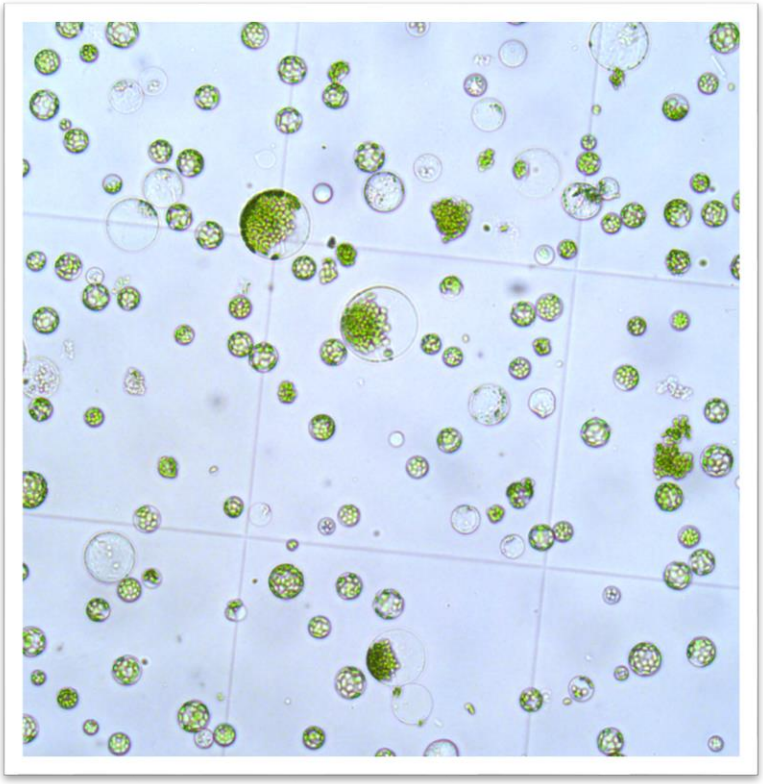
Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Landscape Architecture,
Horticulture and Crop Production Science

Optimization of protoplast methods suitable for transient CRISPR/Cas9 expression in *Lepidium campestre*

Louise Selga



Degree project • 30 hec
Alnarp 2017

Optimization of protoplast methods suitable for transient CRISPR/Cas9 expression in *Lepidium campestre*

Optimering av protoplastmetoder avsedda för transient CRISPR/Cas9-uttryck i *Lepidium campestre*

Louise Selga

Supervisor: Emelie Ivarson, SLU,
Department of Plant Breeding

Examiner: Li-Hua Zhu, SLU,
Department of Plant Breeding

Credits: 30 hec
Project level: A2E
Course title: Master's Thesis Project in Biology
Course code: EX0800
Subject: Biology

Place of publication: Alnarp
Year of publication: 2017
Cover art: Louise Selga
Online publication: http://stud.epsilon.slu.se

Keywords: Protoplasts, *Lepidium campestre*, CRISPR/Cas9

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Breeding

Acknowledgments

I thank my supervisor Emelie Ivarson for the help in this project. I am thankful to Annelie Ahlman for her lab work and guidance. I am grateful to Selvaraju Kanagarajan for providing the plasmid. I thank Li-Hua Zhu for her useful writing advice. Thanks to Mats Grahn for proofreading and to Marcus Hultgren for constant support. This project was supported by Mistra Biotech.

Summary

Lepidium campestre is a wild oil species with a number of traits that are beneficial from an agricultural point of view. CRISPR/Cas9 could be used transiently in protoplasts to accelerate domestication of *L. campestre*. In order for plants in Sweden to be classified as non-GMO they need to be modified without the addition of foreign DNA, therefore transient Cas9 expression is used. Since there are no protoplast methods optimized for *L. campestre* the aim of this project was to develop efficient methods for protoplast isolation, transfection and regeneration suitable for this species. Multiple isolation parameters were compared. When using higher enzyme concentrations significantly more protoplasts were obtained. No significant difference was found between gently shaking the leaves or keeping them stationary during the enzyme digestion. Cutting leaves with razorblades or scissors showed no significant difference in number of protoplasts isolated, and differences in regeneration capacity could not be evaluated due to infections. No significant difference was found when increasing the enzyme incubation time from 15 h to 18 h. Transfection was performed using the plasmid pEAQ-HT-GFP and the PEG incubation time was tested. Transfection was performed successfully using 25 % PEG4000 with incubation times 5 min and 10 min. Two regeneration methods were performed and differences in infection frequency and microcalli production were observed. Method 3.B suffered more infections, possibly due to a higher sensitivity or a contaminated solution. Microcalli were obtained from one plate, which was regenerated according to method 3.A. This shows that regeneration of the protoplasts is possible, and supports further optimization of method 3.A.

Sammanfattning

Lepidium campestre är en vild oljeväxt med ett antal egenskaper som är fördelaktiga inom jordbruk. Domesticering av denna växt kan delvis utföras genom transient uttryck av CRISPR/Cas9 i protoplaster. För att växter i Sverige inte ska bli GMO-klassificerade krävs det

att de blir modifierade utan att främmande DNA tillförs, så därför används transient Cas9-uttryck. Eftersom det inte finns protoplastmetoder anpassade för *L. campestre* så är syftet för detta projekt att utveckla metoder för isolering, transfektion och regenerering av protoplaster lämpliga för denna art. Ett flertal parametrar för protoplastisolering jämfördes och modifierades. När enzymkoncentrationen höjdes isolerades signifikant fler protoplaster. Ingen signifikant skillnad upptäcktes mellan att försiktigt skaka bladen eller att hålla dem stilla under enzymbehandlingen. Att skära bladen med rakblad eller sax gav ingen signifikant skillnad i antal protoplaster isolerade, och eventuella skillnader i regenereringsförmåga kunde ej undersökas på grund av infektioner. Ingen signifikant skillnad hittades mellan antalet protoplaster isolerade efter 15 respektive 18 h enzyminkubering. Transfektionen genomfördes med plasmiden pEAQ-HT-GFP och inkubationstiden i PEG testades. Lyckade transfektioner genomfördes med inkubationstider 5 och 10 min i 25 % PEG4000. Två regenereringsmetoder jämfördes och skillnader i infektionsfrekvens och mikrocalliproduktion noterades. Metod 3.B fick fler infektioner, vilket kan bero på en högre infektionsrisk eller användning av en kontaminerad lösning. Mikrocalli producerades på en platta, som regenererades med metod 3.A. Detta visar att regenerering av protoplasterna är möjlig, och stödjer användning av metod 3.A.

Contents

1 Introduction 1

1.1 *Lepidium campestre* 1

1.2 The CRISPR/Cas9 system 1

1.3 GM crop legislation 2

1.4 Protoplast isolation, transfection and regeneration 4

2 Materials and methods 4

2.1 Plant material 4

2.2 Protocols 5

2.2.1 Protoplast isolation 6

2.2.2 Protoplast transfection 7

2.2.3 Protoplast regeneration 7

2.3 Cutting method 8

2.4 Shaking during enzyme treatment 8

2.5 Method reproducibility 8

2.6 Comparison of enzyme amounts 9

2.7 Isolation incubation time 9

2.8 Transfection incubation time 9

2.9 Statistics 10

3 Results and discussion 10

3.1 Protoplast isolation 10

3.1.1 Cutting method 10

3.1.2 Shaking during enzyme treatment 11

3.1.3 Method reproducibility 11

3.1.3.1 Isolation method 1.A 12

3.1.3.2 Isolation method 1.B 13

3.1.4 Comparison of enzyme amounts 13

3.1.5 Incubation time 15
3.2 Protoplast transfection 15
3.3 Protoplast regeneration 17
3.3.1 Bacterial infections 17
3.3.2 Purple protoplasts 18
3.3.3 Clusters 19
3.3.4 Microcalli 20
4 Conclusions 21
5 References 23
Appendix A. Medias 26
A.1 Medias used in methods 1.A and 2.A 26
A.2 Medias used in methods 1.B and 3.A 27
A.3 Medias used in method 3.B 28
Appendix B. Images 29
B.1 Sample heterogeneity 29
B.2 Enzyme amounts 30

1 Introduction

1.1 Lepidium campestre

Lepidium campestre is a wild species that has multiple potentially beneficial agricultural traits. It grows upright with branching limited to the upper part of the stem, and the seed yield is high but the seeds are prone to shattering (Merker et al., 2010). It is resistant to pollen beetles (Merker & Nilsson, 1995), which are causing big pest problems for oilseed rape in Denmark and southern Sweden (Hansen, 2003). It has a good winter hardiness, which allows it to be cultivated in colder regions than oilseed rape (Merker & Nilsson, 1995). *L. campestre* is biennial which allows it to be cultivated as a catch crop. A catch crop is undersown in an annual crop, and overwinters after the annual crop is harvested. This reduces tilling and thus saves energy and reduces leaching of nutrients (Merker et al., 2010). When grown in combination with barley, *L. campestre* caused a positive effect on the barley seed yield (Merker et al., 2010).

For *L. campestre* to be commercially viable it needs to be domesticated. The oil content in the wild type is low, around 20 % (Ivarson et al., 2017), and the oil composition makes the oil unsuitable for human consumption and prone to oxidation (Ivarson et al., 2016). Seed shattering resistance is another trait that needs to be obtained during domestication (Merker & Nilsson, 1995). Since the genetic variation for oil content is low within *L. campestre*, traditional domestication through intraspecific crossing is an inefficient method for increasing it, and thus genetic engineering is a better alternative (Ivarson et al., 2017). The fatty acid composition has been modified by silencing the two genes *fatty acid elongase 1* (*FAE1*) and *fatty acid desaturase 2 (FAD2*) (Ivarson et al., 2016). Additionally, the oil content was increased by introducing the genes *WRINKLED1* or hemoglobin (*Hb*) from *Arabidopsis thaliana* and *Beta vulgaris* and overexpressing them. Introduction of *A. thaliana* hemoglobin (*AtHb2*) gave the highest oil content increase, 29.9 % (Ivarson et al., 2017).

1.2 The CRISPR/Cas9 system

The domestication of *L. campestre* could partly be facilitated by using the site-directed mutagenesis technique CRISPR (clustered regularly interspaced short palindromic repeats). CRISPR/Cas9 is a new method for genetic engineering which allows for precise editing of genomes. The technique consists of two simple parts: a CRISPR associated protein 9 (Cas9) and a guide RNA (gRNA) sequence designed to match the targeted genome site (Ran et al., 2013). This makes the method comparably simple to use.

Cas9 and other site-specific nucleases (SSNs) such as Transcription activator-like effector nucleases (TALENs) and Zinc finger proteins (ZFNs), act by introducing a double-stranded break (DSB) at the targeted genome site. SSNs then make use of the cell's own mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ acts by re-joining the ends using DNA ligase IV, and if bases have been deleted or modified by nucleases these changes are incorporated into the genome, often resulting in gene silencing. HDR uses a repair template most likely leading to repairs without errors. Under natural conditions the sister chromatid acts as the repair template, but a template encoding desired mutations or insertions can be delivered together with the SSN. HDR is less favored than NHEJ in differentiated cells (Belhaj et al., 2015).

The gRNA directs Cas9 to introduce the DSB at the chosen location in the genome. The gRNA is composed of two parts: a "scaffold" sequence in the 3' end allowing the RNA to bind to Cas9, and a 20-nucleotide long "spacer" sequence in the 5' end matching the target sequence (see Figure 1) which can Watson-Crick-base pair with the target sequence. For Cas9 to introduce a DSB, the 3' end of the genome target sequence must be adjacent to a PAM (Protospacer Adjacent Motif) site. PAM sequences vary depending on which ortholog of Cas9 is used. For the most commonly used *Streptococcus pyogenes*-derived ortholog the PAM is NGG, where "N" is any nucleotide. The DSB is introduced 3 bp upstream of the PAM site, thus the presence of a PAM site adjacent to the DSB target is a prerequisite. However, since NGG is such a short sequence it is also statistically very commonly found in genomes (Ran et al., 2013).

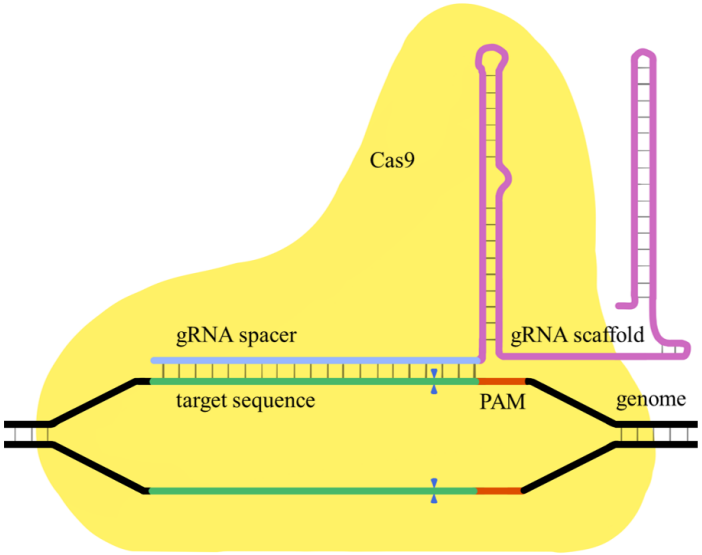


Figure 1: Schematic picture of Cas9 in action. The gRNA binds to Cas9 (yellow) through the scaffold (purple). Genomic DNA (black) is opened by Cas9 and the target sequence (green) base pairs with the spacer sequence (blue). The target sequence is cut (blue triangles) 3 bp upstream of the PAM (red).

1.3 GM crop legislation

According to Jordbruksverket (2015) the current EU legislation concerning GMOs (genetically modified organisms) is outdated and difficult to interpret. In the EU legislation, GMOs are

defined as “organisms in which the genetic material *has been altered* in a way that does not occur naturally by mating and/or natural recombination” (Plan & Van den Eede, 2010). This means that the legislation judges based on the method used to produce an organism, not on the end product.

The regulations on GM crops make it challenging to obtain a permit to grow them, and multinational companies are much better equipped with the regulatory skill sets compared to smaller companies and research institutions (Huesing et al., 2016). This leads to most GM crops being developed by multinational companies (Parisi et al., 2016), and opposes development of minor crops (Huesing et al., 2016). Developing countries are also disproportionately affected due to a lower immediate availability to resources and expertise (Huesing et al., 2016). Most research in GM crops ends without commercial development of the species due to the time and expenses required for deregulation of the crop (Huesing et al., 2016).

Due to NHEJ being the more commonly occurring DNA repair mechanism (Belhaj et al., 2015), CRISPR/Cas9 is most suited to be used to introduce point mutations in a chosen location (Svitashev et al., 2015). Point mutations commonly appear in random locations under natural conditions and can be introduced quickly through radiation. Since one cannot distinguish if a point mutation was introduced using CRISPR/Cas9 or if it appeared naturally, the Swedish Board of Agriculture has decided that if CRISPR/Cas9 is used to modify an organism without inserting foreign DNA to the final product, it will not be classified as a GMO (Jordbruksverket, 2015). Plants fulfilling this can therefore be grown without a permit. This reduces costs and time and simplifies the process, which promotes research (Klarin, 2015). This classification applies until the EU introduces new guidelines regarding GMOs (Jordbruksverket, 2015). Political and public acceptance of GMOs is low, which in addition to regulations prevents GM crops from being utilized to their full potential (Holme et al., 2013). The main concern regarding GMOs is the concept of adding foreign genetic material to a species that cannot obtain this genetic material naturally (Holme et al., 2013). This speaks in favor of not considering gene silencing as genetic modification.

In the case of *L. campestre* this means that introducing *AtHb2* through the HDR pathway would result in a GMO, but silencing *FAE1* and *FAD2* through the NHEJ pathway would not. The gene encoding Cas9 is also foreign DNA and thus it should not remain in the final product. *Agrobacterium* that is commonly used to introduce DNA in plants can therefore not be used, since it is a stable transformation that leaves transfer-DNA in the plant genome (Taiz & Zeiger, 2015). Expressing Cas9 and the gRNA transiently (temporarily) would prevent plasmid DNA

from remaining in the final product, and usage of protoplasts could enable the uptake of such a plasmid (Zhang et al., 2016).

1.4 Protoplast isolation, transfection and regeneration

A protoplast is a cell that has had its cell wall removed (Davey et al., 2005). Protoplast isolation is the act of removing the cell wall and to purify intact protoplasts, removing cell wall debris and damaged protoplasts (Yoo et al., 2007). The cell walls of plants are mainly composed of polysaccharides, namely celluloses, pectins and hemicelluloses (Taiz & Zeiger, 2015). An efficient way to remove the cell wall is thus to dissolve it using enzymes that break down these polysaccharides (Davey et al., 2005).

During transfection the protoplasts are treated to facilitate the uptake of a plasmid. The cell membranes of the protoplasts are hydrophobic and DNA is negatively charged, so polyethylene glycol (PEG) is used to facilitate its uptake. The mechanism of PEG-meditated plasmid uptake is not completely known, but when considering the hydrophobicity of the involved components the process can be interpreted as following: PEG is amphiphilic so its hydrophilic segments can bind to the negatively charged backbone of DNA. An amphiphilic DNA-PEG-complex may form, where the negative charges of DNA are not situated on the surface. This allows the complex to diffuse through the hydrophobic cell membrane.

During the regeneration of the protoplasts they form colonies, regenerate their cell walls and develop microcalli (cell collections that can be seen with the naked eye), and calli, from which shoots can finally be regenerated (Assani et al., 2006). The efficiency of a protocol can vary strongly depending on species or genotype. When Hu et al. (1999) applied the same regeneration protocol on seventeen different genotypes of oilseed rape the calli regeneration rates varied from 1– 24 %, and some genotypes did not regenerate at all.

The aim of this project was to develop efficient methods for protoplast isolation, transfection and regeneration suitable for *L. campestre*, so that genome editing with CRISPR/Cas9 without introducing transfer DNA is made possible in this species.

2 Materials and methods

2.1 Plant material

Different plant parts can be used as source material when producing protoplasts (Wang et al., 2005), and in this project leaves and cotyledons were used. Leaves make a good source since a

small amount can be used to produce a large amount of protoplasts and the donor plant can be kept intact (Wang et al., 2005).

Seeds from *L. campestre* were surface-sterilized with 70 % ethanol for 10 s, then shaken in 15 % calcium hypochlorite for 20 min. The seeds were thoroughly rinsed in sterile water and germinated *in vitro* on medium as described by Ivarson et al. (2013). They were then allowed to grow for 3 weeks in a growth chamber with 16 h day length at light intensity 33 μmol m^{-2} s^{-1} and temperatures 21 °C (day) and 18 °C (night) before protoplast isolation.

2.2 Protocols

Multiple protoplast protocols were tested and modified to determine which functioned best when applied on *L. campestre*. Functionality was determined from yield of protoplasts and microcalli, reproducibility and difficulty of lab work. Brief summaries of the examined protocols outlining their differences can be seen in Table 1 (isolation), Table 2 (transfection) and in Table 3 (regeneration).

*Table 1: Summaries of the isolation protocols examined. Onkokesung, unpublished, adapted from Yoo et al (2007). *The enzyme amounts are described in Table 5.*

Method	Isolation protocol	Species developed for	Cutting method	Enzyme levels*	Shaking during incubation	Incubation time	Purification step
1.A	Onkoke-sung (unpub-lished)	Potato	Razor blade	High (0.6 - 1.5 %)	Yes	15 h	Precipitation on ice
1.B	Chupeau et al. (2013)	*A. thaliana*	Not specified	Low (0.03 - 0.1 %)	No	Not specified	None
1.C	Nicolia et al. (2015)	Potato	Razor blade	Moderate (0.2 - 1 %)	No	14 h	Centrifugation on sucrose interface

Table 2: Summaries of the transfection protocols examined. Onkokesung, unpublished, adapted from Yoo et al (2007).

Method	Transfection protocol	Species developed for	PEG concentration	Incubation time
	Nicolia et al. (2015)	Potato	12.5 %	3 min
2.A	Onkokesung (unpublished)	Potato	50 %	20 min

Table 3: Summaries of the regeneration protocols used. pp refers to protoplasts.

Method	Regeneration protocol	Species developed for	Regeneration phase	Protoplast concentration	Change of medium
3.A	Chupeau et al. (2013)	*A. thaliana*	Liquid	80 000 pp/ml	After 11 days and after 1 month
3.B	Siemens et al. (1993)	*A. thaliana*	Alginate embedded pp, liquid media	1 000 000 pp/ml	Every 10 days

2.2.1 Protoplast isolation

The following enzymes were used for protoplast isolation: Cellulases RS and R10, Macerozyme R10 (a pectinase) and driselase (a mixture of cell wall degrading enzymes) (Creative-enzymes.com, 2017). Cellulase RS is a mutated version of Cellulase R10 that has a higher activity and can dissolve cell walls from a wider range of plants (Duchefa-biochemie.com, 2017).

Isolation methods 1.A and 1.B were tested. Isolation method 1.A was performed as described by Onkokesung (unpublished) and method 1.B according to Chupeau et al. (2013), unless modifications are stated. Protoplast concentration was determined by counting the protoplasts using Leica DM LB microscope. Pictures were taken using Leica Application Suite V4.0.

Method 1.A was performed as follows: leaves were cut in plasmolysis (Appendix A.1) using autoclaved razorblades. Plasmolysis was replaced with enzymatic solution (Appendix A.1). Leaves were gently shaken for 15 h. W5 (Appendix A.1) was added and leaves were gently shaken for 10 min. Mixture was filtered through a 40 μm cell strainer into a 50 ml test tube. Mixture was washed 3 times using 100g centrifugation. Protoplasts were incubated on ice for 30 min, causing precipitation of intact protoplasts. The pellet was resuspended in a specified volume. An aliquot was taken for protoplast counting in a counting chamber using the equation below. Remaining protoplasts were kept on ice.

$$pp = \overline{pp} \cdot 5000\, ml^{-1} \cdot V$$

Where pp refers to the total number of protoplasts, $\overline{pp}$ refers to the average number of protoplasts in a square, 5000 ml^{-1} refers to the volume of a square and V is the batch volume.

Method 1.B was performed as follows: aerial plant parts were chopped in MMG (maceration-glycine-glucose, Appendix A.2) and incubated overnight. Wash solution (Appendix A.2) was added to a 30 ml glass tube and the mixture was filtered into it through an 80 µm mesh filter. Mixture was washed 3 times using 70g centrifugation. An aliquot was taken before the last wash, from which the concentration was determined using equation 1. Protoplasts were kept at 4 °C for 1 h during counting. As Chupeau et al. (2013) does not state an incubation time, 15 h were chosen. Modifications of method 1.B from Chupeau et al. (2013) were as followed: a 40 µm cell strainer was used instead of an 80 µm and the washing took place in a conical 50 ml plastic test tube instead of a 30 ml glass tube.

2.2.2 Protoplast transfection

The marker gene GFP was used in this project to achieve quick results. The plasmid pEAQ-HT-GFP was used. It is approximately 4600 bp in size and enables expression of GFP through the constitutively expressed promoter CaMV 35S (Sainsbury et al., 2009). GFP enables quick determination of whether transfection has occurred or not.

Transfection method 2.A was done according to Onkokesung (unpublished): $4 \cdot 10^5$ protoplasts were mixed with 5-10 µg plasmid. PEG solution (Appendix A.1) was added at a 1:1 ratio. Mixture was incubated for 20 min. Solution was mixed with 1:2 W5 (Appendix A.1). Protoplasts were harvested though 100g centrifugation. Fluorescence was checked after two days. 25 % PEG was used instead of 50 %.

2.2.3 Protoplast regeneration

Regeneration method 3.A was performed according to Chupeau et al. (2013) and method 3.B according to Siemens et al. (1993). Both methods were tested repeatedly to test their regeneration capacity and to identify any problems, with the goal of finding which method is superior for *L. campestre*. Regeneration was also performed on protoplasts isolated with modified isolation methods. This was done with the ambition of identifying if the method of isolation affects the regeneration capacity. Method 3.A was done as follows: Protoplasts were suspended in PIM (protoplast induction medium, Appendix A.2) at $8 \cdot 10^4$ pp/ml. After 11 days PIM was diluted 1:2 with CIM1 (colony induction medium 1, Appendix A.2). 30 days after this the mixture was diluted 1:4 with CIM2 (colony induction medium 2, Appendix A.2). Fe EDTA was used instead of Fe Citrate NH_4 in PIM, CIM1 and CIM2.

Method 3.B was performed as follows: protoplasts ware suspended at 10^6 pp/ml in alginate-mannitol (Appendix A.3). Mixture was placed on calcium agar, forming 2-3 cm discs, and left for 30 min. Polymerization was promoted by adding calcium solution (Appendix A.3) and incubated for 1 h. Discs were then moved to calcium-mannitol solution (Appendix A.3) and kept at 4 °C for 2 days. Solution was replaced with MI (Appendix A.3), which was changed every 10 days. MI was replaced by MII (Appendix A.3) after 30-35 days. Due to the high viscosity of the alginate solution the alginate discs produced had a diameter of 1-2 cm instead of 2-3 cm. The regeneration process was evaluated by using the Leica M 165 FC microscope. Pictures were taken using Leica Application Suite V4.0. Due to a mistake when using this program these pictures do not have scale bars.

2.3 Cutting method

Due to the high air flow in sterile work benches plant material dries out rapidly, which means that cutting the leaves for protoplast isolation needs to be carried out quickly. This was difficult to do with a razorblade, so cutting the leaves with scissors was tested instead. Cutting with scissors was much faster and produced more cuts, but it also lead to more crushed tissue.

The isolation was done according to method 1.B with the razorblade method described by Onkokesung (unpublished). Cutting with razorblades was performed on an autoclaved glass sheet wetted with plasmolysis solution. Cutting with scissors was done in a plastic petri dish containing MGG. Produced protoplasts underwent regeneration method 3.A, but was discontinued due to infections.

2.4 Shaking during enzyme treatment

In method 1.A the plant material is gently shaken during the enzyme treatment while in method 1.B and 1.C it is kept stationary. To see if one method is superior to the others in this regard both were tested. Isolation was done according to method 1.B and the leaves were cut with scissors. The plant material was either gently shaken or left stationary during the enzyme incubation.

2.5 Method reproducibility

It is important that a protocol is reliable and provides reproducible results, thus methods 1.A and 1.B were tested repeatedly. Variations in the results will occur to any protocol due to variations in the plant material and mainly due to experimental errors, but different protocols

can differ in sensitivity to such variations. The aim is therefore to determine a protocol that is robust enough to withstand some degree of variation.

Isolation methods 1.A and 1.B were performed repeatedly as described under section 2.2.1 *Protoplast isolation*, except with all leaves cut using scissors. Method 1.A was performed 11 times under these conditions, and method 1.B was performed 5 times. The total mean value and total standard deviation is calculated in Excel using all the data. The coefficient of variation is calculated by dividing the standard deviation with the mean value.

2.6 Comparison of enzyme amounts

Methods 1.A and 1.B use very different enzyme amounts, see Table 5. To explore what enzyme composition is most suitable for *L. campestre* the enzyme amounts used in methods 1.A, 1.B and 1.C were tested under otherwise identical conditions.

Three enzymatic solutions (Appendix A.1) were prepared using the enzyme amounts shown in Table 5. Isolation method 1.A was performed using the three enzyme solutions. Two replicates were done for each enzyme amount shown in Table 5.

Table 5: Proteins used during protoplast isolation in the different methods. Three enzymatic solutions were prepared using the enzyme amounts corresponding to each method.

Enzymes	Method 1.A	Method 1.B	Method 1.C
Cellulase	1.5 % RS	0.1 % R10	1 % RS
Macerozyme R10	0.6 %	0.03 %	0.2 %
Driselase	-	0.04 %	-
BSA	0.1 %	-	-

2.7 Isolation incubation time

Method 1.A instructs enzyme incubation times between 14-16 h, with 15 h being optimal, while method 1.B simply instructs that the incubation should occur over night. To explore the effects of different incubation times and to see if a long incubation time has any negative effects, two different incubation times were tested. 15 h was chosen since it is used in method 1.A. 18 h was also chosen, since this three-hour long over digestion should provide unambiguous results. Isolation was performed according to method 1.B using incubation times 15 h and 18 h. The test was later performed using method 1.A again using incubation times 15 h and 18 h.

2.8 Transfection incubation time

Transfection method 2.A was performed. The protoplasts used were produced using method 1.A with scissors. The plasmid pEAQ-HT-GFP was used. PEG incubation times 5 min and 10

min were tested. Protoplasts were examined two days after transfection using a Leica M 165 FC microscope equipped with PRIOR Lumen 200 Fluorescence Illumination. Pictures were taken using Leica Application Suite V4.0.

2.9 Statistics

The number of protoplasts isolated when varying the isolation methods were statistically analysed in Excel using one-way ANOVAs. Results were considered significant if $p > 0.05$. Two-tailed t-tests were also performed on the comparison of enzyme amounts. The number of replicates of a test (n) was determined by the amount of plant material available. Reproducibility might be low due to limited amounts of replicates used.

3 Results and discussion

3.1 Protoplast isolation

3.1.1 Cutting method

The method for cutting leaves was compared (Figure 2). Cutting with razorblade or scissors gave no significant difference in number of intact protoplasts isolated. The large standard deviation for scissors is caused by 0.925 million and 0.235 million protoplasts being isolated, and due to there being only two data points an outlier could not be determined. Preferably more than two replicates would have been tested, but the amount of plant material available prevented this.

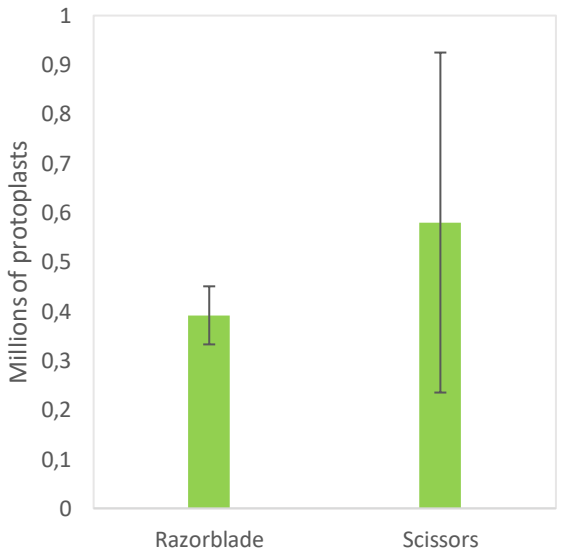


Figure 2: Number of protoplasts isolated when cutting the leaves with razorblades or scissors. Isolation method 1.B was used. n=3 for razorblades, n=2 for scissors. p=0.53.

Since no significant difference was found between using a razorblade or scissors, the easiest method was chosen. Using scissors is quicker, easier and prevents the plant material from drying. Scissors produce more crushed tissue, and it is stated by Nicolia et al. (2015) that crushed tissue is a poor protoplast source. The number of broken protoplasts in the final step appeared to be roughly the same, so the crushed tissue produced by the scissors does not seem to affect this. The microcalli obtained were produced from protoplasts isolated using scissors, which shows that regeneration is possible when using scissors. Regenerations of protoplasts

isolated using razorblades were discontinued due to infections, so it is unknown how well these protoplasts regenerate. Thus a comparison of regeneration capacity of protoplasts isolated using scissors and razorblades would be desired.

3.1.2 Shaking during enzyme treatment

The number of intact protoplasts isolated when gently shaking the leaves or leaving them stationary during the overnight enzyme treatment can be seen in Figure 3. There is no significant difference between the number of protoplasts isolated.

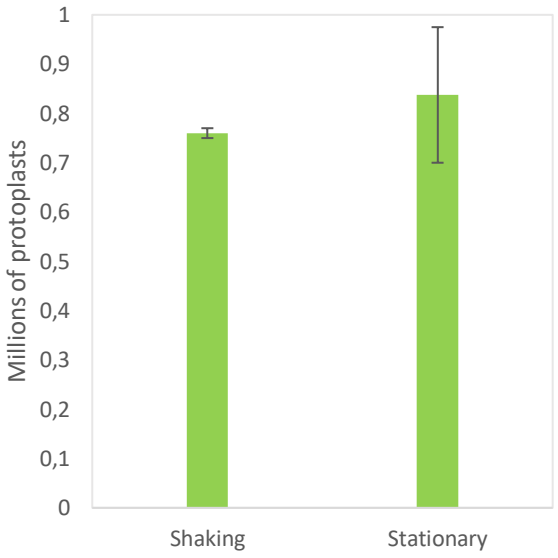


Figure 3: Number of protoplasts isolated when shaking or keeping the leaves stationary during maceration. n=2 for both data series. p=0.63.

Shaking may help release the protoplasts from surrounding cell walls and tissue, but it might also harm the protoplasts. The harming could lead to an increased number of broken protoplasts, which may affect the regeneration capacity. No significant difference in the number of protoplasts isolated was found when comparing gentle shaking to stationary state during the enzyme treatment, and the number of broken protoplasts appeared to be roughly the same. It was therefore decided that the protocols should not be altered on this matter, i.e. gentle shaking was performed when using method 1.A and stationary incubation when performing method 1.B.

3.1.3 Method reproducibility

During this project a lack of homogeneity was observed in the aliquots used to determine the protoplast concentration. A photograph of samples (10 µl) taken from the same aliquot (50 µl) can be seen in Appendix B.1.

A source of replicate variation is heterogeneity in the aliquots used to determine protoplast concentrations. This causes the concentration determined to not be representative of the actual concentration. Sedimentation in the aliquot becomes highly visible after circa 15 min if the protoplast concentration in the sample is high, and visible sedimentation encourages mixing to be performed until sediment is no longer visible. However, the speed of sedimentation is the same regardless of the concentration, so the concentration is equally distorted for lower

concentration aliquots. This may lead to lower concentration batches not being as homogenized since mixing may be less thoroughly performed when the sample already looks homogenous to the eye. It may also cause the first samples placed on the microscope slide having inaccurate concentrations, since no visible sediment has had time to form but enough time has passed from the aliquot being taken for significant sedimentation to have occurred.

Heterogeneity problems should mainly affect the variation between replicates, and affects the different protocols equally. The problem can be minimized by mixing each batch thoroughly immediately before taking an aliquot, and taking the aliquot from the middle of the tube. Aliquots should always be mixed immediately prior to counting by slowly pipetting up and down multiple times at a relatively high volume. It should be kept in mind while mixing that protoplasts are fragile.

3.1.3.1 Isolation method 1.A

The data in Table 6 was all obtained under the same parameters: method 1.A using scissors. Despite this, the mean values for the batches ranged from 0.37 to 9.93. And yet the standard deviation remained low between replicates. This resulted in a large difference between the total standard deviation and the standard deviations for individual batches, and a larger total coefficient of variation compared to the individual coefficients of variation.

Table 6: Number of protoplasts isolated with method 1.A using scissors and a 15 h incubation time.

Replicate batch	a	b	c	d	e	f	g	h	i	j	k
Number of replicates	6	6	3	5	1	2	2	2	3	3	2
Mean value	4.65	5.29	0.37	0.85	1.46	5.49	2.88	9.93	7.67	5.08	8.61
Standard deviation	0.95	0.79	0.19	0.33	-	0.56	0.65	0.32	0.74	0.67	0.28
Coefficient of variation	0.20	0.15	0.50	0.39	-	0.10	0.23	0.03	0.10	0.13	0.03
Total mean value	4.53										
Total standard deviation	2.81										
Coefficient of variation	0.62										

These results points towards the method being robust. The number of produced protoplasts varied a lot between the batches, but not between replicates. This implies that handling errors have a relatively low impact on the number of protoplasts isolated with this method, which is practical. Factors causing variations between batches could be caused by variances in the plant material, human error or differences in the enzyme solution, which is prepared fresh for each batch. Changes over time in the solutions that are stored seems unlikely due to the simple compounds used, and deterioration would most likely manifest as precipitation which would be

immediately visible. Additionally there is no correlation between using new solutions and isolating a high number of protoplasts.

It should be noted that while the results show a high number of protoplasts being isolated using this method, this is not the only parameter determining the effectiveness of a method. The regenerative ability of the isolated protoplasts is a more important parameter when determining which isolation method is suitable for protoplasts utilized for domestication of *L. campestre*.

3.1.3.2 Isolation method 1.B

Table 7 shows the data obtained with the same setup according to method 1.B. The mean values of protoplasts obtained were fairly similar. However the coefficients of variation were fairly large, demonstrating a high variation between replicates within the batch populations.

Table 7: Number of protoplasts isolated with method 1.B using scissors and a 15 h incubation time.

Replicate batch batch	l	m	n	o	p
Number of replicates	2	3	6	1	2
Mean value	0.63	0.56	0.49	0	0
Batch standard deviation	0.30	0.41	0.31	-	0
Coefficient of variation	0.48	0.74	0.63	-	-
Total mean value	0.42				
Total standard deviation	0.37				
Coefficient of variation	0.89				

Unlike methods 1.A and 1.C, method 1.B had no purification step where intact protoplasts are separated from broken cell fragments. An incubation on ice, after counting the protoplasts, was included in the method, but it was done to the pellet, i.e. all the protoplasts were already sedimented, and nothing was discarded after the incubation. This may have caused the larger coefficients of variation compared to method 1.A. A possible cause of the shortage of protoplasts produced could be that a solution has not been correctly prepared. This could for example be a low osmotic concentration causing insufficient plasmolysis. This would explain why no protoplasts are produced in the later batches o and p.

3.1.4 Comparison of enzyme amounts

The number of protoplasts isolated using varying enzyme amounts can be seen in Figure 4. Significantly more protoplasts were isolated with increased enzyme amounts. Photographs of the protoplasts obtained during this test can be seen in Appendix B.2. These photographs show roughly the same quota of broken protoplasts regardless of enzyme concentration. The results encourage the usage of higher enzyme concentrations, and of cellulase RS.

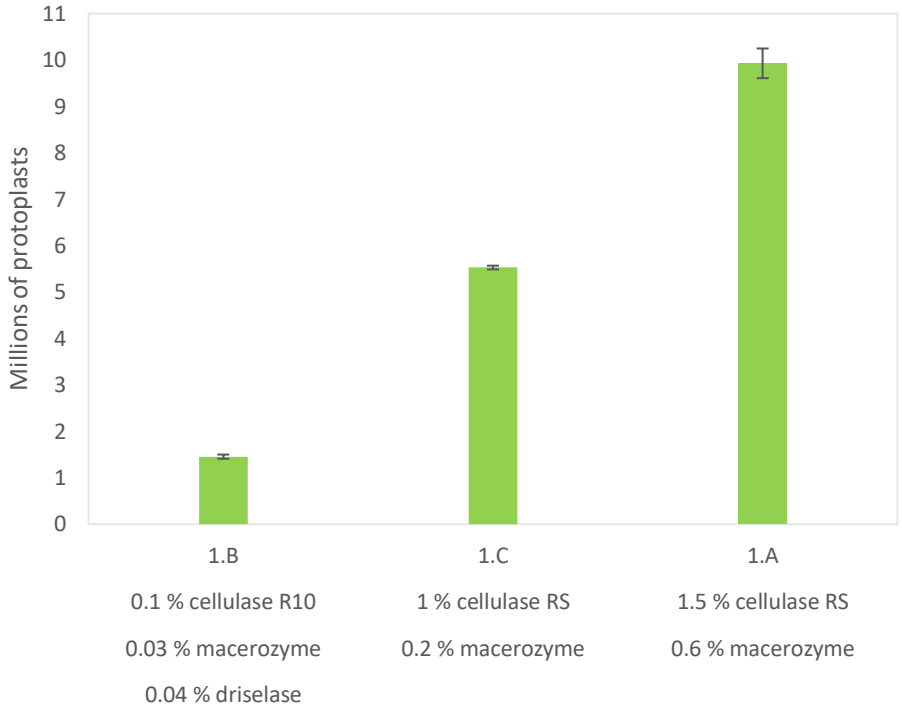


Figure 4: Millions of protoplasts isolated using the different enzyme amounts corresponding to methods 1.A, 1.B and 1.C. The data presented in order of increasing enzyme concentrations. n=2 for all data series. p=0.005 between 1.A and 1.C, p=0.002 between 1.B and 1.C.

From the results displayed in Figure 4 it can be concluded that the previously noted difference in the number of protoplasts isolated using method 1.A and B is caused by the difference in enzyme concentration. Other differences between the methods, such as centrifugation speed or the purification step, have a lesser impact.

Using more enzymes should not be harmful to the protoplasts, since the cell wall is the only cell component that is degradable by the enzymes. A factor to take into account is that using a higher enzyme concentration presents a higher cost, and it should therefore be considered if the increased number of protoplasts isolated is worth this cost. Isolation method 1.A is based on Yoo et al. (2007), and their high enzyme levels are used since short incubation times are desired in their experiments.

Since the enzyme high enzyme levels used in method 1.A originally were used to compensate for a short incubation time, and not intended to be used for achieving a high number of isolated protoplasts, it should be considered if a high number of protoplasts is useful. Regeneration of all produced protoplasts demands a lot of space and medium, and when performing transfections the plasmid quantity quickly becomes the limiting factor. Thus when further optimizing an isolation method suitable for *L. campestre* the effects on regeneration capacity stemming from the isolation method should be prioritized over maximizing the number of protoplasts.

3.1.5 Incubation time

The number of protoplasts isolated with 15 h and 18 h incubation times respectively using method 1.A can be seen in Figure 5. There was no significant difference found in either the number of intact or broken protoplasts. No protoplasts were isolated when performing this test with method 1.B.

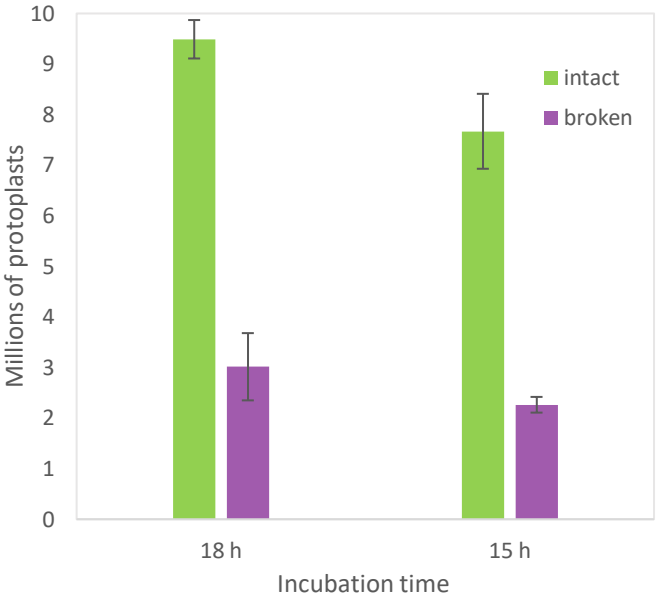


Figure 5: Number of intact (green) and broken (purple) protoplasts isolated at 18 h and 15 h (standard) enzyme incubation times. n=2 for 18 h test and n=3 for 15 h test. p=0.09 for intact protoplasts and p=0.24 for broken protoplasts.

Allowing the enzymes to act during a longer time period should theoretically lead to more cell walls being degraded, and therefore more protoplasts being released. However according to Onkokesung (unpublished), who used a 15 h incubation, over digestion of the protoplasts produced excessive cell debris. This does not seem to apply to the results shown in Figure 5, since there is no significant difference between the number of broken protoplasts obtained at the different incubation times. It should be noted that the number of broken protoplasts is overestimated in the count, since both cell contents and empty cell membranes were counted. A broken protoplast may produce both free cell contents and a cell membrane, i.e. multiple pieces stemming from one protoplast may be counted.

The protoplasts produced have been subjected to regeneration to see if a longer incubation time has a negative effect, but due to time constraints no results have been obtained. The stress of keeping protoplasts in darkness for over 16 h might benefit the dedifferentiation and regeneration (Yoo et al., 2007). Dedifferentiation could potentially lead to homology directed repair becoming the more favored DNA repair mechanism when utilizing CRISPR/Cas9 (Belhaj et al., 2015).

3.2 Protoplast transfection

An untreated control can be seen in Figure 6b, and lit in fluorescent light in Figure 6a. Transfected protoplasts exposed to PEG for 5 min can be seen in fluorescent light in Figure 6c and by comparison in regular light in Figure 6d. Protoplasts exposed to PEG for 10 min can be seen in fluorescent light in Figure 6e and by comparison in regular light in Figure 6f.

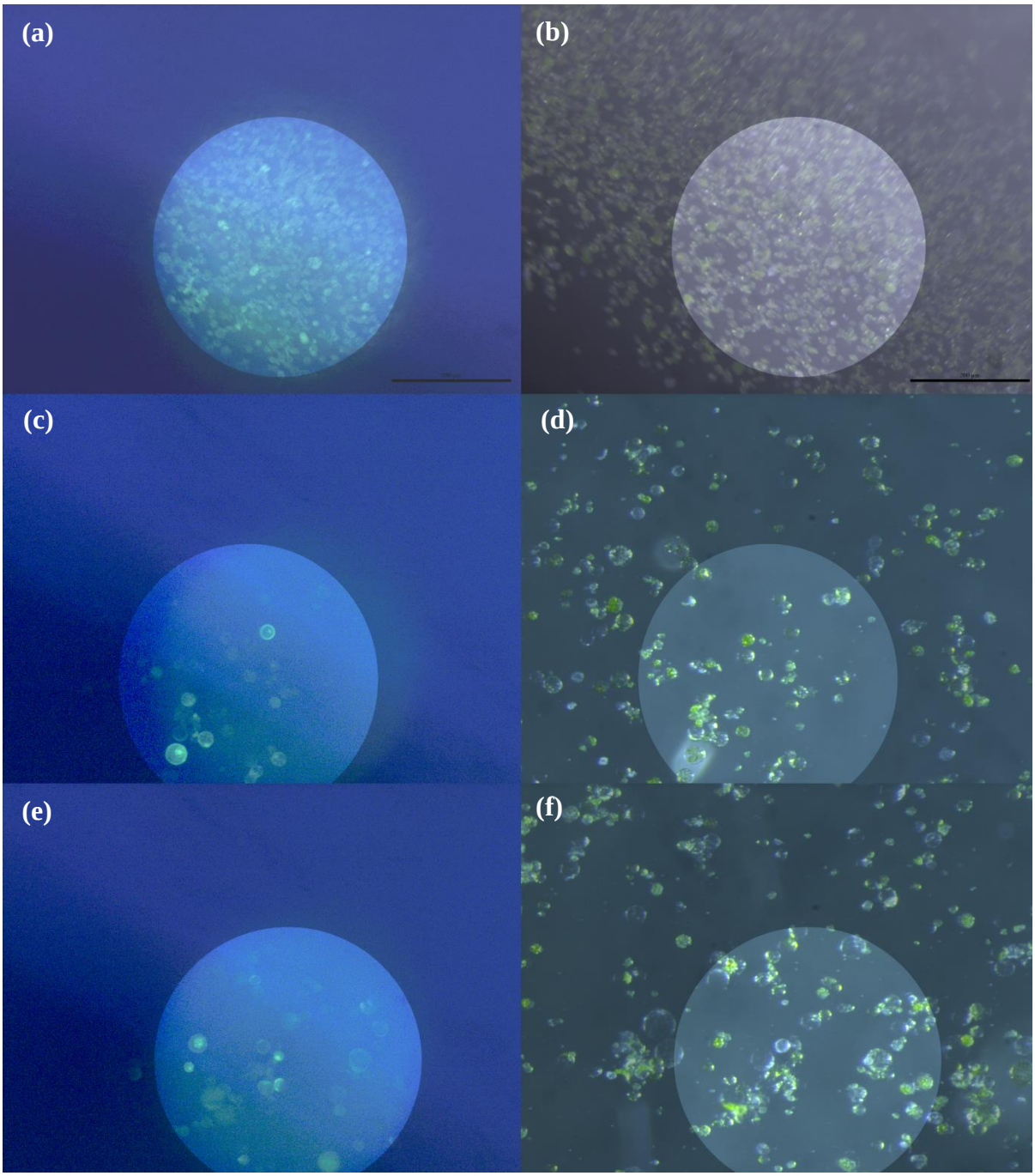


Figure 6: Photos taken with Leica DM LB microscope. Photos a, c and e are shcown in PRIOR Lumen 200 Fluorescence Illumination. Area lit up by fluorescent light is highlighted. (a) untreated protoplasts, all protoplasts reflect some green light. x10 magnification. (b) untreated protoplasts, shown in normal light. x10 magnification. x12 magnification could not be used since this caused the sample to appear too blurred. (c) protoplasts after 5 min of transfection, a few protoplasts appear to glow at levels above control. x12 magnification. (d) protoplasts after 5 min of transfection, shown in normal light. x12 magnification. (e) protoplasts after 10 min of transfection, a few protoplasts seem to glow at levels above control. (f) protoplasts after 10 min of transfection, shown in normal light. x12 magnification.

Determining if transfection had occurred was difficult due to the reflection of green light from all protoplasts, including the non-transfected. The results indicate that successful transfections were performed using both the 5 min and 10 min incubation time. Due to the small area illuminated by the fluorescent light the percentage of protoplasts transfected could not be calculated. This makes any optimization work difficult, so developing a method for analyzing

fluorescence using the Leica DM LB microscope is recommended. Currently the higher exposure time needed would not function to pick up low fluorescence. Yoo et al. (2007), on which method 1.A is based, recommends optimizing the protoplast/DNA ratio, transfection time and PEG concentration empirically, starting at 5-15 min incubation time and 10-20 % PEG. A too high PEG concentration can induce protoplast fusion (Pontecorvo, 1975).

The generally low fluorescence of the protoplasts could indicate that multiple copies of the plasmid have not been acquired. This is not an issue since CRISPR/Cas9 does not require multiple vector copies to function (Svitashev et al., 2015). An alternative pair of plasmids were suggested for this project, which required both plasmids to be absorbed for fluorescence to occur. However results obtained by Svitashev et al. (2015) indicate that by delivering Cas9, gRNA and a HDR template on two different plasmids decreases the mutation frequency. Thus data obtained when using two plasmids is not as relevant when developing a transfection method suitable for CRISPR/Cas9 usage.

3.3 Protoplast regeneration

3.3.1 Bacterial infections

Distinct infections were observed when performing regeneration method 3.B, see Figure 7. When not all plates were contaminated in a batch, the infected plates all stemmed from the same isolation replicate. The infections were opaque white and were observed both as spherical structures imbedded in alginate (Figure 7a-b) and as a film floating in liquid media (Figure 7b-c).

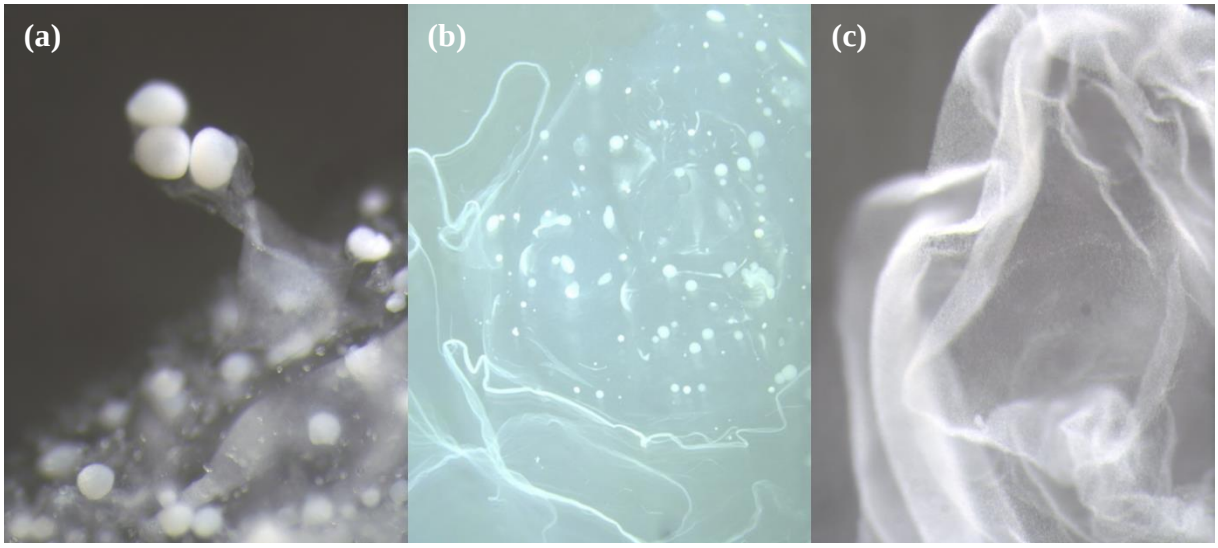


Figure 7: Bacterial growth in regenerations performed with method 3.A. Leica DM LB was microscope used. (a) white opaque spheres growing in and protruding from alginate, x12 magnification. (b) alginate containing big spheres, with a film connected, x0.73 magnification. (c) white opaque film floating in media, x12 magnification.

Structural connections between spheres and film, as can be seen in figure 7a, could indicate that these formations were made from the same bacteria. The white spheres were first observed after 5 days. Some early batches of regeneration method 3.A contained a film, but these were discarded before photos could be taken.

The high infection rate severely hindered the development of a regeneration protocol. Finding out where the infection stems from is important for preventing future infections. Since regeneration plates stemming from the same isolation replicate all were infected the infection seems to occur during the isolation. The source could be a non-sterile work environment or technique, an infected solution used or ineffective sterilization of the plant material. The infections have occurred regardless of whom performed the lab, which could indicate another contamination source. Regeneration method 3.B may infer a higher risk of infections due to the frequent media changes. Sequencing of the infections could be done to determine which organism is causing them, which might help in identifying where they stem from.

3.3.2 Purple protoplasts

Purple protoplasts were observed in three batches. Plates stemming from the same isolation replicate (and thus the same plant material) did not all contain purple protoplasts. Purple protoplasts were observed for both 15 h and 18 h incubation times. The earliest purple protoplast was observed during the concentration determination after the last wash after isolation, see Figure 8a. Purple protoplasts were obtained both in batches regenerated according to method 3.A (Figure 8c) and method 3.B (Figure 8b).

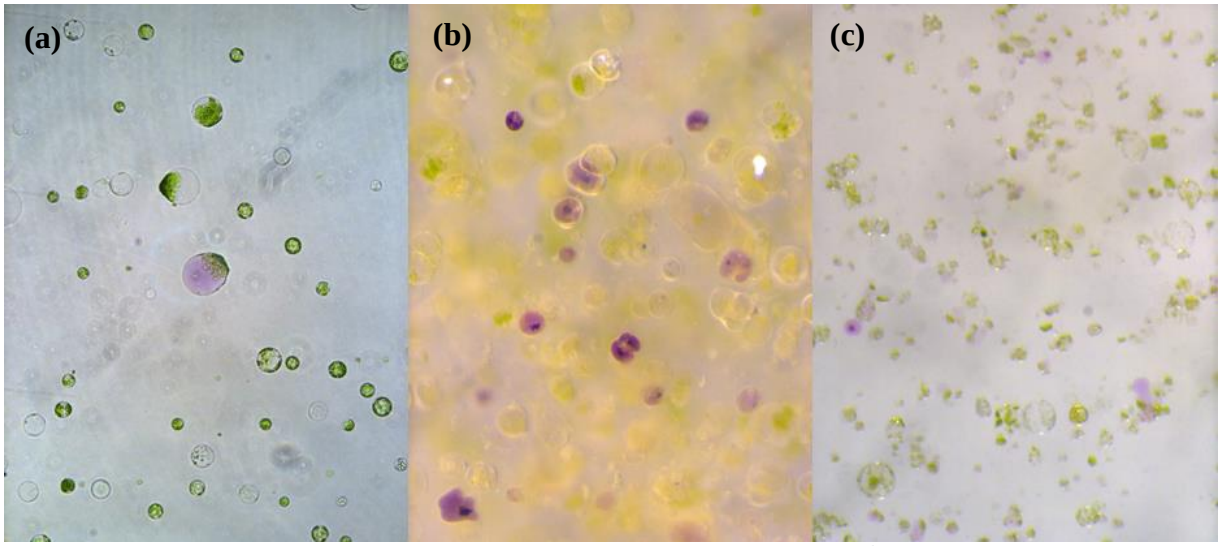


Figure 8: (a) purple protoplast observed during concentration determination. x20 magnification. Leica DM LB microscope used. (b) purple protoplasts in alginate, 25 days old. Cell division and cell enlargement has occurred. x12 magnification. Leica DM LB microscope used. (c) purple protoplasts in liquid media, 5 days old. x12 magnification. Leica DM LB microscope used.

It is unclear what causes their color. The color does not seem to spread to adjacent protoplasts and this could be an indication that it is not an infection. They were observed both when executing modified and non-modified methods, indicating that the phenomenon can occur without extra stress.

3.3.3 Clusters

Protoplast clusters were frequently observed during regeneration according to method 3.A. Some of the clusters did not seem to derive from cell division. The cluster in Figure 9 was observed 5 days after isolation. The number of non-clustered protoplasts was low.

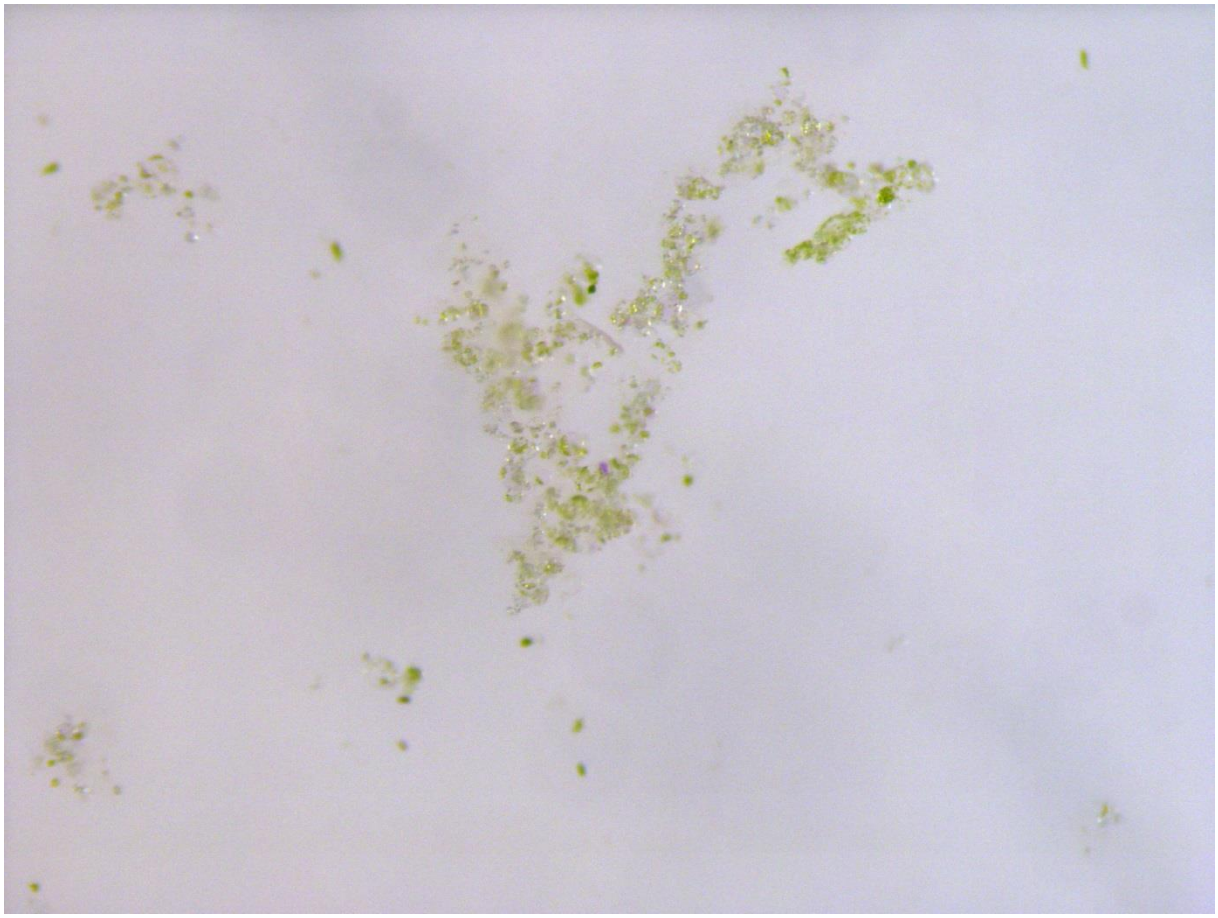


Figure 9: Cluster of protoplasts in PIM, five days old. x5 magnification. Leica DM LB microscope used.

The cluster shown in Figure 9 was most probably formed through aggregation, not through cell division. This, because the number of protoplasts in the petri dish appeared to be unchanged, and if cell division had occurred enough to form large clusters the number of protoplasts would increase noticeably. Protoplasts aggregating is a problem since there will always be a few non-transfected protoplasts. If they are present in a cluster, then there is a risk that a calli composed of both transfected and wild type-cells will be formed. This risk increases when there is a higher

number of protoplasts in the cluster since it is more likely that a larger cluster will contain some of the non-transfected protoplast.

Most clusters observed throughout the project were anchored to the bottom of the petri dish. This likely occurred since the plastic is hydrophobic, and the cell membrane is also hydrophobic relative to the surrounding medium. Thus, when the protoplasts sediment they stick to the bottom. Protoplasts may then sediment onto the fixated protoplasts and bind to them through van der Waal-forces. Binding to a fixated protoplast is more likely than to a free-floating, since the fixated protoplast cannot be repelled away during the collision. Yoo et al. (2007) recommends coating plastic surfaces with 5 % (vol/vol) sterile calf serum for 1-2 s, which prevents protoplasts from sticking to the surface. Petri dishes made of glass instead of plastic are also an alternative. A way to reduce protoplast aggregation is to lower the concentration of protoplasts. However, cells stimulate the division of other cells nearby through release of growth factors, so lowering the concentration could decrease this effect (Davey et al., 2005).

3.3.4 Microcalli

At the end of the project, a total of 6 microcalli had been obtained. All of them originating from one petri dish. These protoplasts were isolated with method 1.A using scissors, and regenerated with method 3.A. The first microcallus was observed 50 days after isolation (Figure 10a). Three months after the isolation six microcalli could be observed, one of which can be seen in Figure 10b.

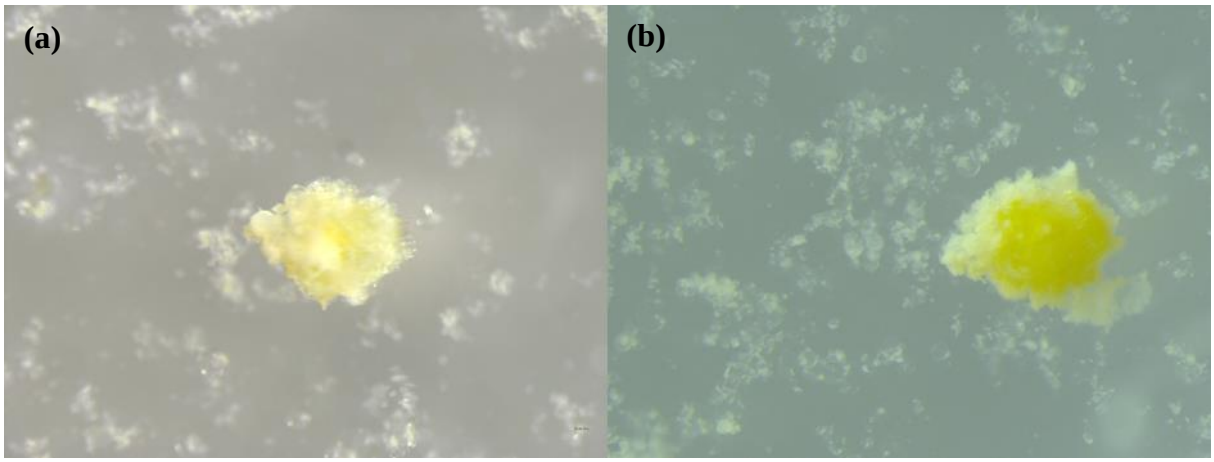


Figure 10: Microcalli from the same plate, x5 magnification. Leica DM LB microscope used. (a) microcallus observed 50 days after protoplast isolation. (b) microcallus observed three months after protoplast isolation.

Microcalli are distinguishable from the previously discussed clusters by being more compact, since aggregation forms more fractal like loose patterns. There is no drastic difference in size

between the microcalli observed after 50 days and those seen after three months. However, the higher opacity of the three months old microcalli compared to the 50-day old could be due to the regeneration of cell walls. The more matte texture of the older microcalli compared to the shinier younger microcallus also points toward this.

According to method 3.A the cells should be transferred from CIM1 to CIM2 after one month. This was attempted, but due to anchorage to the bottom it was made difficult, and as a result the six microcalli observed remained in CIM1 media. This may be the cause of the similarity in size between the young and older microcalli. CIM2 (Appendix A.2) contains twice as much TZ, KNO_3 and NH_4NO_3 as CIM1 (Appendix A.2). Feng & Ouyang (1988) have found that concentrations of KNO_3 up to 2 g/l are favorable for callus induction from wheat anthers, but at higher concentrations the NO_3^- ion becomes harmful. Method 3.A uses a concentration of 1 g/l, so if regeneration proves ineffective during further testing a higher concentration of KNO_3 could be tested.

In method 1.A nutrients are not provided to the cells during the enzyme incubation. It was speculated that this could reduce the produced protoplasts' regeneration capacity. However the microcalli obtained stemmed from protoplasts isolated with method 1.A. This indicates that the absence of nutrients during isolation does not prevent regeneration, although it is entirely possible that the regeneration frequency would be much higher if nutrients had been present during isolation. A systematic comparison would have to be executed for any conclusions to be drawn. It should be mentioned that Yoo et al. (2007), on which method 1.A is based, is developed for observation of transient gene expressions without regeneration in mind.

4 Conclusions

It was found that isolation method 1.A was more impacted by external factors than replicate-specific variations. Mixing aliquots taken for determining protoplast concentration thoroughly immediately prior to counting is crucial to achieving reliable results.

The lack of a purification step in method 1.B may cause problems with this protocol. If protoplasts isolated using method 1.A prove to have a lower regeneration capacity than those isolated using method 1.B, isolation could be performed according to method 1.B using the purification step described in method 1.A. Higher enzyme concentrations could also be used if a high protoplast yield is desirable. An increased incubation time could benefit regeneration due to the increased dedifferentiation caused by the increased stress (Yoo et al., 2007).

Transfections were performed successfully using both 5 min and 10 min PEG incubations. For finding an optimized incubation time a method for analyzing fluorescence using the Leica DM LB microscope with the counting chamber needs to be developed. The PEG concentration should also be optimized. With a high transfection rate the risk of producing a callus composed of both transfected cells and wild type cells decreases. Another way to reduce this risk is to reduce clustering by lowering the protoplast concentration.

Development of a regeneration protocol was hindered by the frequent bacterial infections. Adding antibiotics to the regeneration medias used could fix this, but preventing contamination would be preferable. Microcalli were obtained from one regeneration plate, showing that regeneration of the protoplasts is possible using method 3.A.

5 References

Assani, A., Chabane, D., Foroughi-Wehr, B. and Wenzel, G. (2006). An Improved Protocol for Microcallus Production and Whole Plant Regeneration from Recalcitrant Banana Protoplasts (Musa spp.). *Plant Cell, Tissue and Organ Culture*, 85(3), pp.257-264.

Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N. and Nekrasov, V. (2015). Editing plant genomes with CRISPR/Cas9. *Current Opinion in Biotechnology* 32, pp.76-84.

Chupeau, M., Granier, F., Pichon, O., Renou, J., Gaudin, V. and Chupeau, Y. (2013). Characterization of the Early Events Leading to Totipotency in an Arabidopsis Protoplast Liquid Culture by Temporal Transcript Profiling. *The Plant Cell*, 25(7), pp.2444-2463.

Creative-enzymes.com. (2017). *Driselase - Creative Enzymes*. [online] Available at: http://www.creative-enzymes.com/similar/Driselase_205.html [Accessed 16 May 2017].

Davey, M., Anthony, P., Power, J. and Lowe, K. (2005). Plant protoplasts: status and biotechnological perspectives. *Biotechnology Advances*, 23(2), pp.131-171.

Duchefa-biochemie.com (2017). *Cellulase RS | Duchefa Biochemie*. [online] Duchefa-biochemie.com. Available at: https://www.duchefa-biochemie.com/product/details/number/C8003 [Accessed 23 Apr. 2017].

Feng, G. and Ouyang, J. (1988). The effects of KNO3 concentration in callus induction medium for wheat anther culture. Plant Cell, Tissue and Organ Culture, 12(1), pp.3-12.

Hansen, L. (2003). Insecticide-resistant pollen beetles (*Meligethes aeneus* F) found in Danish oilseed rape (*Brassica napus* L) fields. *Pest Management Science*, 59(9), pp.1057-1059.

Holme, I., Wendt, T. and Holm, P. (2013). Current Developments of Intragenic and Cisgenic Crops. *ISB News Report*, (7), pp. 4-9.

Hu, Q., Anersen, S. and Hansen, L. (1999). Plant regeneration capacity of mesophyll protoplasts from *Brassica napus* and related species. *Plant Cell, Tissue and Organ Culture*, 59, pp.189-196.

Huesing, J., Andres, D., Braverman, M., Burns, A., Felsot, A., Harrigan, G., Hellmich, R., Reynolds, A., Shelton, A., Jansen van Rijssen, W., Morris, E. and Eloff, J. (2016).

Global Adoption of Genetically Modified (GM) Crops: Challenges for the Public Sector. *Journal of Agricultural and Food Chemistry*, 64(2), pp.394-402.

Ivarson, E., Ahlman, A., Lager, I. and Zhu, L. (2016). Significant increase of oleic acid level in the wild species *Lepidium campestre* through direct gene silencing. *Plant Cell Reports*, 35(10), pp.2055-2063.

Ivarson, E., Leiva-Eriksson, N., Ahlman, A., Kanagarajan, S., Bülow, L. and Zhu, L. (2017). Effects of Overexpression of WRI1 and Hemoglobin Genes on the Seed Oil Content of *Lepidium campestre*. *Frontiers in Plant Science*, 7.

Jiang, F., Zhu, J. and Liu, H. (2013) Protoplasts: a useful research system for plant cell biology, especially dedifferentiation. *Protoplasma,* 250(6), pp.1231-1238.

Jordbruksverket, (2015). Svar om ny växtförädlingsteknik ger GMO eller ej - Jordbruksverket. [online] Jordbruksverket.se. Available at: http://www.jordbruksverket.se/pressochmedia/nyheter/nyhetsarkiv20092015/nyheter2015/svaromnyvaxtforadlingsteknikgergmoellerej.5.3eaa64c21510f7c86771c715.html [Accessed 4 Mar. 2017].

Klarin, G. (2015). Genmodifiering med ny teknik kan ske utan tillstånd - Vetenskapsradion. [online] Sverigesradio.se. Available at: http://sverigesradio.se/sida/artikel.aspx?programid=406&artikel=6303669 [Accessed 4 Mar. 2017].

Merker, A. and Nilsson, P. (1995). Some oil crop properties in wild *Barbarea* and *Lepidium* species. *Swedish J Agric Res.* 25, pp.173-178.

Merker, A., Eriksson, D., and Bertholdsson, N. (2010). Barley yield increases with undersown *Lepidium campestre*. *Acta Agric. Scand. B Soil Plant Sci.* 60, pp.269-273.

Nicolia, A., Proux-Wéra, E., Åhman, I., Onkokesung, N., Andersson, M., Andreasson, E. and Zhu, L. (2015). Targeted gene mutation in tetraploid potato through transient TALEN expression in protoplasts. *Journal of Biotechnology*, 204, pp.17-24.

Onkokesung, N. (unpublished). Protoplast isolation in dicot plants. Unpublished protocol.

Parisi, C., Tillie, P. and Rodríguez-Cerezo, E. (2016). The global pipeline of GM crops out to 2020. *Nature Biotechnology*, 34(1), pp.31-36.

Plan, D. and Van den Eede, G. (2010). The EU Legislation on GMOs - an overview. Luxembourg: Publications Office of the European Union, p.3.

Pontecorvo, G. (1975). Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. *Somatic Cell Genetics*, 1(4), pp.397-400.

Ran, F., Hsu, P., Wright, J., Agarwala, W., Scott, D. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols* 8(11), pp.2283-2308.

Sainsbury, F., Thuenemann, E. and Lomonossoff, G. (2009). pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnology Journal*, 7(7), pp.682-693.

Svitashev, S., Young, J., Schwartz, C., Gao, H., Falco, S. and Cigan, A. (2015). Targeted Mutagenesis, Precise Gene Editing, and Site-Specific Gene Insertion in Maize Using Cas9 and Guide RNA. *Plant Physiology*, 169(2), pp.931-945.

Taiz L, Zeiger E, 2015. Plant Physiology 6th Edt, Sunderland, USA: Sinauer Associates Inc., 732-737, 762.

Wang, Y., Sonntag, K., Rudloff, E. and Han, J. (2005). Production of fertile transgenic *Brassica napus* by Agrobacterium-mediated transformation of protoplasts. *Plant Breeding*, 124(1), pp.1-4.

Yoo, S., Cho, Y. and Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols*, 2(7), pp.1565-1572.

Zhang, Y., Liang, Z., Zong, Y., Wang, Y., Liu, J., Chen, K., Qiu, J. and Gao, C. (2016). Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nature Communications*, 7:12617.

Appendix A. Medias

A.1 Medias used in methods 1.A and 2.A

Plasmolysis solution

0.4 M mannitol

Enzymatic solution

0.4 M mannitol

10 mM MES (pH 5.7)

0.6 % macerozyme R10

1.5 % cellulase RS

0.1 % BSA

1 mM $CaCl_2$

1 mM β-mercaptoethanol

Sterile MilliQ water

W5 solution

154 mM NaCl

125 mM $CaCl_2$

5 mM KCl

2 mM MES (pH 5.7)

Sterile MilliQ water

MMG

0.4 M mannitol

15 mM $MgCl_2$

4 mM MES (pH 5.7)

Sterile MilliQ water

WI solution

4 mM MES (pH 5.7)

0.4 M mannitol

20 mM KCl

Sterile MilliQ water

PEG solution

50 % PEG4000

0.4 M mannitol

0.1 M $CaCl_2$

Sterile MilliQ water

(Onkokesung, unpublished)

A.2 Medias used in methods 1.B and 3.A

	GM[a]	MGG[b]	PIM[c]	CIM1[d]	CIM2[d]	SIM[e] Col-0	SIM[e] Ws	PDM[f]
Macrosalts								
KNO_3	950	250	505	505	1010	1010	1010	950
NH_4NO_3	825		160	400	800	0	800	825
$CaCl_2, 2H_2O$	220	15	440	440	440	220	220	220
$MgSO_4, 7H_2O$	185	25	370	370	370	185	185	185
KH_2PO_4	85		170	170	170	85	85	85
$(NH4)_2SO_4$		13.4						
NaH_2PO_4		15						
Microelements								
Fe Citrate NH_4[g]	50		30	30	30	50	50	50
KI	0.4	0.75	0.01	0.01	0.01	0.8	0.8	0.01
H_3BO_3	1.5	3	1	1	1	3	3	1
$MnCl_2, 4H_2O$	15					30	30	
$MnSO_4, 4H_2O$		10	0.1	0.1	0.1			0.1
$ZnSO_4, 7H_2O$	6	2	1	1	1	12	12	1
$Na_2MoO_4, 2H_2O$	0.45	0.25				0.9	0.9	
$CuSO4, 5H_2O$	0.045	0.025	0.03	0.03	0.03	0.09	0.09	0.03
$CoCl_2, 6H_2O$	0.045	0.025				0.09	0.09	
$AlCl_3$			0.03	0.03	0.03			0.03
$NiCl_2, 6H_2O$			0.03	0.03	0.03			0.03
Vitamins								
Inositol	100	100	100	100	100	100	100	100
Panthotenate Ca	1	1	1	1	1	1	1	1
Biotin	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Niacin	1	1	1	1	1	1	1	1
Pyridoxin	1	1	1	1	1	1	1	1
Thiamin	1	1	1	1	1	1	1	1
Folic Acid			0.2					
Other constituants								
Glucose		45 000	40 000	0				
Sucrose	10 000		0	30 000	20 000	20 000	20 000	10 000
Mannitol			60 000	70 000	60 000	40 000	40 000	
Glycine		25 000						
2, 4-D			1	0	0	0	0	
Thidiazuron (TZ)			0.022	0.11	0.22			
Indole-3-butyric acid (IBA)						0,1	0,1	
Meta-topolin						0.2	0.2	
MES	700	700	700	700	700	700	700	700
Bromocresol purple (BCP)[h]	8	8	8	8	8	8	8	8
Vitro Agar	6 000					6 000	6 000	4 000
Onozuka R10		1 000						
Macerozyme		300						
Driselase		400						
pH of fresh medium	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.6

[a]Germination medium. Half macrosalts of Murashige and Skoog (1962). Vitamin composition based on Morel and Wetmore (1951).
[b]Maceration-glycine-glucose medium. One tenth of macrosalts. Microsalts based on Gamborg et al. (1968).
[c]Protoplast-induction medium. Microsalts based on Heller (1953).
[d]Colony-induction medium.
[e]Shoot-induction medium.
[f]Plant development medium.
[g]FeCitrate NH_4 is less toxic than FeEDTA, solutions at pH 5.6 make easy to test ranges.
[h]BCP is a convenient, non-toxic pH indicator (Roscoe and Bell, 1981).

(Chupeau et al., 2013)

A.3 Medias used in method 3.B

Alginate-solution

1.3 % (w/v) sodium alginate

0.4 M mannitol

Calcium-solution

50 mM $CaCl_2$

0.4 M mannitol

Calcium-mannitol-solution

10 mM $CaCl_2$

0.4 M mannitol

M1

Nitsh

100 g/l mannitol

10 g/l glucose

10 g/l sucrose

100 mg/l casein hydrolase

0.5 mg/l 2,4-D

0.5 mg/l NAA

0.5 mg/l BAP

MII

MS

54 g/l glucose

20 g/l sucrose

400 mg/l inositol

500 mg/l glutamine

0.5 mg/l 2,4-D

0.5 mg/l NAA

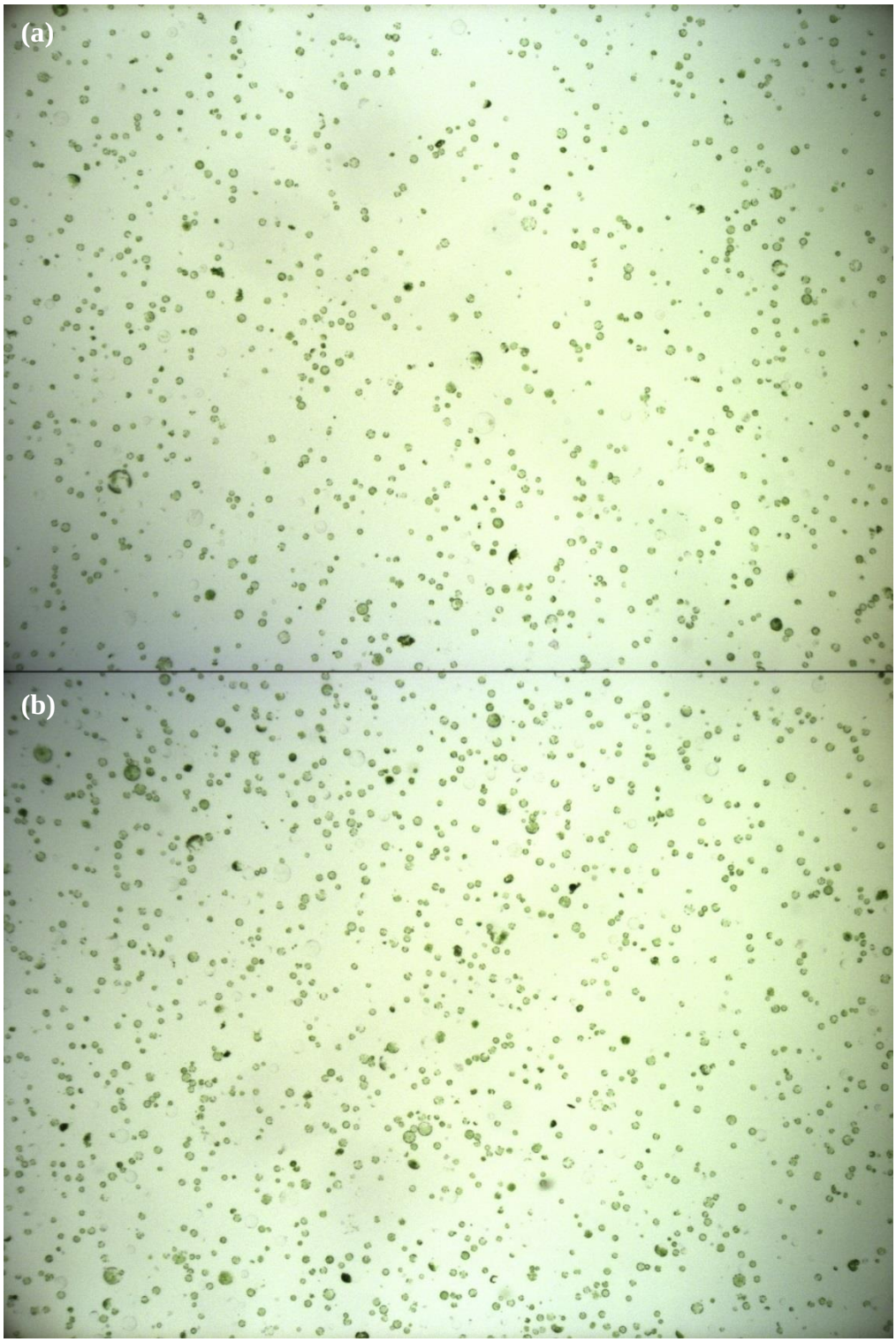
0.5 mg/l BAP

(Siemens et al., 1993)

Appendix B. Images

B.1 Sample heterogeneity

The first sample (b) was taken from the lower part of the sample tube immediately after mixing the sample through pipetting up and down, and the second sample (a) was taken from the upper part of the sample tube 3 min after mixing. x5 magnification. Leica DM LB microscope used.



B.2 Enzyme amounts

a-b: Protoplasts isolated using enzyme amounts from method 1.B.
c-d: Protoplasts isolated using enzyme amounts from method 1.C.
e-f: Protoplasts isolated using enzyme amounts from method 1.A.
The quota of broken protoplasts appear to be roughly the same for all batches. x10 magnification. Leica DM LB microscope used.