultivation. Therefore, Fe-EDDHA was added to the raspberry medium throughout the whole experiment. A comparison of plants grown on the two different media is shown in Figure 6.

Figure 6: In vitro cultures of 'Veten' grown on medium with Fe-EDDHA (left) and without an added iron source (right) after approximately 3 weeks.

Callus induction

After about four weeks callus started forming on petioles as well as leaf explants (Figure 7 and 8). Since the explants produced a lot of phenols, a lot of the calli were brown and not the desired

Figure 7: Approximately 4-week old petiole segment from 'Veten' on callus induction medium 3

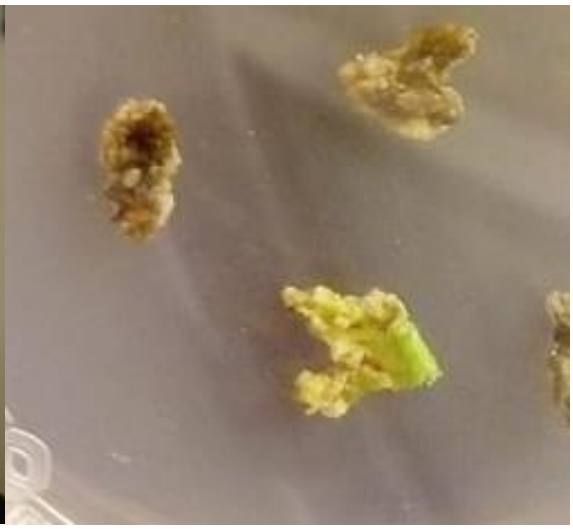


Figure 8: Approximately 4-week old leaf segments from 'Veten' on callus induction medium 2

healthy white colour. Therefore, every time the callus was transferred to new medium the phenolic parts were removed as much as possible until white healthy callus was obtained after about 16 weeks (Figure 9).

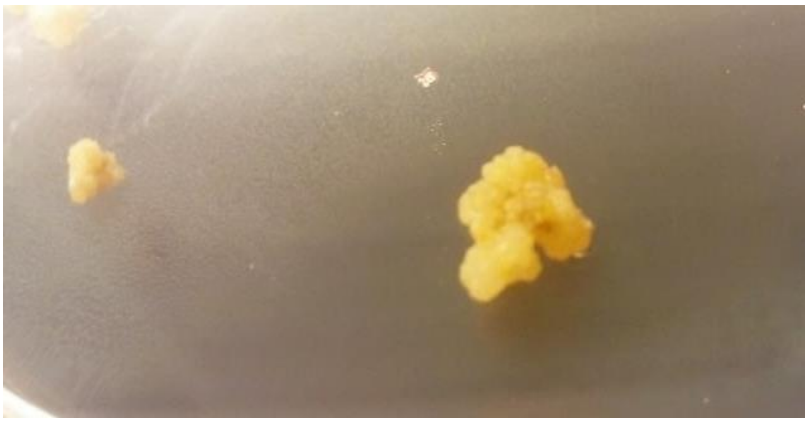


Figure 9: Callus from 'Veten' petiole segments 16 weeks after induction.

Sequencing *F3'H*

The sequences obtained from 'Ninni' and 'Veten' were blasted against the sequences obtained from Jahn Davik. The sequence obtained from 'Veten' matched the scaffold sequence from Jahn Davik to 98.4% and the two sequences from 'Ninni' matched it to 98.5% and 98.9 %. The rest of the sequencing reads were of insufficient quality and were therefore discarded. It was assumed that the small discrepancy between the scaffold sequence and the sequences of 'Ninni' and 'Veten' originated from sequencing errors.

Testing the dose response to glufosinate

After three weeks, all explants cultivated on medium with 3 or 5 mg/L glufosinate and 80% cultivated of the plants on medium with 1 mg/L glufosinate had died. On the explants cultivated on medium containing 0.5 mg/L glufosinate necrosis occurred on most of the explants and 20% had died completely, whereas the control stayed green and started forming shoots (Figure 10). Because 3 mg/L glufosninate was the lowest concentration where all explants died, this concentration was used in the regeneration medium to select for plants transformed by *Agrobacterium*.

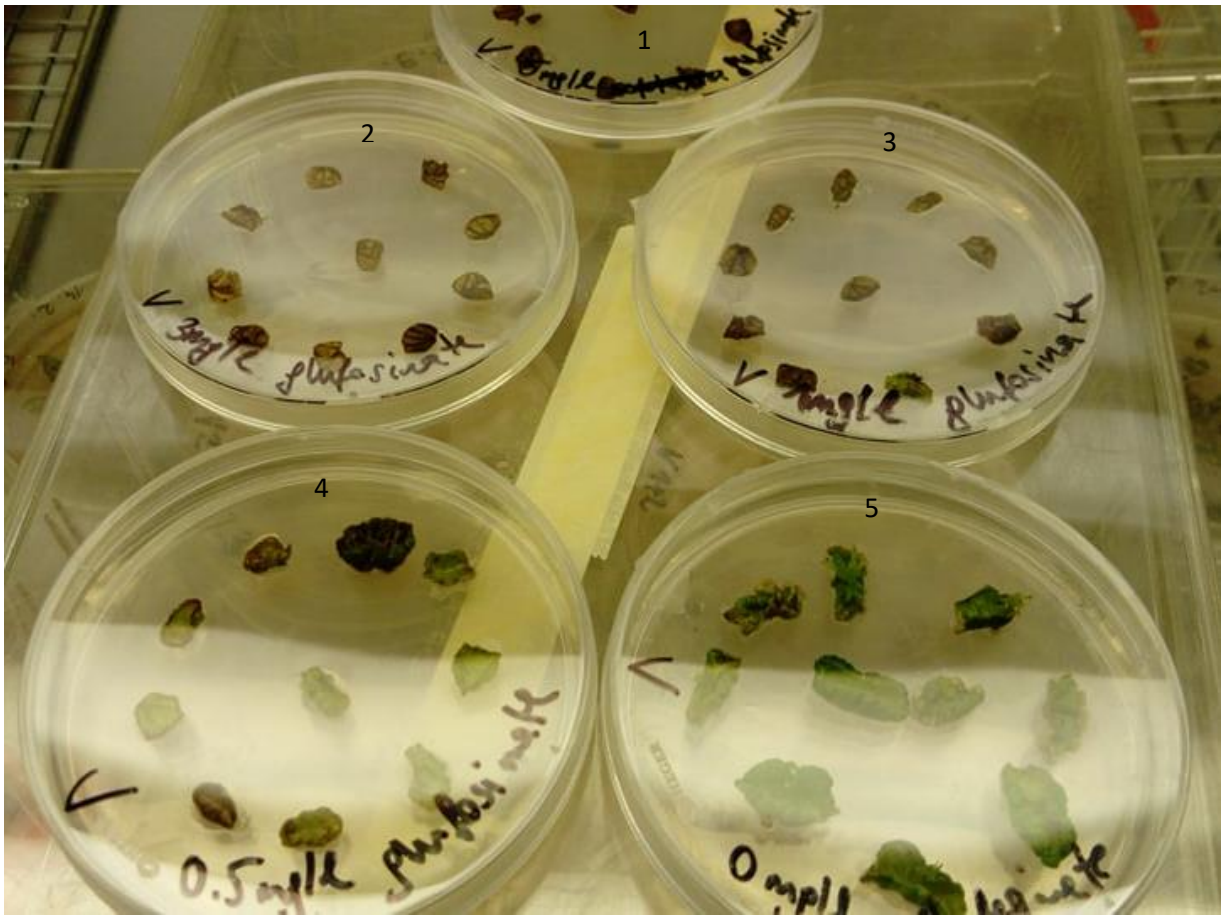


Figure 10: 'Veten' leaf discs on medium with different concentrations of glufosinate after 3 weeks. 1. 5 mg/L; 2. 3 mg/L; 3. 1 mg/L; 4. 0.5 mg/L; 5. 0 mg/L (control)

Verifying the integration of the expression cassettes into pFGC-pcoCas9

The sequences obtained from pFGC-pcoCas9 gRNA 1, pFGC-pcoCas9 gRNA 3, pFGC-pcoCas9 gRNA 1+2 and pFGC-pcoCas9 gRNA 3+4 were blasted against the corresponding plasmid sequence constructed with a SnapGene simulation. The sequences from pFGC-pcoCas9 gRNA 1, pFGC-pcoCas9 gRNA and pFGC-pcoCas9 gRNA 1+2 matched the simulation to 100% and 99.8%. The only sequencing read of good quality from pFGC-pcoCas9 gRNA 3+4 was a 100% match. This means that all four gRNA expression cassettes have been successfully integrated into pFGC-pcoCas9.

Testing the guide RNAs with agroinfiltration

The infiltration of the tissue did not work very well; the *Agrobacterium* solution did not spread very far in the tissue and the necrotic areas caused by the syringe covered almost the whole infiltrated area (Figure 11).

Figure 11: A leaf of a greenhouse grown 'Veten' plant one week after infiltration with the infiltrated areas circled with black marker

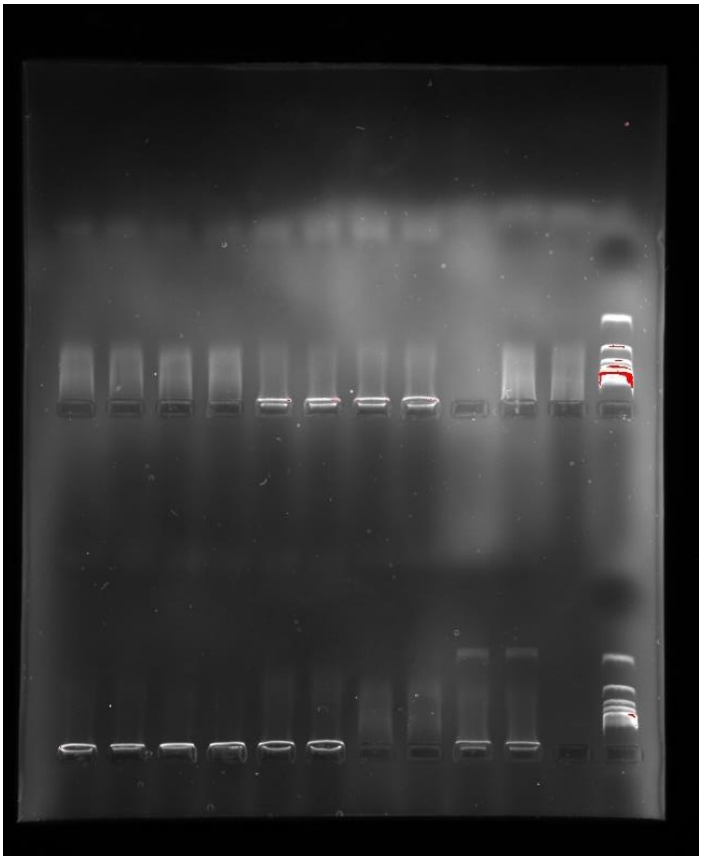


Figure 12: PCR amplification of DNA from leaf discs infiltrated with agrobacterium harbouring pFGC-pcoCas9 gRNA 1+2 (bottom) or pFGC-pcoCas9 gRNA 3+4 (top) using the REDExtract-N-Amp™ Tissue PCR Kit. Every sample was distributed into two wells. The ladder used was the 100 bp ladder.

Even though the infiltration did not function as expected the infiltrated areas were analysed with a REDExtract-N-Amp™ Tissue PCR Kit by Sigma Aldrich. This did not yield any usable results since the primers designed to amplify the target regions of the four gRNAs did not work well and only yielded a weak band for one sample (Figure 12). The primers were later tested on DNA extracted with a QIAGEN DNeasy Plant Mini Kit and this did not produce results either. The REDExtract-N-Amp™ Tissue PCR Kit was also later tested with newly designed primers that had previously been proven to work (Figure 13 and 14) in 'Veten', but again did not yield any results. The lack of results can possibly be attributed to poor primer design or the use of a kit not optimal for the plant material.

This method was not successful in raspberry especially since the infiltration of the leaves proved to be a challenge. It could be possible to use a different infiltration technique like using vacuum infiltration as in Santos-Rosa et al. (2008). With successful infiltration and the right detection

method, agroinfiltration could be an interesting method to test CRISPR/Cas9 constructs, but also to produce transgene-free gene edited plants by regenerating shoots from infiltrated areas.

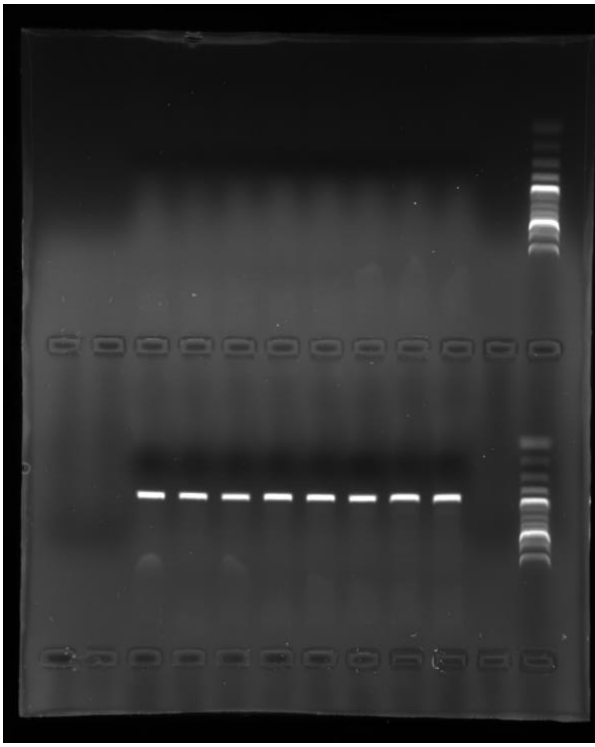


Figure 13: DNA from 'Veten' extracted with QIAGEN DNeasy Plant Mini Kit and amplified with the primers Ru_gRNA1+2_2 (bottom) and Ru_gRNA1+2_3 (top) with a gradient from 51.7 to 58.3 °C. The ladder next to samples is a 100 bp ladder.

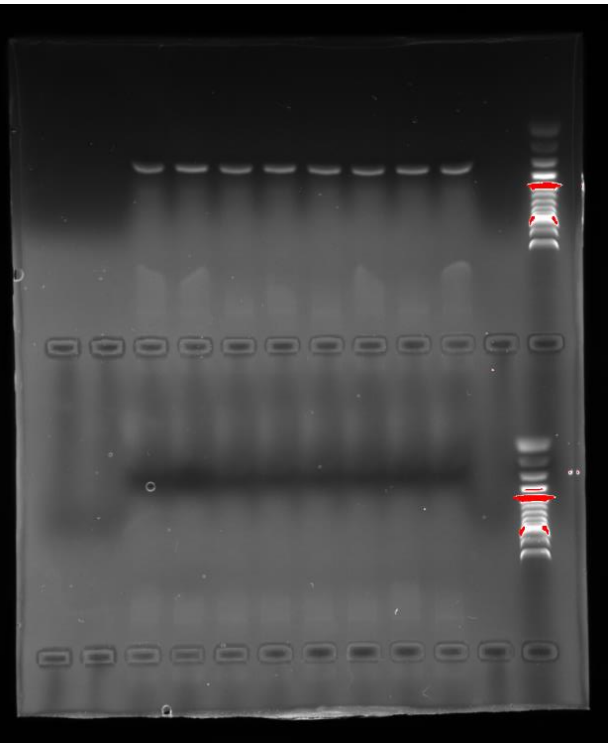


Figure 14: DNA from 'Veten' extracted with QIAGEN DNeasy Plant Mini Kit and amplified with the primers Ru_gRNA3+4_2 (bottom) and Ru_gRNA3+4_3 (top) with a gradient from 51.7 to 58.3 °C. The ladder next to samples is a 100 bp ladder.

Regeneration of transformed plants

Explants transformed with *Agrobacterium*

Three to four weeks after the *Agrobacterium* transformation shoots started forming on the edges of the explants, first on the leaf discs (Figure 15 and 16) and then also on the petiole segments (Figure 18). After that, when glufosinate was added to the medium, the shoots only grew very slowly and a lot of them (the non-transformed ones) died due to the herbicide. Since even the

Figure 15: Leaf disc from ’Ninni‘ after four weeks of transformation with pFGC-pcoCas9 gRNA1+2

Figure 16: Leaf discs from ‘Veten‘ after four weeks of transformation with pFGC-pcoCas9 gRNA1+2

shoots, that did not die from the glufosinate, considerably slowed down their growth on the medium with glufosinate, it is very likely their growth was also inhibited by the herbicide. This can also be inferred from comparison with non-transformed explants, which grew much quicker (Figure 17).

Figure 17: Non-transformed leaf from ’Ninni’ on regeneration medium without glufosinate and cefotaxime after about three weeks

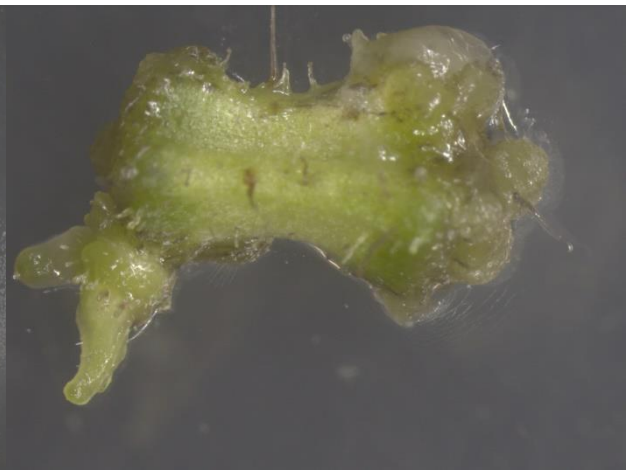


Figure 18: Petiole segment from ‘Veten’ transformed with pFGC-pcoCas9 gRNA3+4 after approximately four weeks

Explants transformed by particle bombardment

After 8 weeks on the regeneration medium no shoots had formed from the calli. Some parts of the callus turned green, whereas most of the calli turned brown and a very small portion of the

Figure 16: 'Veten' callus a little more than eight weeks after bombardment with pFGC-pcoCas9 gRNA1+2 showing mainly brown and some vividly green cells

Figure 17: 'Veten' callus a little more than eight weeks after bombardment with pFGC-pcoCas9 gRNA1+2 showing mainly light green and white cells

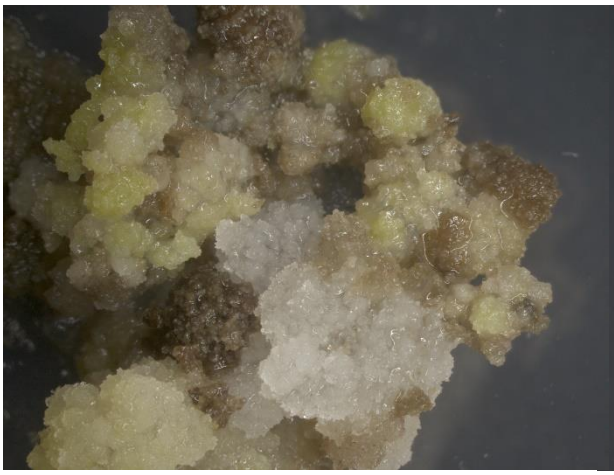


Figure 18: 'Veten' callus a little more than eight weeks after bombardment with pFGC-pcoCas9 gRNA1+2 showing a mix between pale green, white and brown cells

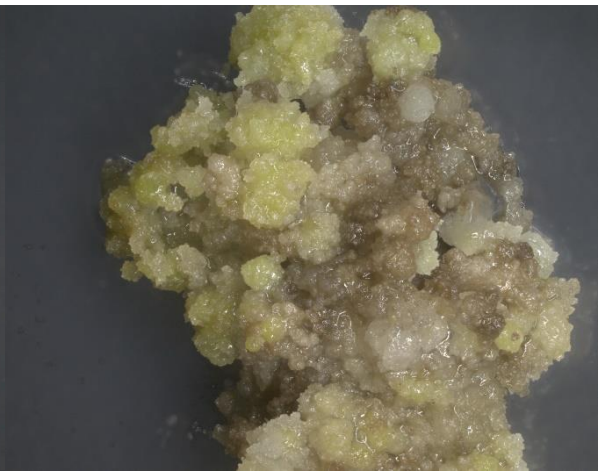


Figure 19: 'Veten' callus a little more than eight weeks after bombardment with pFGC-pcoCas9 gRNA1+2 showing a mix between pale green, brown and some white cells

callus stayed white (Figure 19 to 22). This suggests that regenerating shoots from raspberry callus requires a different hormone composition in the medium than the regeneration of shoots from leaf discs and petiole segments. There are very few protocols for callus regeneration in raspberry since most experiments use direct organogenesis in raspberry (Ambrozic Turk et al., 1994, Cousineau and Donnelly, 1991, Graham et al., 1997, Mathews et al., 1995, McNicol and Graham, 1990, Mezzetti et al., 1997, Zawadzka and Orlikowska, 2006). Maybe the protocol for

shoot regeneration from raspberry callus by Popescu and Valentina (2000) could be used to successfully regenerate shoot from transformed raspberry callus.

Analysing transformants

Explants transformed with *Agrobacterium*

By the time the experiment ended the plants were not big enough to extract DNA from them without destroying them. About 12 weeks after the transformation, 25 plants remained which had not been killed by infections or selected against by the glufosinate. It cannot be said with certainty however, that these 25 plants are actually transformed without testing them on a molecular level or waiting until the plants display fruits with a colour change.

This shows for once, that shoot regeneration takes some time, especially with a selection agent that slows down the growth of the explants. It is possible that an alternate selection agent to glufosinate would yield better results. Another possibility would be not to use any selection, but a more efficient screening method like in Liu et al. (2012) and Yi et al. (2016). This would also be useful to detect transformants created by transient expression by for example, particle bombardment or agroinfiltration.

Explants transformed by particle bombardment

Random samples were taken from the bombarded callus and DNA extracted from it with a QIAGEN DNeasy Plant Mini Kit. The target regions of the gRNAs were amplified with the same primers as used in Figure 12 and 13 and the DNA was visualised on a gel (Figure 23 and 24). Only one sample showed a visible difference to the wild type (Figure 24); a band that shows an approximately 1.5 kb long fragment in addition to the expected band with a fragment with a length of around 300 bp with 284 bp being the exact length in the wild type. This indicates that in this one sample there were cells that carried DNA with a big insertion in the target region which shows that at least one of the gRNAs worked.

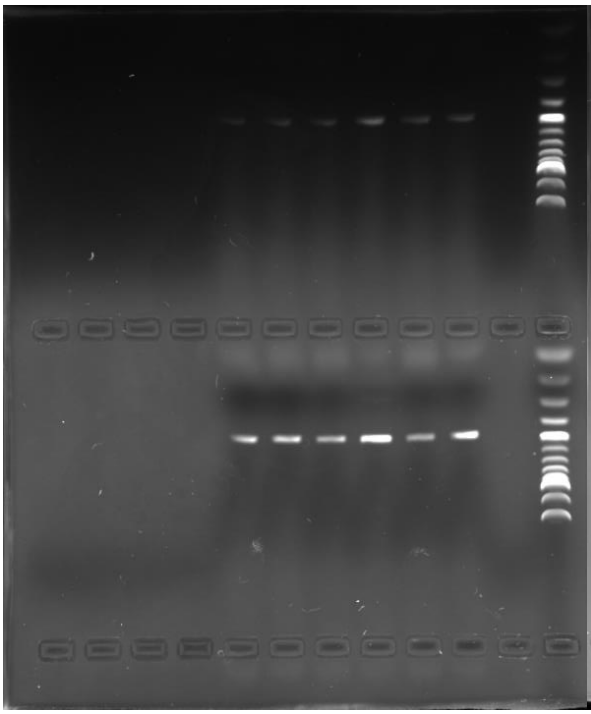


Figure 20: DNA from 'Veten' callus transformed with pFGC-pcoCas9 gRNA 3+4 (bottom) and pFGC-pcoCas9 gRNA 1+2 (top). The target region of gRNA 1+2 was amplified. The ladder used was a 100 bp ladder.

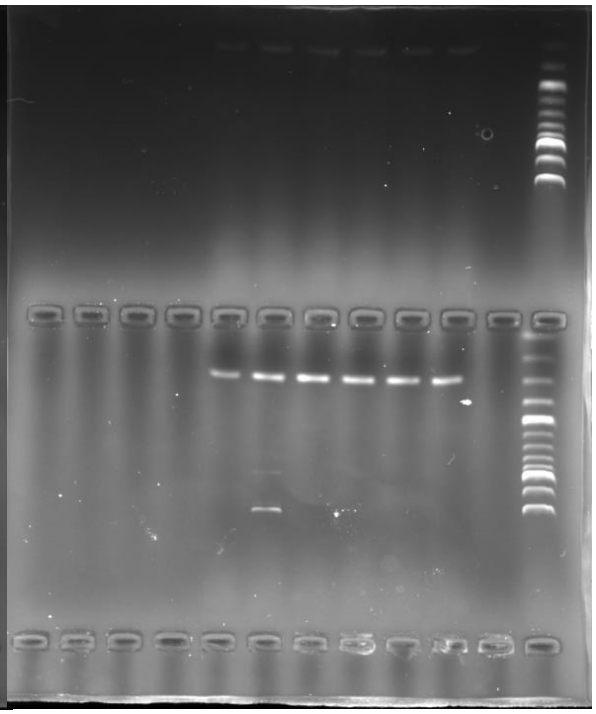


Figure 21: DNA from 'Veten' callus transformed with pFGC-pcoCas9 gRNA 3+4 (bottom) and pFGC-pcoCas9 gRNA 1+2 (top). The target region of gRNA 3+4 was amplified. The ladder used was a 100 bp ladder. The second sample from the left on the bottom shows a clear band at ca. 1500 bp in addition to the band at 284 bp.

The bands from the gel (Figure 23 and 24) were purified with a GeneJET Gel Extraction Kit from Thermo Fisher. The obtained DNA was not of good enough quality for sequencing, so it was amplified with the same primers again and purified with a QIAquick PCR Purification Kit from Qiagen and sequenced. The callus transformed with gRNA 1 and 2 did not show any difference to the wild type. All the samples of sufficient quality from the callus transformed with gRNA 3 and 4 however, showed a substitution of one base at the same place within the target region of gRNA 3. There were two types of substitutions; the substitution of an A for a T (Figure 25) and the substitution of the same A for a C (Figure 26). The second type of substitution was more common with 73% of the samples showing it. Finding transformants in every sample of the six random samples taken suggests that particle bombardment is a very efficient delivery method for CRISPR/Cas9 in raspberry.

The 1500 bp long band visible on the gel did not yield a usable sequencing result within the time frame of the thesis, but it is possible that the insertion happened at the same place where the other samples show a substitution since that seems to be the place where the DSB in gRNA 3 is induced.

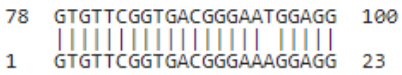


Figure 23: DNA sequence from callus transformed with gRNA 3 showing a substitution of one base (A for T) (top) blasted against the target region of gRNA 3 (bottom)

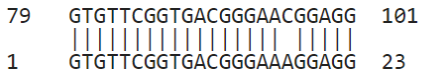


Figure 22: DNA sequence from callus transformed with gRNA 3 showing a substitution of one base (A for C) (top) blasted against the target region of gRNA 3 (bottom)

Although the regeneration of plants from callus was not successful, the transformation of the obtained callus by particle bombardment was successful, therefore it would be interesting to experiment more with this method and optimise a regeneration protocol for transformed raspberry callus. Callus is definitely an interesting explant for biolistic transformation, because there are a lot of cells packed in one place which make a good target for the gold particles. The problem with callus is however that it takes time to initiate it and that it may have a high frequency of somaclonal variation (Skirvin et al., 1994). An alternative to bombarding callus could be using leaf explants instead (Klein et al., 1988). This could present a good solution for raspberry since direct organogenesis from leaves or leaf discs has been proven to work well in this species (Ambrozic Turk et al., 1994, Cousineau and Donnelly, 1991, Graham et al., 1997, McNicol and Graham, 1990, Mathews et al., 1995). Particle bombardment is not only interesting because of its success in this experiment, but also because it harbours the possibility of transient expression of CRISPR/Cas9 which can produce transgene-free gene edited plants (Yi et al., 2016), another reason to explore this method further.

Conclusions

The experiment was successful since a CRISPR/Cas9 construct, that successfully edited the genome of raspberry, was created and therefore a proof of concept for CRISPR/Cas9 in raspberry has been provided. There is however much more room for improvement in regard to an efficient regeneration of transformants.

The process of callus induction was not optimal, and it took very long to obtain the desired white porous callus, this is often the case in many plants (Hoque and Arima, 2002, Laukkanen et al., 1999). Regenerating plants from the callus did not work with the medium and hormone concentration tested here. Since the callus experiment was not the main focus of the experiment, not enough time was invested in finding a regeneration medium with a suitable hormone concentration. Nevertheless, to optimise callus induction and shoot regeneration from transformed

callus of raspberry, it is recommended to set up a whole new factorial experiment to tests the different conditions suitable for this.

Another method that needs more exploration is the agroinfiltration of raspberry leaves. If this method is improved and made functional, it could be a quick way to test a CRISPR/Cas9 construct.

The *Agrobacterium*-mediated transformation has not yielded any solid evidence yet since the potential transformants were still too small for analysis. However, since a number of plants survived the selection by glufosinate it seems likely that there may be some successfully transformed plants. When those plants are analysed later, it would also interesting to determine possible off target effects, but this requires a full genome sequence of our raspberry cultivars to do the analysis needed.

For future work, it would be interesting to further explore biolistic transformation of raspberry since the method seems to be very promising in terms of efficiency and due to its potential of producing transgene-free gene edited plants by transient expression to allow the CRISPR/Cas9 complex to do its job without being integrated in the genome.

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Appendix

Media

Lysogeny broth (LB) 1 L

Reagent	Amount
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
Water	to 1 L
pH 7	

Low-salt lysogeny broth (low-salt LB) 1L

Reagent	Amount
Tryptone	10 g
NaCl	5 g
Yeast extract	5 g
Water	to 1 L
pH 7	

Sequence alignments

Figure 24: Sequence 1 obtained from DNA from 'Veten' amplified with the primer RuF3'H_1sc blasted against the scaffold sequence obtained from Jahn Davik

Range 1: 5 to 190 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
333 bits(180)	2e-96	184/186(99%)	0/186(0%)	Plus/Minus

```
Query  2    AAGGGTCCACTCTCCTGGTCAATGTATGGGCCATATCGCGTGACCCGGCTGAATGGGCCG  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  190  AAGGGTCCACTCTCCTGGTCAATGTATGGGCCATATCGCGTGACCCGGATGAATGGGCCG  131

Query  62   ATCCGCTTGAGTTCAGGCCCGAAAGGTTCCTACCGGGCGGCGAAAAGCCCAACGTGGATA  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  ATCCGCTTGAGTTCAGGCCCGAAAGGTTCCTACCGGGCGGCGAAAAGCCCAACGTGGATA  71

Query  122  TTAGAGGCAATGATTTTGAAGTCATACCGTTTGGTGCTGGGCGAAGAATATGTGCTGGGA  181
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  70   TTAGAGGCAATGATTTTGAAGTCATCCCGTTTGGTGCTGGGCGAAGAATATGTGCTGGGA  11

Query  182  TGAGCT  187
            ||||||
Sbjct  10   TGAGCT  5
```

Figure 25: Sequence 1 obtained from DNA from 'Ninni' amplified with the primer RuF3'H_1sc blasted against the scaffold sequence obtained from Jahn Davik

Range 1: 2 to 194 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
340 bits(184)	1e-98	191/194(98%)	1/194(0%)	Plus/Plus

```
Query  36   ATCGCGTGACCCGGCTGAATGGGCCGATCCGCTTGAGTTCAGGCCCGAAAGGTTCCTACC  95
            |||||||||||||| || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  2    ATCGCGTGACCCGGATG-ATGGGCCGATCCGCTTGAGTTCAGGCCCGAAAGGTTCCTACC  60

Query  96   GGGCGGCGAAAAGCCCAACGTGGATATTAGAGGCAATGATTTTGAAGTCATACCGTTTGG  155
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  61   GGGCGGCGAAAAGCCCAACGTGGATATTAGAGGCAATGATTTTGAAGTCATCCCGTTTGG  120

Query  156  TGCTGGGCGAAGAATATGTGCTGGGATGAGCTTGGGCTTGCGTATGGTCCATTTAATGAC  215
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TGCTGGGCGAAGAATATGTGCTGGGATGAGCTTGGGCTTGCGTATGGTCCATTTAATGAC  180

Query  216  TGCAACCTTGGTCC  229
            ||||||||||||||
Sbjct  181  TGCAACCTTGGTCC  194
```

Figure 26: Sequence 2 obtained from DNA from 'Ninni' amplified with the primer RuF3'H_1sc blasted against the scaffold sequence obtained from Jahn Davik

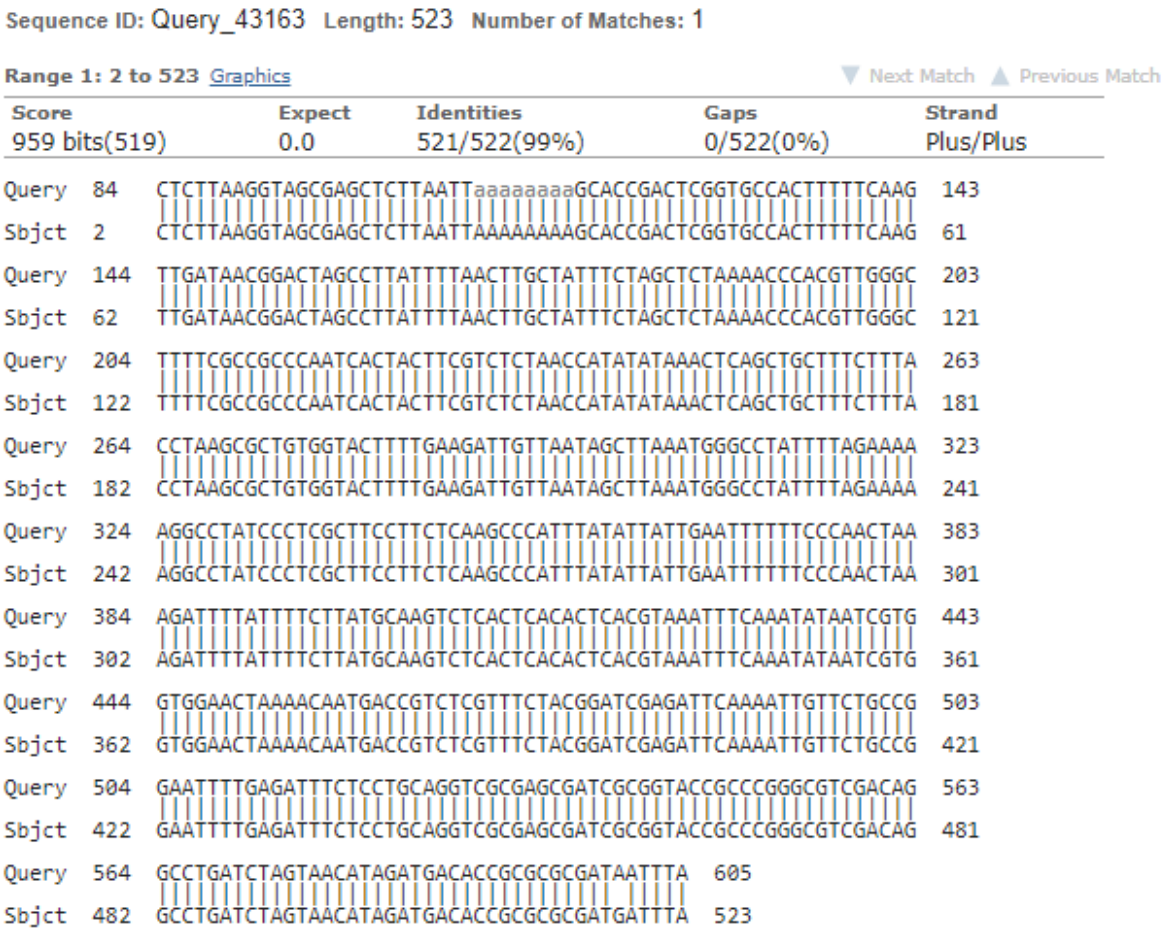

Figure 27: Sequence 1 obtained from pFGC-pcoCas9 gRNA 1 through Sanger Sequencing blasted against the sequence obtained from the SnapGene simulation

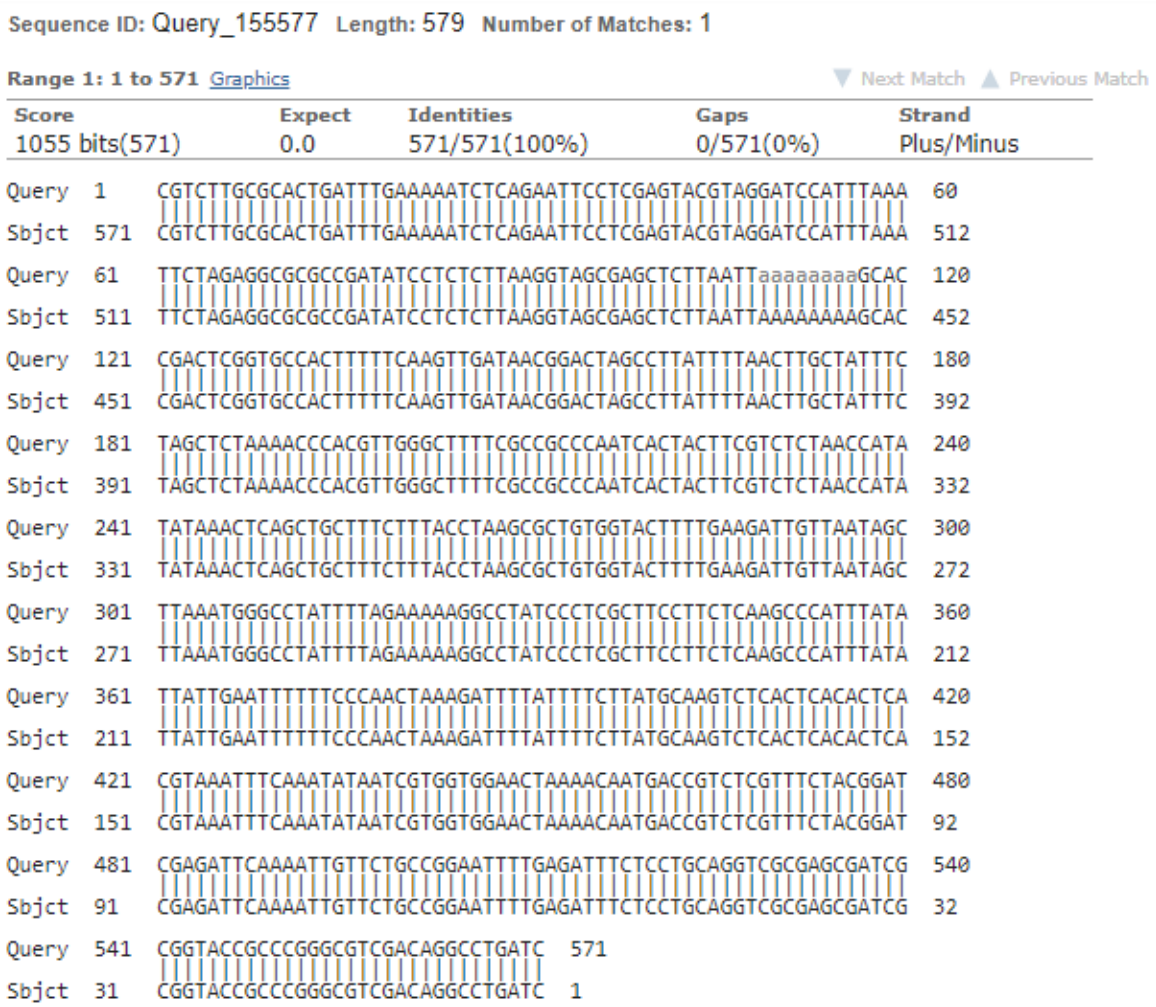

Figure 28: Sequence 2 obtained from pFGC-pcoCas9 gRNA 1 through Sanger Sequencing blasted against the sequence obtained from the SnapGene simulation

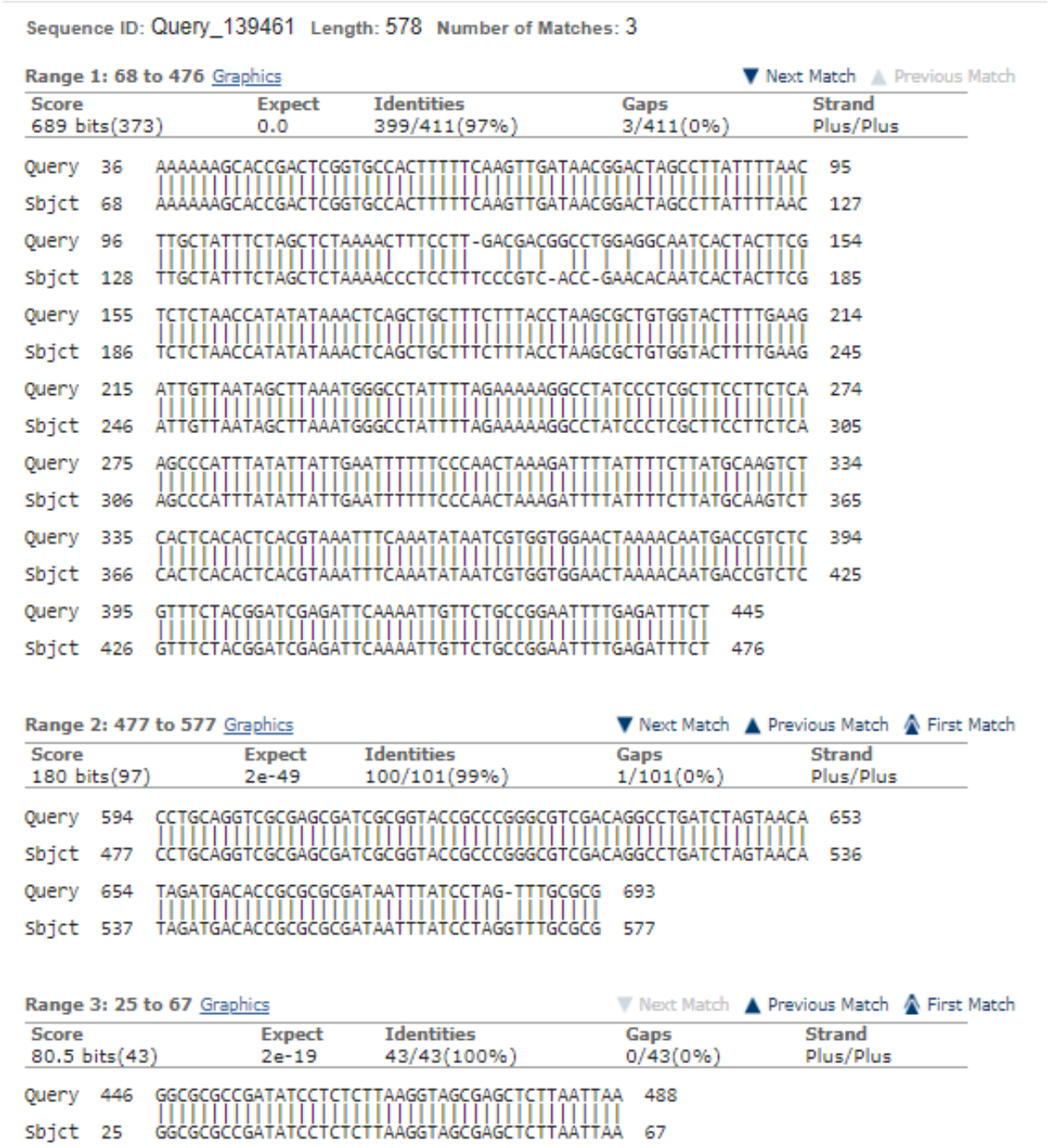

Figure 29: Sequence 1 obtained from pFGC-pcoCas9 gRNA 3 through Sanger Sequencing blasted against the sequence obtained from the SnapGene simulation

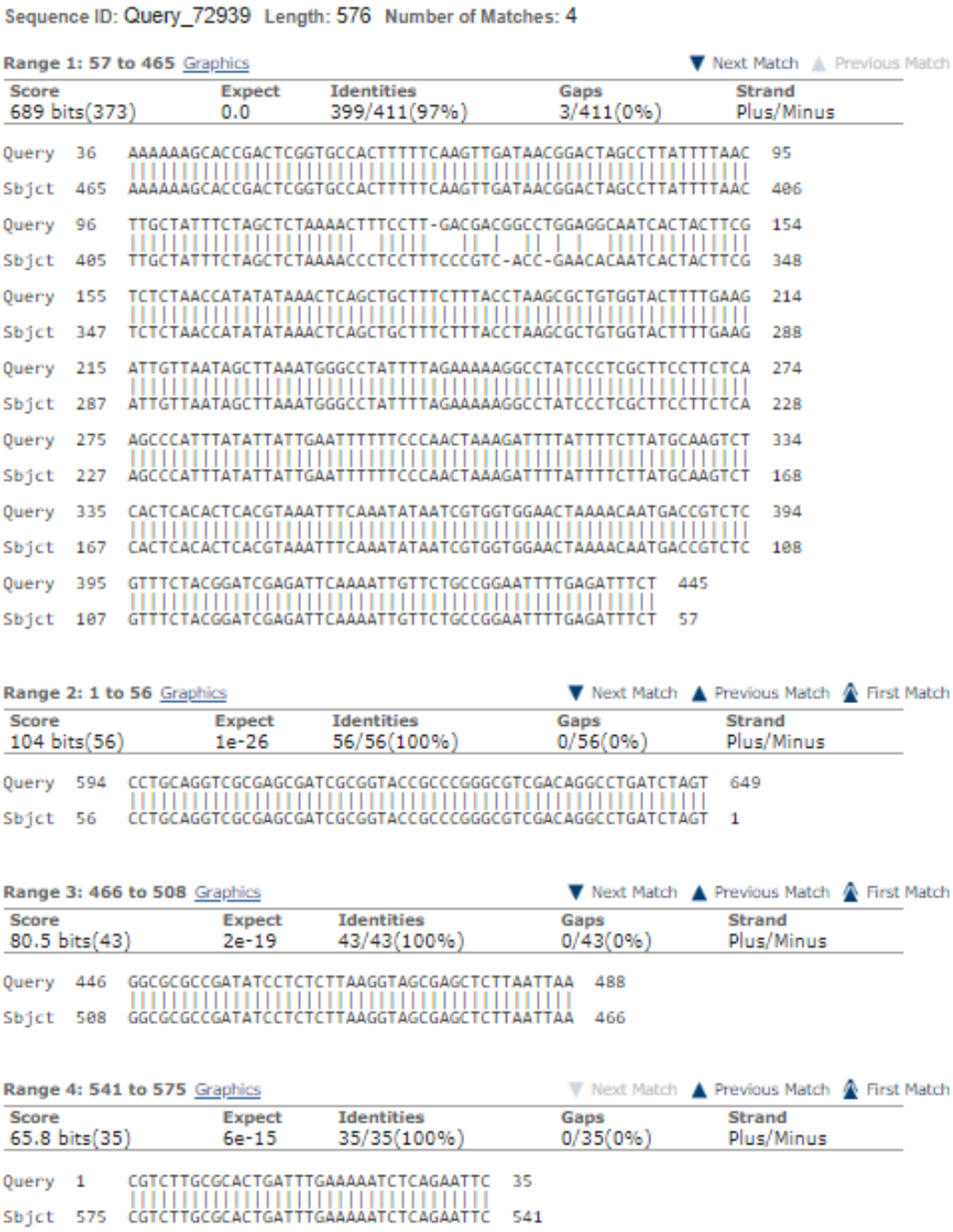

Figure 30: Sequence 2 obtained from pFGC-pcoCas9 gRNA 3 through Sanger Sequencing blasted against the sequence obtained from the SnapGene simulation

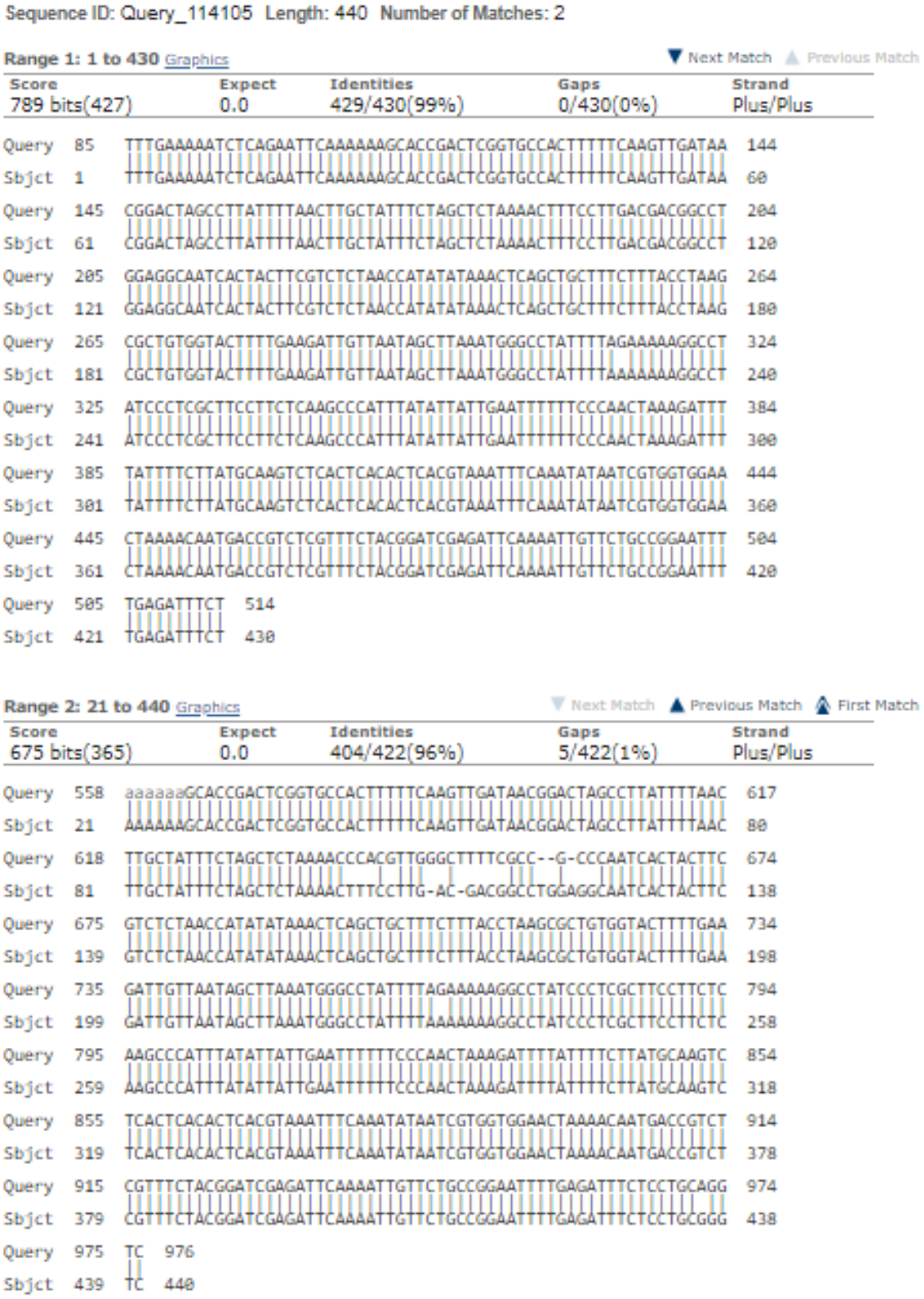

Figure 31: Sequence 1 obtained from pFGC-pcoCas9 gRNA 1+2 through Sanger Sequencing blasted against the sequence obtained from the SnapGene simulation

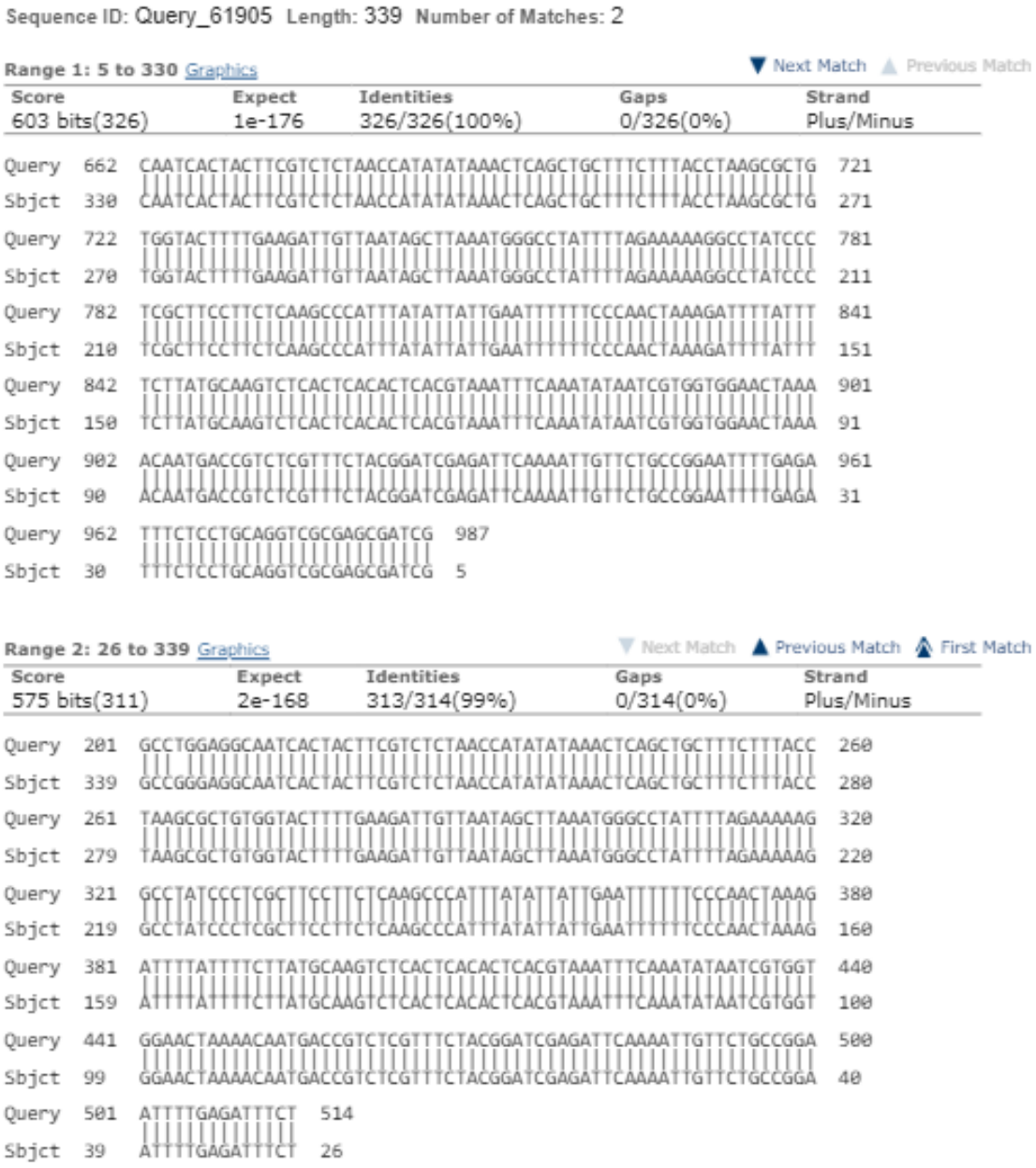

Figure 32: Sequence 2 obtained from pFGC-pcoCas9 gRNA 1+2 through Sanger Sequencing blasted against the sequence obtained from the SnapGene simulation

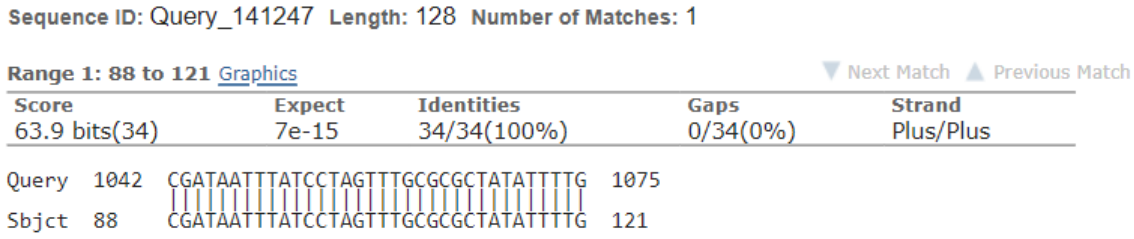

Figure 33: The one sequence with the best quality obtained from pFGC-pcoCas9 gRNA 3+4 through Sanger Sequencing blasted against the sequence obtained from the SnapGene simulation

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