Design of single guide-RNAs targeting the *LcMyb28* gene, and isolation and regeneration of protoplasts from *Lepidium campestre*

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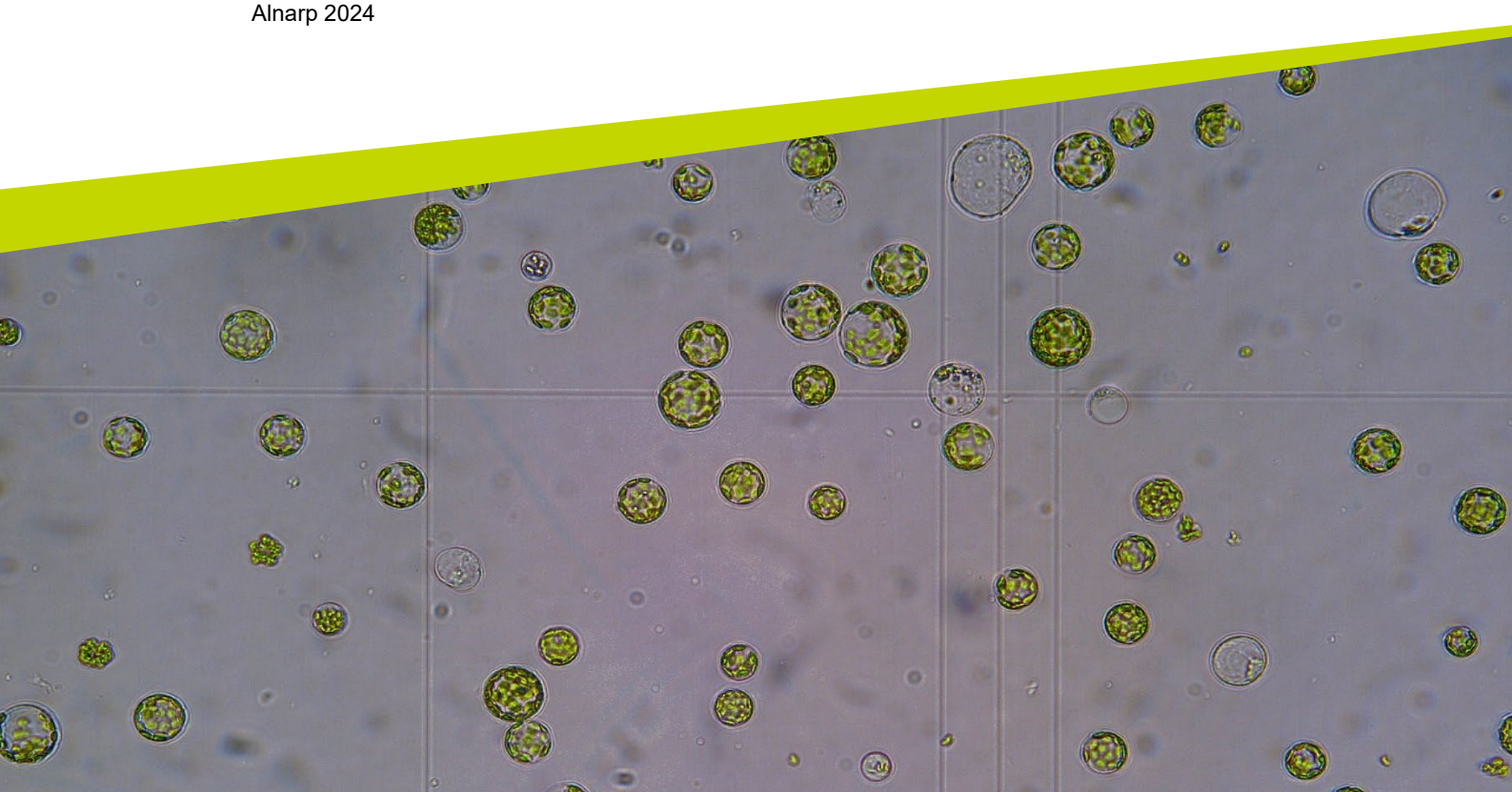

Design of single guide-RNAs targeting the *LcMYB28* gene, and isolation and regeneration of protoplasts from *Lepidium campestre*

Design av guide-RNA för geneditering av *LcMYB28*, samt isolering och regenerering av protoplaster från *Lepidium campestre*

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Abstract

Field cress (*Lepidium campestre*) is a potential new oilseed crop which for a long time has been seen as a good alternative to rapeseed in the northern parts of Sweden due to its high tolerance to cold and potential for high yield. During recent decades *L. campestre* has been under domestication but traditional plant breeding is a slow and limited process, and while traditional methods for genomic modifications are quicker, plants bred using these methods are unlikely to be allowed in the EU for a foreseeable future. This is due to the addition of foreign DNA into the plant's genome resulting in the plant being classified as transgenic. Due to resent developments in CRISPR/Cas9 techniques involving ribonucleoprotein approaches it is now possible to mutate specific genes in the plants genome without leaving any trace of foreign DNA in the targeted cell. One way of introducing the ribonucleoprotein complex is by transfection of protoplasts, which first need to be isolated before being transfected and finally regenerated into plantlets. This is a time-consuming task that requires a lot of delicate and precise work.

This Bachelor project is part of a bigger project with the goal of improving the seed cake quality of field cress by lowering the amount of glucosinolates. This will be done by using a ribonucleoprotein approach to silence *Lepidium campestre MYB28* (*LcMYB28*) which is a transcription factor involved in the biosynthesis of glucosinolates, using a ribonucleoprotein approach. For this single guide RNAs were designed targeting the Lc*MYB28* gene. In the future these guide RNAs will be used to transfect protoplasts of *L. campestre*, regenerate them and to analyze the glucosinolate content to determine if *LcMYB28* is a good candidate for future breeding programs for *L. campestre.*

Keywords: *L. campestre, Protoplast isolation, sgRNA, MYB28, Glucosinolates*

Sammanfattning

Fältkrassing eller *Lepidium campestre* är en vild växt i familjen Brassicaceae och en potentiell oljeväxt som under de senaste årtiondena har varit under domesticering på grund av sin höga köldtolerans och tvååriga livscykel. Tyvärr är traditionell växtförädling en långsam och begränsad process. Traditionella metoder för genomisk modifiering är snabbare men det är osannolikt att växter som tagits fram med dessa tekniker kommer att tillåtas i EU inom en överskådlig framtid på grund av inkorporeringen av främmande DNA i växternas genom. Tack vare CRISPR/Cas9 och ribonukleoprotein är det nu möjligt att mutera specifika gener i växternas genom utan att lämna några spår av främmande DNA i växten. Nackdelen med dessa tekniker är att de måste utföras på protoplaster som först måste isoleras innan de transfekteras och slutligen regenereras till småplantor. Detta är ett väldigt tidskrävande arbete som också kräver stor kunskap och erfarenhet.

Det här kandidatprojektet är en del av ett större projekt med målet att använda metoder med ribonukleoprotein för att tysta *LcMYB28*-genen, en transkriptionsfaktor som är involverad i biosyntesen av glukosinolater. För detta skapades i denna studie flera single guide RNAs som ska matcha *LcMYB28*-genen. I framtiden kommer dessa att användas för att transfektera protoplaster av *L. campestre* och regenerera dessa till plantor som sedan kan användas för att analysera glukosinolathalten för att avgöra om *LcMYB28* är en bra målgen för framtida förädlingsprogram.

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Abbreviations

At	*Arabidopsis thaliana*
bp	Base pair
GFP	Green fluorescent protein
Lc	*Lepidium campestre*
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
pps	Protoplasts
sgRNA	Single guide RNA
RNAi	RNA interference
RNP	Ribonuclear protein

1. Background

1.1 Lepidium campestre

Field cress (Lepidium campestre) is a plant in the *Brassicaceae* family that has been studied during the last 30 years as a potential oil crop due to its high cold tolerance, potential high yield as well as its biennial life cycle (Merker & Nilsson 2010). Two studies (Merker & Nilsson 2010, Nilsson *et al*. 1998) points out that due to the high cold tolerance, it could be a good oil-crop for the cold northern parts of Sweden where rapeseed cannot be grown. The authors also suggest that farms focusing on no- or reduced tilling could use *L. campestre* for reduced nitrogen leaching and to lower ground disturbance due to its ability to keep the ground covered for two years without the need of using heavy machinery. The biennial lifecycle allows the farmer to undersow a cereal (barley for example) with *L. campestre*, where barley is harvested the first year and *L. campestre* can then be grown for another year and harvested the second year. By doing so, the total combined yield has been shown to increase (Merker *et al*. 2010). In a study done in 1999 by Andersson *et al.* three different wild plants were tested as potential new oilseed crops, one of them being *L. campestre*. The study found that *L. campestre* was a good potential oilseed crop with a long list of positive properties, but some changes had to be made regarding the quality and the content of the seed oil. The researchers also addressed the early and uneven pod shattering as well as the glucosinolate content of the crop as problems. The pod-shattering is a problem due to harvest loss (Liljegren *et al*. 2004), and glucosinolates remain in both oil and seedcake are problematic due to their bad taste, pungent smell and potential toxicity for animals and humans (Tan *et al*. 2011).

1.2 Glucosinolates

Glucosinolates are a large group of secondary metabolites that act as one of the plants natural defences against pests and herbivores and are commonly found in cruciferous plants (Prieto *et al*. 2019). Glucosinolates are in their natural form mainly health beneficial due to their antioxidant and phase 1 metabolic activities as well as direct antimicrobial properties (Index 2021). Glucosinolates can however be problematic in too high concentration and have been shown to cause reduced growth and feed intake as well as causing anemia, gastrointestinal irritation, goiter and renal lesions in cattle (Index 2021). However, when the leaves and cells of the plant are broken, usually due to chewing or cutting, the glucosinolates are converted into an array of breakdown products with the aid of myrosinase, epithiospecific protein (ESP) and Fe II (Parker 2015). Some of these mentioned compounds are biologically inert nitriles and isocyanates with a long list of health promoting effects but these compounds also give the plant the unwanted pungent smell and bad taste (Tan *et al*. 2011). The seedcake that remains after the process of extracting oil would preferably be used for animal feed but due to the mentioned negative effects of the remaining glucosinolates the value of the seedcake as feed is low.

1.3 MYB28

In an earlier study by Augustine *et al.* (2013) with *Brassica juncea*, it was found that the *MYB28* and *MYB29* genes were transcription factors that amongst other genes controlled the amount of glucosinolates in the plant. Their research showed that plants of *B. juncea* with all homological genes of *MYB28* mutated had an 86 % lower glucosinolate content in the seeds. The same study determined however that the *MYB29* gene did not seem to be as impactful when knocked out. Another study (Hirai *et al.* 2007) overexpressed the *MYB28* gene in cell suspension cultures and found that it caused the cells to produce a large amount of glucosinolates. It is therefore of interest to determine if mutating the *MYB28* gene in *L. campestre* would result in a lowered glucosinolate content of the seeds.

1.4 Previous modifications

During the last 30 years work has been done at SLU to domesticate *L. campestre* and thanks to funding by MISTRA and SLU the work has intensified during the last decade. Some of the traits that have been studied and controlled is the oil quality by lowering the quantity of erucic acid (22:1) and linolenic acid (18:3) as well as greatly increasing the quantity of oleic acid (18:1) to increase the shelf life and decrease oxidation problems when heated (Ivarson *et al.* 2016, Sandgrind *et al.*

2023). In the study by Ivarson *et al.* (2016) the increase in oleic acid was from 11% in wildtype (WT) to 80% in transgenic lines, the big difference is that her study was based on RNAi-technology while the study by Sandgrind *et al.* (2023) used CRISPR-technology. Ivarson *et al*. (2017a) also managed to increase the general oil content by 25% in *L. campestre.* The genes for pod shattering and uneven seed maturation have been identified and tested in *Arabidopsis thaliana* (Liljegren *et al.* 2004). One of these genes, *INDEHISCENT* have successfully been silenced in *L. campestre* by Ivarson *et al*. (2023) using RNAi technique, which resulted in increased pod shatter resistance. Other projects like testing if *L. campestre* can be used to produce wax esters has also been done at SLU (Ivarson *et al.* 2017b).

1.5 CRISPR/Cas9

Clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 is a gene-editing technology that consists of two parts, a guide RNA of 18-29 base pairs that can easily be fabricated and swapped out to match the targeted gene, and the endonuclease CRISPR-associated protein 9 (Cas9) (Redman 2016). The Cas9 protein will, when delivered to the desired gene, cause a double strand break in the genome. After the cut the gene can then be repaired following two different pathways. Either through homology direct repair where a DNA template, homologous to the desired gene is used as a template to repair the break Or through non-homologous end joining. In this case of non-homologous end joining the cell repairs the double strand break without a template, and this typically causes an insertion or deletion of a few base-pairs, which if targeted correctly will cause the gene to be knocked out (Redman, 2016). Thanks to the easy customization, low cost and versatility of this technique CRIPSR/Cas9 has in recent years quickly superseded older methods for genomic modification (Woo 2015). These CRISPR-complexes are traditionally incorporated into the plant cell via *Agrobacterium tumefaciens*-mediated transformation or direct transfection with plasmids coding for the CRISPR-complex with binding sites for specific gRNA. There are more methods for transfection but they all share the same problem, these methods leave traceable foreign DNA segments in the plant genome. This would lead to the plant being classified as transgenic and therefore not allowed to be grown in some countries (Woo 2015). A newer method to get around this problem is transfection of prebuilt gRNA and Cas9 complexes, so called RNPs via polyethylene glycol (PEG) mediated transfection. With this method the RNPs can be inserted into the protoplast to cause a double strand break in the genome before it is broken down by the cells natural defences. Once broken down there is no trace of the RNP in the genome (Woo 2015) and the mutation cannot be distinguished from a random natural mutation. By using this method, modified crops could potentially be allowed to be grown in more countries around the world.

2. Project aim

The aim for this project is to learn how isolate intact protoplasts from *L. campestre* and regenerating them into plantlets. The aim is also to design a sgRNA targeting the *LcMYB28* gene.

3. Materials & Methods

3.1 Plant material

The seeds of *L. campestre* used for this study originated from accession no. NO94-7. The plants were propagated in the biotron and were originally collected in Öland by late professor Arnulf Merker.

3.2 Seed sterilization and germination

Seeds of *L. campestre* were shaken with 70% ethanol for 30s after which the seeds were sterilized for 20 min in 15% v/v calcium hypochlorite and a drop of tween 20 under gentle shaking. The seeds were then rinsed with sterile water and placed on growing medium (pH 5.7) containing ½ MS (Murashige, T. and F. Skoog 1962), 10 g L^{-1} sucrose and 7 g L^{-1} Bacto agar. Before being used for the protoplast extraction the seeds were grown for 3-4 weeks in a climate chamber with a temperature of 23 °C/18 °C (day/night) and a photoperiod of 16 h with a light intensity of 40 µmol m^{-2} s^{-1}.

3.3 Protoplast isolation and regeneration

Most of the protoplast isolations were carried out as practice to perfect the technique and to understand the protocol. These "test runs" where repeated once every week for a few months before transfections using plasmid DNA (pDNA) coding for green fluorescent protein (GFP) were added to the protocol. The protoplast isolation and regeneration were carried out according to the protocol by Sandgrind *et al.* (2021). In short, leaves of 3–4 week old *L. campestre* were cut up into thin strips using razor blades and placed in a enzymatic solution (0.4 M mannitol, 10 mM MES, 0.6 % (w/v) macerozyme R-10, 1.5 % (w/v) Cellulase R-10, 0.1 % (w/v) BSA, 1 mM $CaCl_2$*$2H_2O$ and 1 mM B-mercaptoethanol, pH 5.7) under gentle shaking in dark

for 14-16 hours. The enzymatic solution containing the leaf material was then filtered, washed and centrifuged with the addition of W5 solution (9 g L^{-1} NaCl, 18.4 g L^{-1} $CaCl_2*2H_2O$, 372.5 mg L^{-1} KCl, 426 mg L^{-1} MES, pH 5.7) in multiple steps to remove any unwanted material or chemical residue, leaving mostly intact protoplasts in the sample while the rest was discarded. The protoplast quality and concentration were then checked by loading 15 µl of the protoplast suspension on a hemocytometer and intact protoplasts were counted using a 20x bright field microscope to determine the concentration. The samples were then centrifuged, the supernatant removed and then the remaining pellet of protoplasts was diluted. Two concentrations were tested, 600 000 pp/ml and 750 000 pp/ml. For diluting the protoplasts, both 0.5 M mannitol and MMG solution (0.4 M Mannitol, 7.5 ml 2 M $MgCl_2*6H_2O$ L^{-1}, 4 mM MES, pH 5.7) was tested.

Alginate solution (2.8 % (w/v) sodium alginate, 0.4 M mannitol) was then added at an equivalent volume to the 750 000 pp/ml solution. After mixing, the solution was pipetted as 400 µl droplets onto ca-agar plates (10 g L^{-1} phyto agar, 72.88 g L^{-1} mannitol, 2,94 g L^{-1} $CaCL_2*2H_2O$) and left for 30 minutes to produce alginate disks. Superseding this, roughly 2 ml calcium solution (50 mM $CaCL_2*2H_2O$, 0.4 M mannitol) was added onto each disk and left for 60 minutes to complete the polymerization. These disks where then transferred to 6-well plate with 3 ml M1-media (2.18 g L^{-1} Nitsch medium inc. vitamins, 100 g L^{-1} mannitol, 10 g L^{-1} glucose, 10 g L^{-1} sucrose, 100 mg L^{-1} casein, 0.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} NAA, pH 5.7) in each well. These plates were covered with aluminum foil and kept in the climate chamber for 24 hours before replacing the aluminum foil with a twice folded fiber cloth. After 5 days the liquid media was changed to MII (2.18 g L^{-1} Nitsch medium inc. vitamins, 100 g L^{-1} mannitol, 10 g L^{-1} glucose, 10 g L^{-1} sucrose, 100 mg L^{-1} casein, 0.05 mg L^{-1} 2,4-D, 1.1 mg L^{-1} TDZ, pH 5.7) and this was repeated for 3 weeks before changing media every 7 days. After ~40 days the protoplast had clumped together and grown into microcallus that could be transferred to solid regeneration media (30 g L^{-1} sucrose, 0.5 mg L^{-1} $AgNO_3$, 1.1 mg L^{-1} TDZ, 2.5 g L^{-1} gelrite, 4.9 g L^{-1} MS + MES & Vitamins, pH 5.7). The cultures were subcultured to fresh media every third week until shoot formation starts.

3.4 Design of single guide RNA for RNP

To ensure that the right gene was used as well as enabling creating a suitable sgRNA for the CRISPR/Cas9 complex the *LcMYB28*-gene first had to be sequenced. This was done through genomic DNA-extraction from *in vitro* leaves of *L. campestre* using a NucleoSpin - Plant II kitfrom MACHEREY-NAGEL. The extracted DNA was then used to run multiple samples in a PCR with different combinations of

primers that were designed based on published *Lepidium campestre MYB28* (*LcMYB28*) genome sequence (Primers are listed in appendix 1). The samples were then run on gel electrophoresis through a 1% Agarose gel together with a ladder to determine the length of any possible successful PCR-runs and visualized with UV. After finding one successful combination and determining that it had a length matching the calculated length of MYB 28 the PCR-product was cleaned up using NucleoSpin - Gel and PCR Clean-up kit, (MACHEREY-NAGEL). The DNA was mixed with *LcMYB28*-specific primers and sent to Eurofins for Sanger sequencing. The results were then compared to the published *L. campestre* sequence for *MYB28*. Knowing the correct sequence was used, we could now compare the sequence to the cDNA of the *Arabidopsis thaliana MYB28 (AtMYB28)* sequence to roughly determine the locations of exons and introns of *LcMYB28*, this could then be used to design a few fitting single guide RNAs (sgRNAs) with as few mismatches as possible. These were designed using Geneious Prime[1] and then ordered from Thermo Fisher Scientific (list in appendix 2).

3.5 GFP-test using plasmid transfection

To test the protocol, check the quality of the protoplasts and to gain experience with the protocol a few PEG-mediated transfections were carried out using pDNA coding for a green fluorescent protein (GFP). Protoplasts were diluted to 600 000 or 750 000 Protoplasts(pps)/ml in 0.5M mannitol or MMG-solution (see Protoplast isolation 3.3). To a 2 ml Eppendorf tube 40 µg pDNA was added, to the same tube 200 µl protoplast solution was added and the total volume was then doubled by adding 25 % PEG-Ca^{2+} solution (25 % w/v PEG4000, 0.4 M mannitol, 0.1 M $CaCL_2$*$2H_2O$). The sample was then incubated in the dark for 5 minutes and W5 solution was then added to stop the reaction. Following this the samples were centrifuged and the supernatant removed to be exchanged by 1000 µl M1 medium. The whole volume was then transferred to a 12-well plate, covered with aluminum foil for 24 hours in a growth chamber before it was exchanged by a twice folded fiber-cloth. After a total of 48 hours a UV microscope was used to check for fluorescence indicating a production of GFP, indicating successful protoplast transfection. By counting or estimating the number of florescent protoplasts and dividing them by the total number of intact protoplasts a rough transfection efficiency could be calculated.

[1] Geneious Prime 2024.0.4 (https://www.geneious.com)

4. Results & Discussion

According to Reed & Bargmann (2021) protoplast isolation and regeneration are techniques that consumes time and require a lot of delicate work, knowledge, and specialized tissue culture expertise. The aim of this study was to learn the skills and knowledge required for protoplast isolation, regeneration and transfection. A secondary purpose of this study was also to create sgRNAs to be used with the Cas9 protein in order to knock out the *LcMYB28* gene. This would enable future work on the actual RNP-based transfection of *L. campestre.*

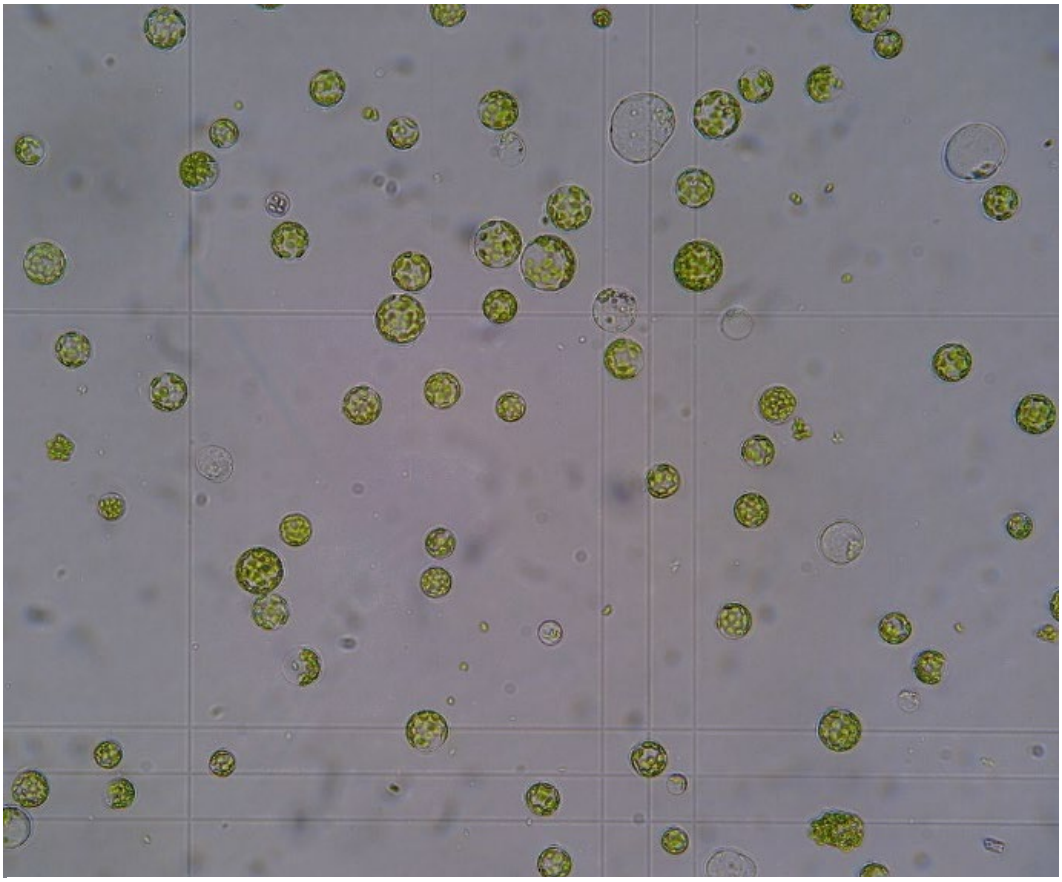

Figure 1. Intact protoplasts on a hemocytometer isolated from 4-week-old leaves of L. campestre. Intact protoplasts can be seen as spheres while raptured ones are empty or deformed.

4.1 Protoplast isolation and regeneration

As mentioned by Reed & Bargmann (2021) learning the techniques for protoplast isolation and regeneration take a long time to learn and are hard to master. This could clearly be seen in the results from the varying batches of isolated and regenerated protoplasts. Even though the first batch was successful in that protoplasts where isolated (fig 1) and managed to be regenerated into callus (fig 4) the regeneration efficiency was low. From 12 samples each containing roughly 120 000 pps only 17 micro calluses were regenerated. This was partially due to few micro calluses emerging from every sample but also the result of bacterial infections that ruined multiple samples. In subsequent batches a clear improvement could be noted in the number of calluses regenerated from each 200 µl sample. This can be credited to learning and getting an overall better understanding of the protocol and all steps involved, leading to faster and more efficient work together with better sterile technique.

The first step of the protocol as mentioned earlier is to cut leaves from 4 week old *L. campestre* seedlings and let them soak in enzymatic solution for 14-16 hours. On day 2 clearer indications of the result of the technique could be seen, mostly indicated by the level of broken protoplasts and debris that had to be washed away in the many steps of washing and centrifuging. Another factor to note is the number of protoplasts remaining in the end of the isolation step, this varied from 1.25*10^6 – 4.20*10^6 pps per batch. This generally increased over time with a few exceptions (not shown). As mentioned earlier, after the final centrifugation the protoplasts were diluted in 0.5 M mannitol or MMG solution, both were tested but no clear difference could be seen between the two solutions.

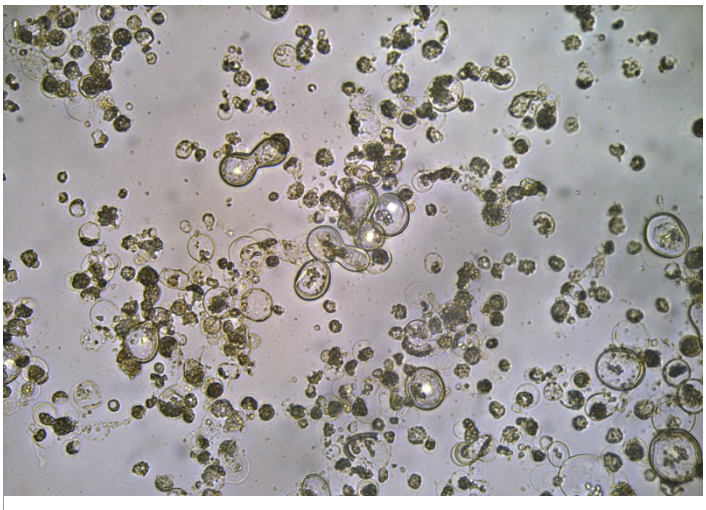

Figure 2. Protoplasts in liquid culture lumping together and starting to duplicate into micro-callus, photo taken 27 days after isolation

Four days after the protoplast isolation, the MI medium was replaced by liquid M11 medium, and if any contaminations had occurred during the isolation step it could generally be seen at this

stage. After roughly 16 days in M11 medium the protoplasts had started to grow into micro calluses that could be seen in the protoplast discs as small yellow dots. When cultured free-floating in a liquid medium these tiny microcalluses could be seen under a microscope (fig 2) These miccrocalluses then grew into bigger calluses and by roughly day 50 they were big enough to transfer to solid regeneration medium (fig 3). Once placed on solid regeneration medium the calluses started growing fast. According to the protocol shoots should begin to emerge after 30 days. But after 47 days after being placed on solid medium the first batch (fig 4) still showed no indications of shoot differentiation. If the project would have lasted longer, shoots would possibly had emerged, since the callus looked healthy.

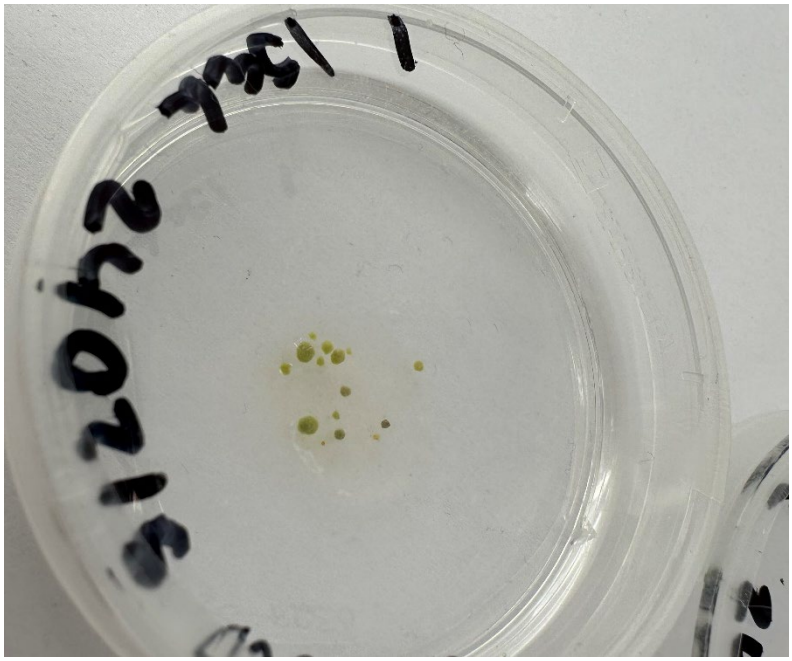

Figure 3. Microcallus in an alginate disk cultured in liquid medium MII.

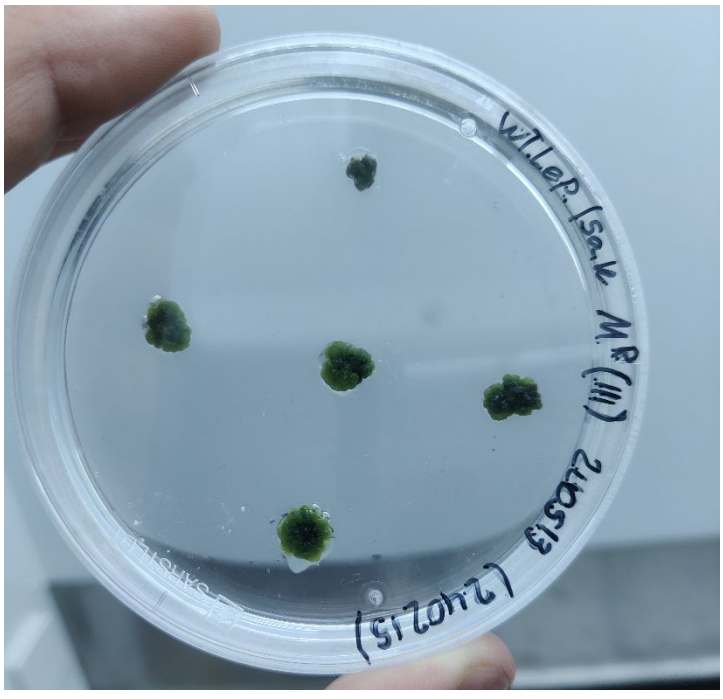

Figure 4. Callus 47 days after being moved to solid regeneration media. In total 95 days after isolation.

4.2 Transfection for GFP

As described, protoplast-quality was also checked by using protoplasts for PEG-mediated transfection with GFP-coding pDNA. Successful transfections requires pps of high quality, thus high transfection rate indicates high-quality protoplasts. The results of the first two attempts at this turned out to be non-conclusive, a few fluorescent intact protoplasts could be found (1-5 pps/1.5 µl sample, not shown) but mostly fluorescent debris and broken parts of protoplasts. After studying the samples in normal brightfield we found that almost no intact protoplasts remained in the samples. While trying to find a way to explain it we found that the protoplasts where tightly stuck to the bottom of the well where they had been resting for the last 48 hours, sadly these samples where in small 12-well plates so attempts to looking directly at them in the plate where futile due to the distance from the lens. Also, any attempts at moving the protoplasts resulted in them breaking.

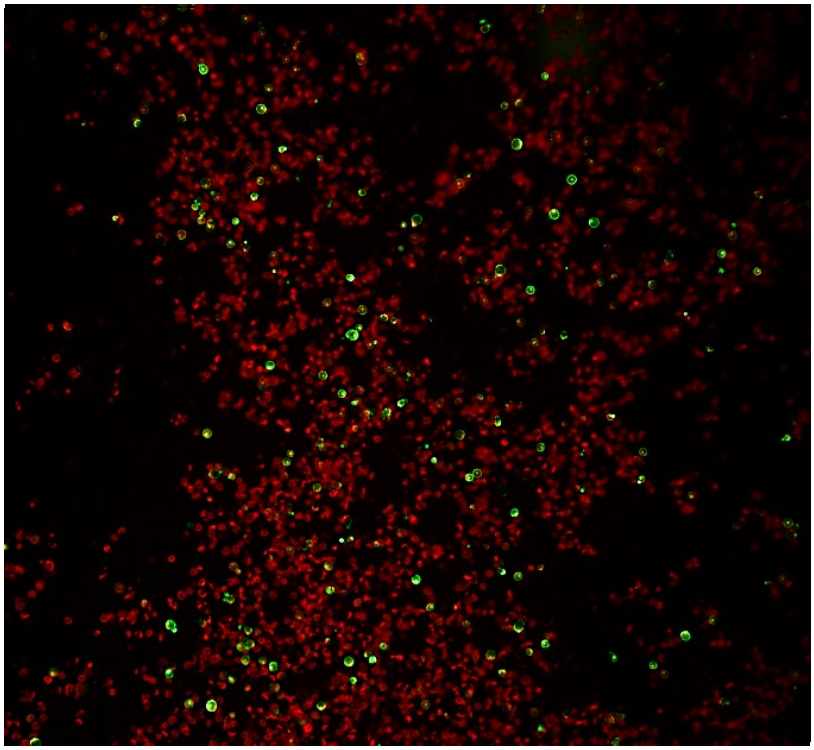

Figure 5. Picture of protoplasts transfected with GFP-coding plasmids 48 hours after transfection, successfully mutated protoplasts can be seen as green on a background of non-fluorescent protoplasts showing a red colour due to autofluorescence.

We therefore did another round of transfections with the aim of looking at them on the bottom of the Petri dishes without moving them, therefore 3.6 cm Petri dishes where used. For this round of transfections 3 different combinations of pDNA-amounts and protoplast concentrations were tested. The combinations tested where 600 000 pps/ml + 40 µg pDNA, 750 000 pps/ml + 40 µg pDNA and 750 000 pps/ml + 100 µg pDNA. These combinations where all tested with two different samples of pDNA, generating 6 different transfections in total. After 48 hours these samples were then studied under UV and clear fluorescence could be seen (fig 5). While the number of protoplasts that showed fluorescence was not the 60% wanted for successful transfections it was still higher than previous attempts. A clear visual difference could be seen between the different combinations of pps and pDNA amounts (not shown), from a visual assessment the samples containing 100 µg of

pDNA had higher fluorescence and amongst them the sample with 750 000 pps/ml had a higher number of fluorescent protoplasts than the samples containing 600 000 pps/ml. If this was due to there being more protoplasts in the sample or the efficiency being higher is impossible to tell without counting the efficiency in percent.

4.3 Creating the sgRNA

As mentioned earlier the first step in creating a sgRNA for the RNP complex was to confirm that we had the correct sequence for *LcMYB28*. To achieve this, we extracted DNA from *L. campestre* and designed 3 pairs of forward and reverse primers based on the published *LcMYB28* gene sequence. We used these to run multiple PCRs with different combinations of forwards and reverse primers. The results showed that primer combination 3 + 6 gave rise to a band matching the *LcMYB28* gene in length at roughly 1300 bp. To make sure this was the right gene the product was sent to Eurofins for Sanger sequencing. After the sequence returned (appendix 3) it was compared with the published *LcMYB28* sequence that the primers had been based on and showed an almost 100% match (appendix 4) except for a few uncertain reads from the sequencing. Using the cDNA sequence for published *AtMYB28,* the location of exons and introns in *LcMYB28* was estimated. Based on the location of the exons and using simulations from Biolib[2] to study the conserved domain, *LcMYB28* protein function could be gained, and Genious Prime [3]could then be used to find sgRNAs targeting the functional domain of the sequence. Due to many mismatches in the suggested sgRNAs we had to settle for three sgRNAs at 20 bp length, one of them targeting close to the functional domain and the second and third targeting exon two and three (see appendix 5). These can then be used as a mock transfection to remove a big part of the gene due to two sgRNAs targeting sequences early in the gene and the third targeting sequences in the back of the gene, leading to the functional gene mutating. The sgRNAs could also be used one at a time to determine their effectiveness on their own. Since this study did not conduct the actual transfection there is no way to tell if these sgRNAs will work as intended but for this project it counts as a success.

[2] The library of biological data science 2024.03.01 (https://dtu.biolib.com/DeepTMHMM)
[3] Geneious Prime 2024.0.4 (https://www.geneious.com)

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Appendix 1

Primers based on the suggested *LcMYB28*-gene used for PCR of the DNA extracted from our plant material.

Primer 1 F	TTCGGGTAAGATCCAAGAGCG
Primer 2 F	AGCGTTTCTCGATCAGTCTCA
Primer 3 F	ATGTCAAGAAAGCCATGTTGTGTC
Primer 4 R	TCAGATGAAATGCTTTTCGAGT
Primer 5 R	CGACTAGTAGTCTTGGTTACAGAAC
Primer 6 R	TGGGATCAATAACCATGAGATCTTG

Appendix 2

The three different sgRNAs that were designed to target the *LcMYB28*-gene.

Lc*MYB28*sgRNA1	TACATCCATGAGCACGGCGA	1.5 nmol
Lc*MYB28*sgRNA2	AAGAGGCGAGTTTAGTTCGG	1.5 nmol
Lc*MYB28*sgRNA3	TAGCAAAACATTTACCTCGA	1.5 nmol

Appendix 3

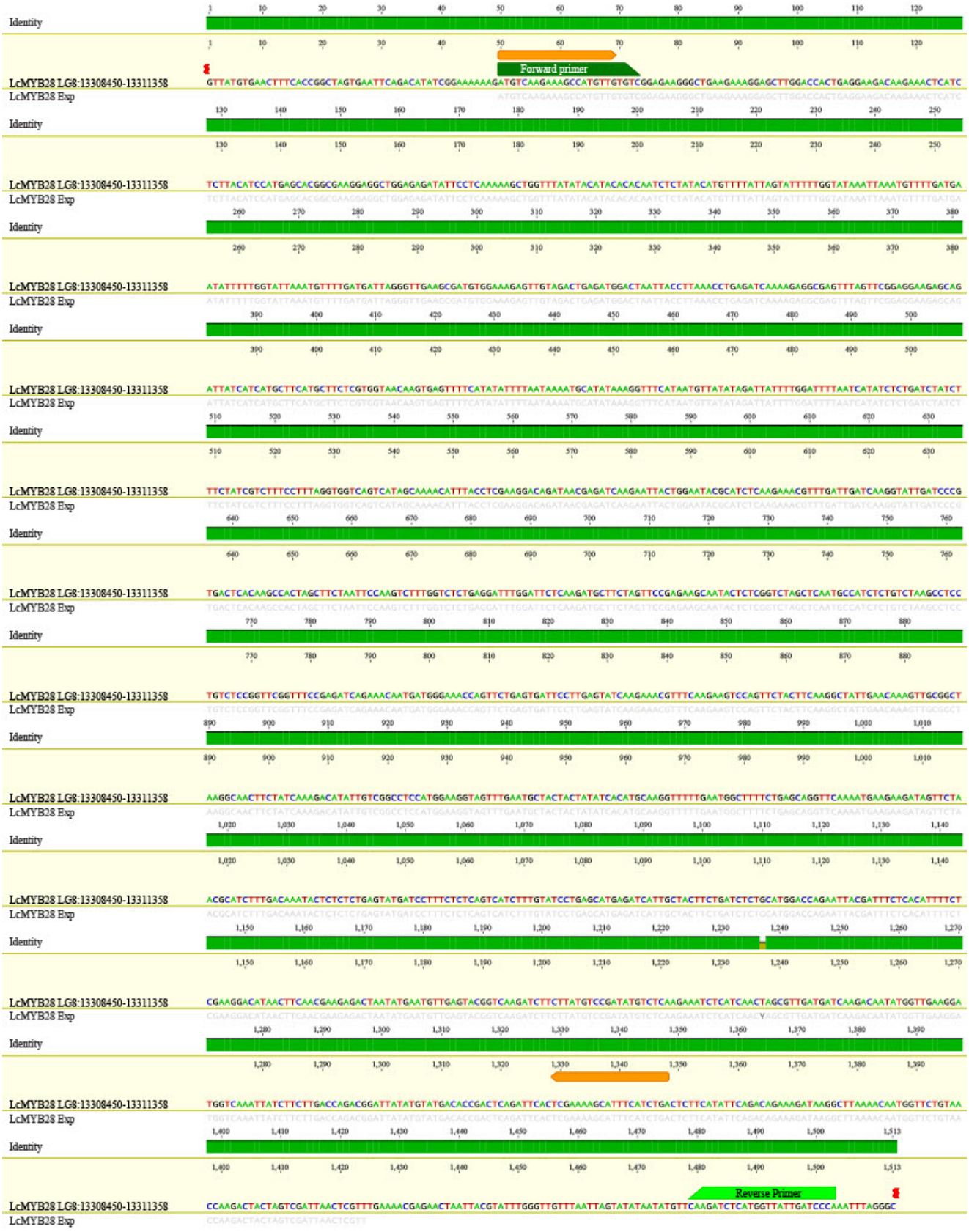
The *LcMYB28* sequence after combining the forward and backwards read from the Sanger sequencing.

ATGTCAAGAAAGCCATGTTGTGTCGGAGAAGGGCTGAAGAAAGGAGC
TTGGACCACTGAGGAAGACAAGAAACTCATCTCTTACATCCATGAGCA
CGGCGAAGGAGGCTGGAGAGATATTCCTCAAAAAGCTGGTTTATATAC
ATACACACAATCTCTATACATGTTTTATTAGTATTTTTGGTATAAATTA
AATGTTTTGATGAATATTTTTGGTATTAAATGTTTTGATGATTAGGGTT
GAAGCGATGTGGAAAGAGTTGTAGACTGAGATGGACTAATTACCTTA
AACCTGAGATCAAAAGAGGCGAGTTTAGTTCGGAGGAAGAGCAGATT
ATCATCATGCTTCATGCTTCTCGTGGTAACAAGTGAGTTTTCATATATT
TTAATAAAATGCATATAAAGGTTTCATAATGTTATATAGATTATTTTGG
ATTTTAATCATATCTCTGATCTATCTTTCTATCGTCTTTCCTTTAGGTGG
TCAGTCATAGCAAAACATTTACCTCGAAGGACAGATAACGAGATCAA
GAATTACTGGAATACGCATCTCAAGAAACGTTTGATTGATCAAGGTAT
TGATCCCGTGACTCACAAGCCACTAGCTTCTAATTCCAAGTCTTTGGTC
TCTGAGGATTTGGATTCTCAAGATGCTTCTAGTTCCGAGAAGCAATAC
TCTCGGTCTAGCTCAATGCCATCTCTGTCTAAGCCTCCTGTCTCCGGTT
CGGTTTCCGAGATCAGAAACAATGATGGGAAACCAGTTCTGAGTGATT
CCTTGAGTATCAAGAAACGTTTCAAGAAGTCCAGTTCTACTTCAAGGC
TATTGAACAAAGTTGCGGCTAAGGCAACTTCTATCAAAGACATATTGT
CGGCCTCCATGGAAGGTAGTTTGAATGCTACTACTATATCACATGCAA
GGTTTTTGAATGGCTTTTCTGAGCAGGTTCAAAATGAAGAAGATAGTT
CTAACGCATCTTTGACAAATACTCTCTCTGAGTATGATCCTTTCTCTCA
GTCATCTTTGTATCCTGAGCATGAGATCATTGCTACTTCTGATCTCTGC
ATGGACCAGAATTACGATTTCTCACATTTTCTCGAAGGACATAACTTC
AACGAAGAGACTAATATGAATGTTGAGTACGGTCAAGATCTTCTTATG
TCCGATATGTCTCAAGAAATCTCATCAACYAGCGTTGATGATCAAGAC
AATATGGTTGAAGGATGGTCAAATTATCTTCTTGACCAGACGGATTAT
ATGTATGACACCGACTCAGATTCACTCGAAAAGCATTTCATCTGACTC
TTCATATTCAGACAGAAAGATAAGGCTTAAAACAATGGTTCTGTAACC
AAGACTACTAGTCGATTAACTCGTT

Yellow highlighted: Prediction of the first 67 base pairs 5’ CDS with lower certainty due to the forward primer being located close to the start codon
Blue highlighted: 3’ UTR

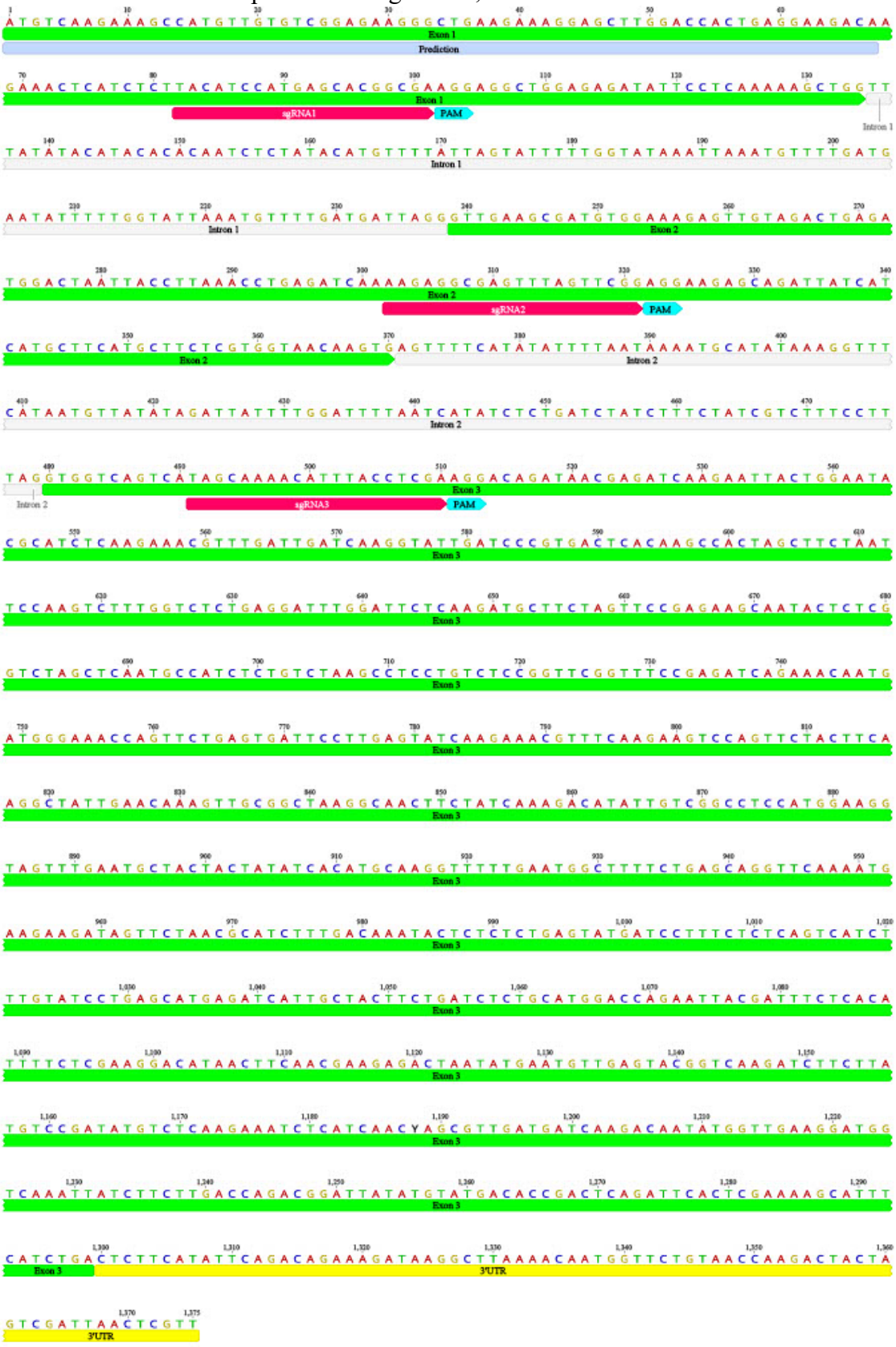
Appendix 4

Comparison between the sequence we got after sending our *LcMYB28* DNA for Sanger sequencing (top) and the published sequence we based the primers on (bottom). Primers 3 and 5 are marked out.



Appendix 5

The full *LcMYB28* nucleotide sequence from our material with exons and introns marked out as well as positions of sgRNA 1,2 and 3.



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