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Final Thesis

Gene editing of grapevine protoplasts using a microfluidic platform

Supervisor: Prof. Claudio Moser

Co-supervisor: Doct. Tobias Weil

Student: Paolo Viviani

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SUMMARY

TABLES AND FIGURES

RIASSUNTO

L'obiettivo di questa tesi è stato quello di stabilire un nuovo metodo per migliorare l'efficienza dell'editing genetico nella vite attraverso la microfluidica. Le Tecnologie di Evoluzione Assistita (TEA), tra cui l'editing genetico, sono delle tecniche di miglioramento genetico molto promettenti per efficienza e specificità e i primi prototipi arriveranno a breve dal laboratorio al campo offrendo un'opportunità di un'agricoltura più sostenibile e resiliente ai cambiamenti climatici e con maggiore sostenibilità ambientale. Infatti, la vite è una delle colture più importanti a livello mondiale e alcune varietà sono gravemente colpite dal riscaldamento globale. Inoltre, la sua coltivazione richiede grandi quantità di fitofarmaci e quindi ha un impatto considerevole sull'ambiente e sulla biodiversità. Le TEA sono uno strumento promettente per lo sviluppo di nuovi cloni di varietà coltivate che ne mantengano l'elevata qualità e le caratteristiche specifiche e al contempo richiedano un numero inferiore di trattamenti contro le principali malattie della vite e offrano una maggiore resilienza agli stress abiotici. Al fine di produrre piante geneticamente modificate prive di DNA esogeno, sono di fondamentale importanza nuovi approcci che migliorino l'efficienza del rilascio del complesso CRISPR-Cas9 nei protoplasti vegetali. A questo scopo, nel corso di questa tesi è stata testata con successo una tecnologia innovativa che utilizza un nuovo sistema di microfluidica, ottenendo il trasferimento altamente efficiente della proteina Cas9-GFP nei protoplasti della vite. Inoltre, la tesi ha previsto la generazione di callo da tralci acclimatati di vite, il miglioramento del protocollo per la purificazione dei protoplasti e la rigenerazione fino ad embrione di protoplasti che hanno attraversato il sistema microfluidico.

ABSTRACT

The aim of this thesis was to establish a new method to improve the efficiency of gene editing in grapevine through microfluidics. Assisted Evolution Technologies (TEA), including gene editing, are very promising genetic improvement techniques in terms of efficiency and specificity and the first prototypes will soon arrive from the laboratory to the field, offering an opportunity for more sustainable and resilient to climate change and with greater environmental sustainability. In fact, the vine is one of the most important crops worldwide and some varieties are seriously affected by global warming. Furthermore, viticulture requires enormous quantities of antimicrobials and pesticides and thus, has a considerable impact on the environment and biodiversity. TEAs are a promising tool for the development of new clones of cultivated varieties that maintain their high quality and specific characteristics and at the same time require fewer treatments against the main vine diseases and offer greater resilience to abiotic stress. In order to produce genetically modified plants without exogenous DNA, new approaches that improve the efficiency of the release of the CRISPR-Cas9 complex into plant protoplasts are of fundamental importance. To this end, during this thesis an innovative technology using a new microfluidics system was successfully tested, achieving a highly efficient introduction of the Cas9-GFP protein into grapevine protoplasts. Additionally, the thesis comprised the generation of callus from woody cane, an improvement of the protocol for protoplasts purification and the regeneration of embryos from protoplasts that passed through the microfluidic device.

INTRODUCTION

The growing interest in the new gene technologies (NGTs) has revolutionized all biotechnology sectors and nowadays, a mindset change is taking place in agriculture leading a new green revolution aiming at providing plants resistant to disease and resilient to climate change. However, currently just a few edited plants have been obtained using NGTs. Consequently, new methods to deliver CRISPR-Cas9 are needed to promote a more sustainable future for agriculture.

GENOME EDITING TECHNOLOGIES

During recent years, the emergence of highly versatile genome-editing technologies has provided investigators with the ability to introduce sequence-specific modifications rapidly and economically into the genomes of a broad spectrum of cell types and organisms (Gaj T, 2016 Dec). In particular, the ease with which CRISPR-Cas9 can be configured to recognize new genomic sequences has accelerated scientific breakthroughs and discoveries in disciplines as diverse as synthetic biology, human gene therapy, disease modeling, drug discovery, neuroscience, and agricultural sciences. (Gaj T, 2016 Dec)

Traditionally genome engineering is performed by polyethylene glycol (PEG) mediated transfection, because PEG enhances stability and transfection efficiency (Simone Scintilla, 2022). However, the toxicity of PEG causes cells death (David A. Herold ∗, 1982), and this limitation has halted the scalability of this technology and limited the engineering of custom cell lines. In contrast, microfluidics allows for scalability because this technology is modular, enabling an easy integration with automation capabilities for high-throughput and single-cell studies. (T. Luo, 2019). Indeed, recent advances in microfluidics allowed for a gentle and highly efficient delivery of CRISPR-Cas9 ribonucleoprotein complexes into human cells (Yip, 2020).

IMPORTANCE OF GENE EDITING FOR VITICULTURE

DISEASES AND ENVIRONMENTAL ISSUES

The development of NGT-based technologies is crucial for agriculture and in particular for viticulture as it will lead to a more sustainable and eco-friendly viticulture (Johnny Vicente Montalvo-Falcón, 2023). Considering their high pedoclimatic adaptation capacity, the cultivation of *Vitis vinifera* cultivars is only possible between 30° to 50 °N and S latitude (Anton, 2023). When weather conditions are favorable (mild temperatures and high humidity) during the crop cycle, almost every organ of the plant is susceptible to the main fungal and oomycete diseases, such as downy mildew, powdery mildew, and grey mold, that are caused by *Plasmopara viticola* (Berk. and Curtis) Berl. and De Toni, *Erysiphe necator* Schwein., and *Botrytis cinerea* Pers., respectively (Luca Capriotti, 2020). According to recent global surveys conducted in the main winegrowing regions of the world, these diseases were considered by researchers and production professionals as the most harmful for grape production (Bois, Zito, & Calonnec, 2017).

Moreover, treatment of fungal and oomycete diseases requires a high number of fungicides, which cause contamination of nearby soils and waters (Santa Olga Cacciola, 2019). For example, since the end of the nineteenth century Cu-based fungicide treatments (i.e. Bordeaux combination Ca(OH)2 + CuSO4) have been widely used in vineyards to treat downy mildew resulting in an overall increase of Cu concentrations in vineyard soils often ranging from 200 to 500 mg/kg (Brun et al., 1998, Schramel et al., 2000, Chaignon et al., 2003). Great quantities of such trace elements can cause significant environmental problems, especially when they accumulate and contaminate soils, vegetation, animals, surfaces or groundwaters. (Samaneh Najafi, 2023) Consequently, improving disease resistance in grapevine by gene engineering may lead to a more sustainable and environment compatible viticulture.

THE CLIMATE CHANGE CALLENGE FOR TRADITIONAL VINE BREEDING

Gene editing is going to be important not only for the development of new disease tolerant grapevine varieties, but also to keep local and international varieties resilient to climate change. Indeed, genetic improvement of crops is an important technique for adapting agricultural production to climate change, greater product quality and quantity demands, and product diversification. (Massel K, 2021) (Webb LB, 2007) (Zhang Y, 2018). Traditional breeding is a long-lasting process for crop improvement, as it can takes decades to bring unique crosses to the market. In contrast, direct transfer of genes and other genetic components into elite crops yields genetically modified (GM) varieties with desirable characteristics faster than conventional breeding, however GM goods are impeded by mainly unverified health and environmental safety concerns. (Samaneh Najafi, 2023). Therefore, NGTs are a promising approach for modifying plant genomes regarding their resistance to climate change.

ACCEPTANCE OF GENE EDITING BY THE PUBLIC

During recent years, there have been numerous complains about the introduction of edited crops into agricultural fields because they were considered as GMO. However, on the 5th of July 2023, the European Commission (EC) adopted a proposal to regulate plants obtained by certain new genomic techniques (NGTs) and their use for food and feed. This new European regulation is regarded as a game changer, because the proposal represents the end of a long process that started on the 25th of July 2018 when the European Court of Justice determined that organism created through NGTs were to be regulated as “GMOs” and covered by the EU’s “GMO Directive” of 2001 (Faltus, 2023). However, in 2021 a legislative initiative was started upon a study from the EC concluded that the GMO Directive is not “fit for purpose”. For these reason newer products can be used outside workshops. The NGT or genome editing proposal was

finally presented as part of the adopted package of measures for sustainable use of key natural resources, and it will now be evaluated by the European Parliament and Council of the EU. (USDA)

Following the EC, also the Italian parliament formulated a new proposal under the lead of local agricultural associations and authorities such as CREA, recommending testing the edited plants not only in greenhouses but also in open fields. Hence, promoting a public-private system of genetic improvement based on the most advanced genomic technologies is crucial for keeping the national agricultural sector future-proof and for maintaining it sustainable and competitive. The NGT, within an Italian agricultural context, can contribute to an increase of sustainability and to the production of healthier foods. On this basis, some recommendations are drawn up so that Italy can seize this opportunity, and, to this end, three actions are suggested to political actors:

"•Allow field testing of NGTs in a short time: NGTs are radically different from the GMOs of the past, they cannot be regulated in the same way.

• Relaunch a research program on clean biotechnologies for tomorrow's agriculture: a change in the authorization framework at European level is expected in the coming months and it would be serious if Italy did not present itself at the meeting with an adequate investment program, yes it would risk nullifying all the work done so far.

• Preparing tools for the technological transfer of results from research to the production world, also involving private industries, to renew the varietal panorama and make it suitable for the new climate scenario." (Giannetti, 2023)

Therefore, edited or cis-genesis plants capable of increasing the sustainability of crops through the reduction of phytosanitary treatments as well as advanced knowledge and specialist skills

in an innovative and emerging sector within the agricultural research panorama, will put Italy on a par with other more advanced European countries. (Giannetti, 2023)

In conclusion, the new EU regulation and the pressure on Italian authorities, will pave the way for a promising future of gene editing in plants, and for the development of more efficient gene delivery methods into plant cells.

STATE OF THE ART OF THE GENOME EDITING TECHNOLOGY

Several gene editing techniques are available for modifying the DNA of a living organism and one validated methodology to produce an edited plant is described in the following paragraphs.:

STEP 1: DESIGN OF SYNTHETIC GUIDE RNA (sgRNA)

Nowadays, designing unique synthetic guide RNAs is simple thanks to computational techniques that utilize empirical data derived from previous large-scale studies using genome-wide libraries for genetic screening.. (J. G. Doench, 2016) (Y. Fu, 2013). With other words, computational tools to construct guides with the highest predictive on-target activity and the fewest off-target effects are available.

STEP 2: DELIVERY OF Cas9 AND sgRNAs

Chaotic advection via serpentine channels and co-encapsulation of single cells with plasmids and lipofectamine were described to induce transfection via endocytosis (X. Li, 2018). However, endocytosis has low efficiency in incorporating CRISPR Cas9 into cells (T. Luo, 2019). Microfluidic mechanoporation on the other hand has the advantage to increase significantly the delivery of external macromolecules into cells (Alena Uvizl R. G., 2021) and additionally offers general benefits of microfluidics as it requires low quantities of reagents for

high-throughput investigations, and improved spatiotemporal control of target cells (Jeongsoo Hur, 2021)

STEP 3 AND 4: VALIDATION AND PROPAGATION OF GENE-EDITED CELLS

Gene-edited cells are often isolated via an antibiotic selection method or by using a fluorescent marker for fluorescence-activated cell sorting (FACS). Identifying genetically modified cells is essential to determine whether the desired modification has been incorporated into the target gene. The confirmation of successful deletions or insertions can involve different strategies, ranging from phenotypic (e.g., fluorescence microscopy) and genotypic profiling (e.g., mismatch detection) to genomic sequencing or even disease modeling using "organ-on-chip" devices (L. A. Low, 2020). Finally, to ensure healthy cellular expansions, numerous cell clones must be maintained. In this thesis the microfluidic mechanoporation was tested for its potential to deliver Cas9-GFP protein into grapevine protoplasts.

PRODUCTION OF GRAPEVINE PROTOPLAST CELLS AND THEIR REGENERATION

The production of protoplast, which are cells without cell wall, is an essential step for the correct delivery of CRISPR-Cas9 RNPs into grapevine cells. The procedure to obtain protoplasts from grapevine is given hereafter:

PLANT MATERIAL AND INDUCTION OF EMBRYOGENIC CALLI

Leaf segments cut from *in vitro*-sub-cultured plantlets were initially used to create friable brownish white calli with embryogenic potential of *V. vinzjkra* cv. *Koshusanjaku* according to the method of Matsuta and Hirabayashi (N. Matsuta and T. Hirabayashi, 1989) with several modifications (M. Nakano, 1994). The embryogenic calli were maintained by subculturing

monthly onto Nitsch's medium (J.P. Nitsch and C. Nitsch, 1969) lacking vita mins, inositol and glycine but supplemented with 1 PM 2,4-D and 30 g/l sucrose and solidified with 2 g/l gellant gum (Wako Pure Chemical Indus- tries). The friable callus cultures containing no appreciable embryos established after several subcultures were used as the source of protoplasts (Yan-Ming Zhu", 1997).

However, to obtain grapevine calli, which are able to produce plants, they must be obtained by flowers as described in (Simone Scintilla, 2022). Calli of grapevine are obtained as described below: Crimson seedless and Sugraone embryogenic calli were started from immature inflorescences that were gathered from a vineyard in San Michele all'Adige. (Trento, Italy). Flowers were surface sterilized for 20 minutes in diluted bleach (3% active hypochlorite), subsequently rinsed with sterile distilled water for 20 minutes and stored in the fridge at 5°C. After two to four days, anthers with filaments and ovaries were cut under a stereomicroscope and processed as described in Martinelli et al. (Martinelli, 2001) (Simone Scintilla, 2022)

PROTOPLAST ISOLATION AND CULTURE

Protoplasts were isolated from 1 g of embryogenic callus of either Sugraone or Crimson S. in 13 ml of enzymatic mixture composed of 1% (w/v) cellulase Onozuka R-10 and 0.3% (w/v) macerozyme R-10 (Duchefa Biochemie, Haarlem, The Netherlands) plus 0.2% (w/v) hemicellulase (Merck KGaA, Darmstad, Germany) dissolved in Gamborg B5 including vitamins (Duchefa Biochemie, Haarlem, The Netherlands) and 0.45 M mannitol (Table 1), in sterile conditions. The suspension was mixed on a tilt shaker at 25°C for 16 hours in the dark, and then filtered through a 60 mm nylon sieve (Millipore, Burlington, MA, U.S.A). Protoplasts were collected by centrifugation at 80 g for 4 minutes without brake, washed in MMG solution (Table 1), and further purified on a 16% w/v sucrose cushion by centrifugation (90 g, 4 minutes,

no brake). Protoplasts were then checked for plasma membrane integrity through FDA staining as described before (Huang et al., 1986). Briefly, a 50X stock solution of FDA in acetone was prepared at 5 mg ml^{-1}, added to protoplast suspension in MMG and incubated for 5 minutes before observation in the microscope. Viability of protoplasts was assessed with FDA staining (Widholm., 1972).

Purified protoplasts were cultured at 1 x 10^5protoplasts/ml in a 60 x 15 mm plastic Petri dish (Falcon 1008, Becton Dickinson, New Jersey) by embedding in 2 g/l gellant gum-solidified Nitsch's medium containing 2 mg/l NAA, 0.5 mg/l BA, 0.3 M glucose and 0.09 M sucrose. The method used for embedding the protoplasts in gellant gum medium was the same as reported in (M. Mii, 1991).

Protoplasts were mainly cultured using the disc-culture method described by Dons and Bouwer (J.J.M. Dons and R. Bouwer, 1986) with following minor modifications: five small droplets (each 0.8 ml) containing protoplasts in culture medium were quickly poured with a pipette into 60 x 15 mm plastic Petri dish. After solidification, 4 ml of liquid Nitsch's medium containing 2 mg/l NAA, 0.5 mg/l BA, 0.3 M glucose and 0.09 M sucrose was added as a reservoir. The liquid medium was replaced every 2 weeks by fresh medium described above but lacking glucose. The pH of culture media was adjusted to 5.7 prior to autoclaving. All dishes were sealed with Parafilm@ and maintained at 25°C in the dark. Plating efficiency, defined as the percentage of dividing protoplasts, was estimated after 3 weeks of culture. The percentage of colonies, each of which consisted of more than 5 cells, was estimated after 6 weeks of culture. The number of somatic embryos was recorded after 3 or 4 months of culture (Yan M, 2010).

PLANT REGENERATION

After 4 months of culture, protoplast-derived torpedo embryos (l-2 mm long) were transferred to Nitsch's medium supplemented with 30 g/l sucrose and 2 g/l gellan gum for germination. Regenerated plantlets with well-expanded five to six leaves were washed carefully to remove the gellan gum and transferred to pots (9 x 9 cm) containing vermiculite. Potted plants were acclimatized in a transparent plastic cabinet covered with polyethylene bags at 20℃ under 24 h illumination (45 pmol/m' per s) with fluorescent lamps. After 2-3 weeks, acclimatized plants were transferred to the greenhouse. (Yan-Ming Zhu", 1997)

Table 1 Reagents

Name/Abbreviation	**Composition**
W5	2mMMES·H_2O,154mMNaCl,125mMCaCl2 ·$2H_2O$,5mMKCl,pH5.7
MMG	4 mM MES · H2O, 0.4 M mannitol, 15 mM MgCl2, pH 5.7
WI	4 mM MES · H2O, 0.5 M mannitol, 20 mM KCl, pH 5.7
PEG-Calcium	0.2 M mannitol, 100 mM CaCl2 · 2H2O, 40% (w/v) PEG4000
B5 solution for callus digestion	Gamborg's B5 salts including vitamins, 0.45M mannitol, 1% (w/v) Cellulase Onozuka R-10, 0.2% (w/v) Hemicellulase, 0.3% (w/v) Macerozyme R-10, pH 5.7
Sucrose solution	16% (w/v) sucrose, pH 5.7
Alginate solution	0.5M mannitol, 1.6% (w/v) sodium alginate
Calcium-agar	0.4 M mannitol, 50 mM mM CaCl2 · 2H2O, 1.4% (w/v) plant agar, pH 5.7
Media	
Nitsch and Nitsch based liquid medium for protoplast culture (NNp)	Nitsch and Nitsch salts including vitamins, 88mM sucrose, 300mM glucose, 0.1% (w/v) activated charcoal, 0.93 mM kinetin, 2.22 mM 6-BAP, 10.7 mM NAA, pH 5.7

Solid medium for embryo development (GISCA)	Nitsch and Nitsch salts including vitamins, 132mM sucrose, 300 mM glutathione, 0.25% (w/v) activated charcoal, 2.22 μM 6-BAP, 10.7 μM NAA, pH 5,7
Nitsch and Nitsch solid medium for plant Nitsch and growth and propagation	Nitsch and Nitsch salts including vitamins, 66mM sucrose, 0.67% (w/v) plant agar, pH 5.75

Chemicals	**Supplier**
Plant agar	Duchefa Biochemie Cat. P1001
Low melting agarose PPC (LMPA)	Duchefa Biochemie Cat. L1204
GelriteTM	Duchefa Biochemie Cat. G1101
MES monohydrate	Duchefa Biochemie Cat. M1503
Active charcoal	Duchefa Biochemie Cat. C1302
Sucrose	Duchefa Biochemie Cat. S0809
Glucose monohydrate	Duchefa Biochemie Cat. G0802
Nitsch	Duchefa Biochemie Cat. N0224
Gamborg's B5 medium including vitamins	Duchefa Biochemie Cat. G0210
Cellulase Onozuka R-10	Duchefa Biochemie Cat. C8001
Macerozyme R-10	Duchefa Biochemie Cat. M8002
Hemicellualse from A. niger	Sigma-Aldrich Cat. H2125

6-Benzylaminopurine (6-BAP)	Duchefa Biochemie Cat. B0904
Kinetin	Duchefa Biochemie Cat. K0905
a-Naphtalene Acetic Acid	Duchefa Biochemie Cat. N0903
b-Naphtoxyacetic Acid	Duchefa Biochemie Cat. N0912
Indole-3-acetic acid (IAA)	Duchefa Biochemie Cat. I0901
Polyethylene glycol (PEG) 400	Sigma-Aldrich Cat. 8.07490
Cas9 protein	ThermoFisherTM Cat. A36499
Single-guide RNA (sgRNA)	Merck Custom gRNA
Cas9-GFP protein	Sigma-Aldrich Cat. CAS9GFPPRO
Fluorescein diacetate (FDA)	Sigma-Aldrich Cat. F7378

Table 1 chemicals used for gene editing and plant regeneration

GENE-EDITING AND MICROFLUIDICS

NUCLEIC ACIDS DELIVERY VIA NANOPARTICLES.

The cell membrane is largely impermeable to macromolecules. To facilitate membrane poration or endocytotic delivery of the target molecule, several techniques utilize polymeric nanoparticles (Slowing II, 2007) (Pack DW, 2005), liposomes (J, 1997), or chemical modifications (Verma A, 2008) such as cell-penetrating peptides (CPPs) (Heitz F, 2009) (Duan H, 2007). The efficacy of the delivery vehicle by traditional methods often depend on the structure of the target molecule and the cell type, making them efficient for the delivery of structurally uniform materials such as nucleic acids, but often ineffective for the delivery of

more structurally diverse materials such as proteins and some nanomaterials (Yan M, 2010) (Shi Kam NW, 2004) (Derfus AM, 2004).

Furthermore, the endosome escape mechanism, on which most of these approaches rely, is frequently inadequate, resulting in a large amount of material remaining trapped in endosomal and lysosomal vesicles (Varkouhi AK, 2011). However, more effective gene delivery methods, such as viral vectors (Waehler R, 2007) (Y-C, 2008), often risk chromosomal integration and are limited to DNA and RNA delivery (Anton, 2023). Nevertheless, PEG is still the most commonly used chemical used to transfect grapevine protoplasts, yet, as motioned before it is toxic to all organisms and reduces cell proliferation (David A. Herold ∗, 1982).

Here, a rapid mechanical deformation of cells might represent a suitable and innovative method for high-throughput cytosolic delivery of macromolecules, such as CRISPR-Cas9 RNPs, into living grapevine cells. Microfluidic mechanoporation enables gentle and efficient transfection of cells by rapid contact-based cell deformation due to fast passing cells through constrictions with a minimum dimension lower than the cell diameter, resulting in transient membrane pores

that promote passive diffusion of molecules into the cytoplasm. (Figure 1). (Armon Shareia, 2012)

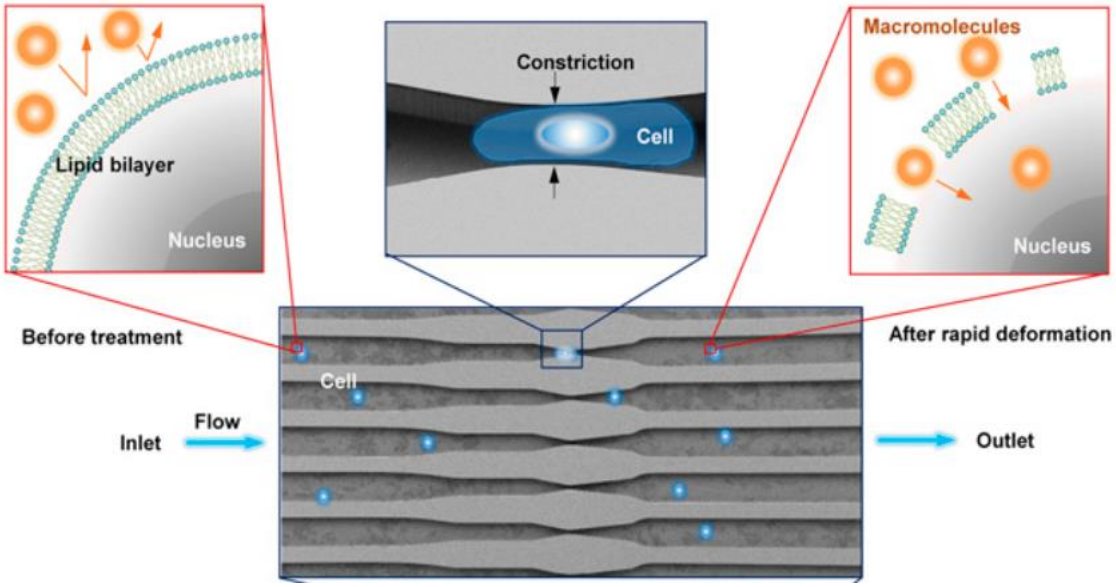


Figure 1 Schematic representation of microfluidic mechanoporation (reprinted from Armon Shareia et al, 2023 under creative commons attribution 4.0)

AIM OF THE THESIS

The aim of this thesis was to investigate a new method to deliver clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9) ribonucleoprotein complexes into grapevine protoplasts using a microfluidic platform. This was possible by modification of protoplasts purification protocol described in (Simone Scintilla, 2022) and a new method developed to obtain callus from woody canes.

MATERIAL & METHODS

WORK DESIGN

A basic requirement for the development of an efficient microfluidic technique to deliver CRISPR-Cas9 RNPs into grapevine cells was to study in detail also all steps of protoplast regeneration starting from the production of embryogenic callus. Initially, it was necessary to determine differences between calli produced from flowers' anthers of different grapevine varieties, that have been either obtained from acclimated shoots (flowering woody cuttings) or from flowers collected from vineyards. Then, the protocol for callus purification needed to be modified because the original protocol resulted in a great quantity of cell debris and in cells with nonuniform shapes.

EMBRIOGENIC CALLUS OBTAINED FROM VINE WOODY CANES

New embryogenic vine callus can only be obtained from anthers, and flowers can be collected only in a few months of the year. In addition, finding the perfect conditions in the field is difficult and sometimes challenging due to the presence of high phenological heterogeneity. Consequently, to maximize the chances to obtain a good quality callus and to have the possibility to start new embryogenic callus along the year we have investigated the use of woody canes as starting material.

TEST

Firstly, shoots were cut from the point of insertion and stored at 4°C in sealed plastic containers for three months (figure 2-A), to allow a complete differentiation of the buds. After that period shoots were cut in woody cuttings of 3 buds length (figure 2-B). Then, the cuttings were processed by immersion in chests containing a fungicide to prevent gray mold (figure2-,D). After 48 hours the woody canes were initially lacked with paraffin, introduced in baker filled

Figure 2 Preparation of protoplasts from woody cane A) woody cuttings, B) shoots cut from point of insertion, C) prevention of gray mold using an anti-Botrytis compound.

with water and cultured into perlite under cool fluorescent day light (15 h photoperiod at 30 J.lmol m·2 s·1 tight intensity). The cuttings were first cultured at 28 ± 2°C at 100 % humidity

for 2 weeks and then exposed to 24 ± 2 ·c at 50-70 % humidity. Immature inflorescences were obtained within 6-8 weeks using the method described by Mullins (MULLINS, 1141 - 1148). Then, new inflorescences are formed with a diversification in times of budburst (figure 3-A,B). A few inflorescence were cut out from the canes and single flowers were selected under binocular microscope and dissected to separate anthers and ovary using tweezers and bistoury.

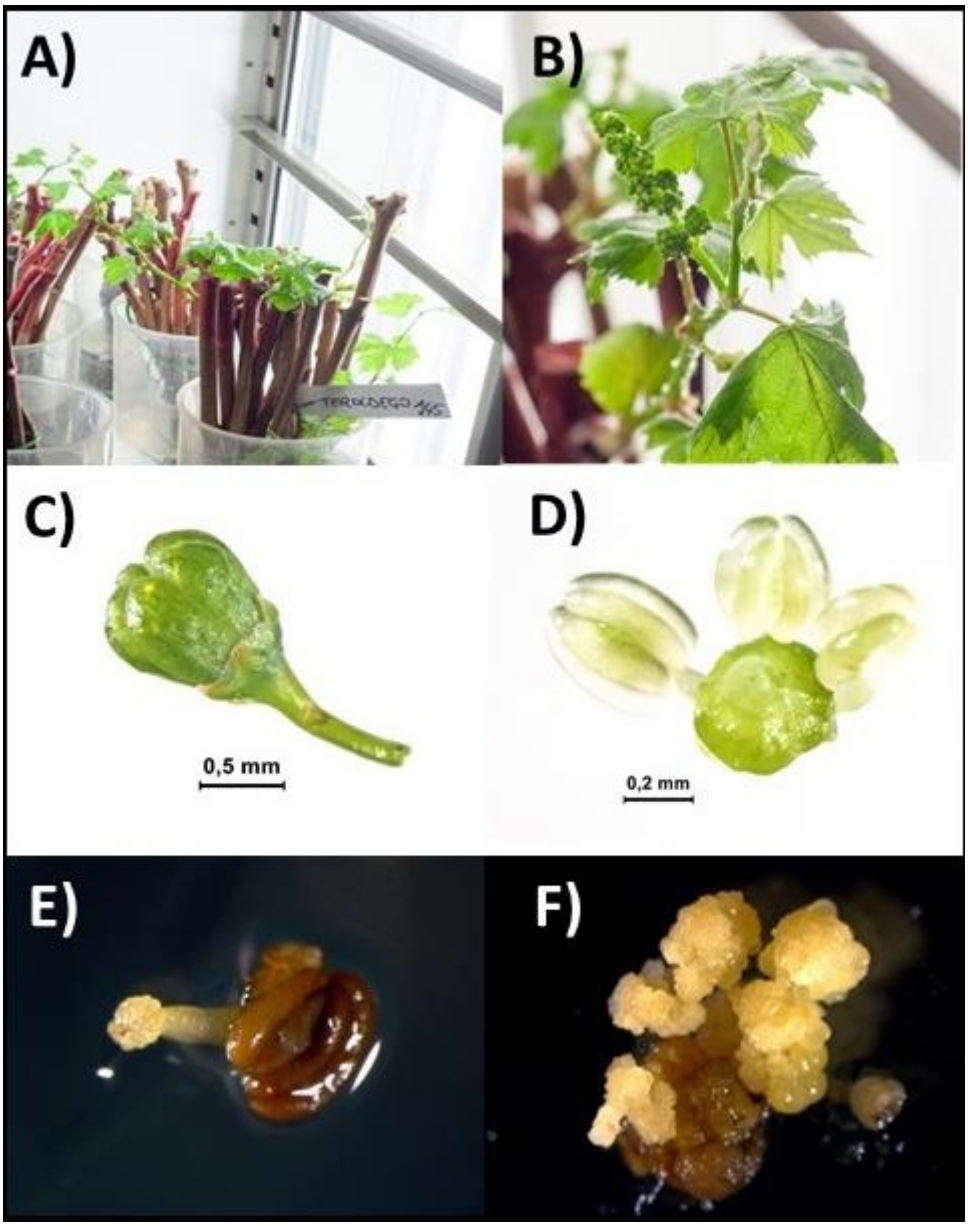


Figure 3 Procedure for obtaining flowers from cuttings till formation of embryogenic callus, A) woody cuttings budburst, B) inflorescence formation, C) flower with caliptra, D) ovary and anthers after flower dissection, E) callus formation from anthers phase 1, F) callus formation from anthers phase 2

Then anthers were placed on three different cultural media with different hormones concentration as described in Table 1. The phases leading to callus formation are shown in Figure 3: first phase was flower isolation (fig.3-C), the second step (figure 3-D) was anthers extraction from flowers by calyptra removal, last step (figure 3-E,F) consists in the formation of new undifferentiated cells (callus) from the anthers.

PROTOPLAST PURIFICATION FROM CALLUS

The existing protocol for purification of protoplasts had to be adapted for microfluidics, in order to reduce the clocking of microchannels, mainly caused by non-uniformly sized cells and the presence of cell debris.

TEST

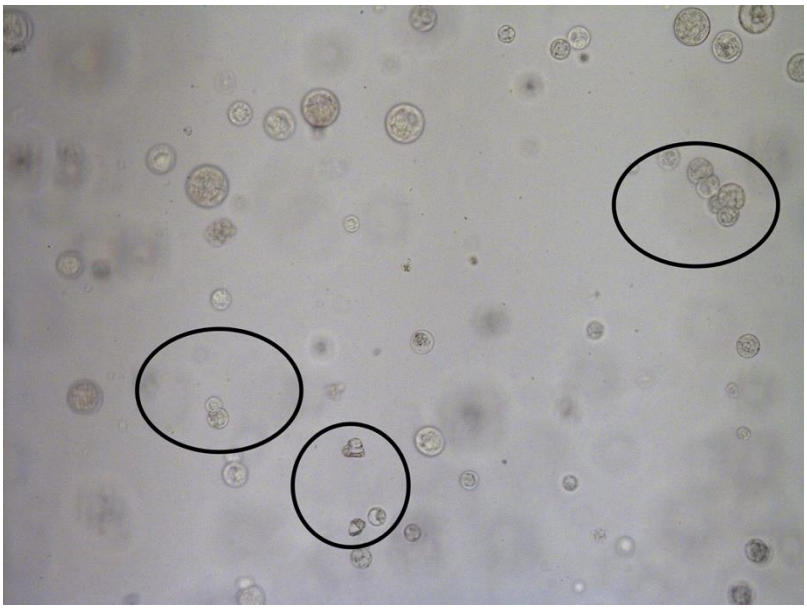


Figure 4 Optical microscopy of debris (highlighted by black circles)

To determine the quality of each protoplast purification, the quantity of debris (figure 4, highlighted by circles), which includes cell aggregates and damaged protoplasts which were all

evaluated as debrides. For this purpose, the established protocol was modified by the addition of two essential steps: A) a second sucrose purification to reduce cell debris and B) a double filtration with 40 µm CORNING cell strainers to obtain uniform cells. These modifications yielded in good quality protoplast with uniform cell size and a significant reduction of debris. In addition, Fluorescein diacetate (FDA) staining has been used to monitor protoplasts viability as described elsewhere (HESLOP-HARRISON J, 1970) (JM, 1972), (PJ, 1976), (EVANS DA, 1981).

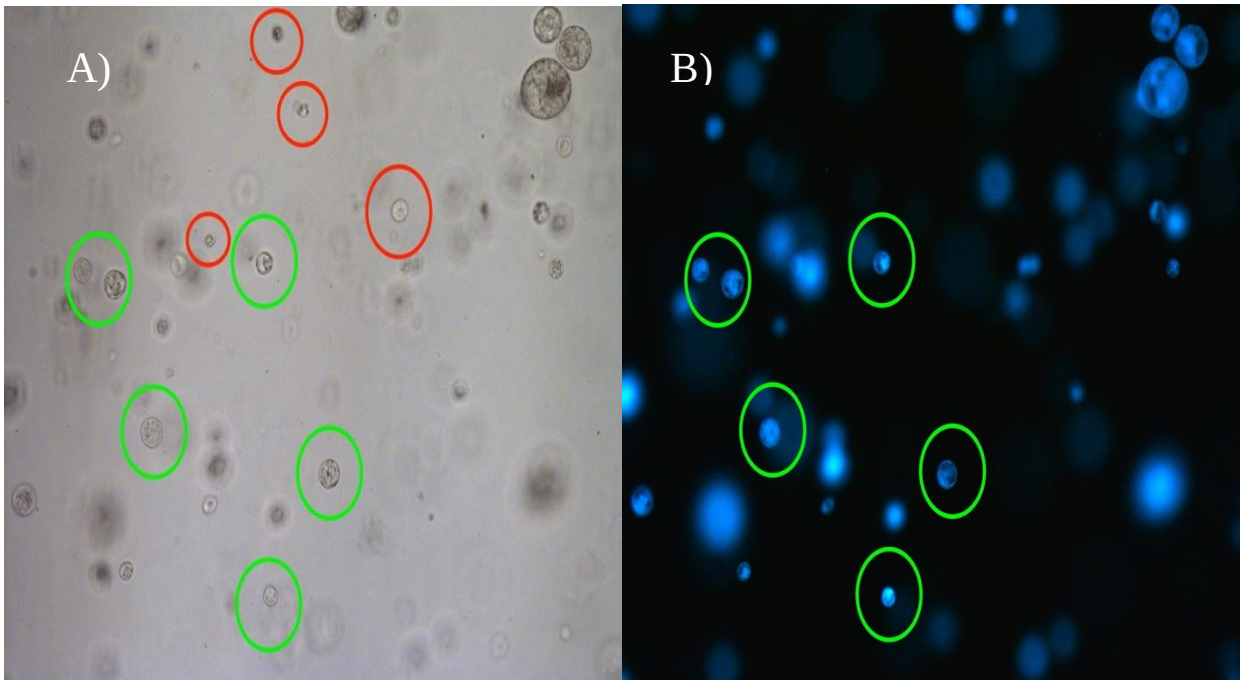


Figure 5 FDA staining of protoplast. Green circles: viable protoplasts; red circles: dead protoplasts

The principle of staining with FDA relies on the nonpolar FDA molecule crossing the plasma membrane and its ester bonds being hydrolyzed in the cytoplasm to release fluorescein. The polar fluorescein molecule remains in the cytoplasm because it cannot pass through either the plasma membrane or the tonoplast of living cells (ROTMAN B, 1966), (HESLOP-HARRISON J, 1970). Living cells are therefore distinguished by their bright fluorescence when excited with blue light (Figure 5 B). Red circles highlight protoplasts that are not viable as they show no

fluorescence (Figure 5 B) and are only visible on the brightfield image (Figure 5 A) while green circles highlight vital protoplasts (Figure 5) (C.-N. HUANG 1, 1986)

All tests were performed with protoplast obtained from 3 different grapevine varieties Merlot, Crimson and Chardonnay. Negative controls were produced as described in the original protocol (one filtration and a single washing step with sucrose) to ensure if there was a real difference from standard method, but in addition to the original protocol protoplasts were filtered using filters 40 μm CORNING filter to ensure to have the same dimension of protoplasts. Then, purified protoplasts were embedded in alginate disks as previously described (Simone Scintilla, 2022).

Finally, alginate disks were observed in a Zeiss Axio Imager Z2 fluorescence microscope and images were taken using an Axio Cam (Zeiss). For each alginate disk 10 spots were analyzed. Debris were counted using a counting chamber (FAST-READ 102®, Biosigma s.r.l.) at 10X magnification.

MICROFLUIDICS

Single-cell analyses are of paramount importance to understand biological processes in detail. Furthermore, the regeneration of plants from a single cell will eliminate chimerism. To study protoplast regeneration on single-cell basis a microfluidic device was tested that enabled the encapsulation of single protoplasts into individual monodisperse droplets.

The establishment of the microfluidic mechanoporation technique for grapevine protoplasts required several steps including but not limited to the choice and composition of the running buffer as well as the adjustment of flow parameters and cell concentrations. For this purpose, a series of different saline solutions with adjusted osmolarity and pH has been tested for their suitability. While most of the tested solutions affected either flow parameters or cell viability,

the MMG solution proved to be an ideal choice for the stability and longevity of protoplasts after the passage through the microfluidic device. The age and general condition of the starting material was another important factor; with other words, the better the condition of the callus used for protoplast purification, the better was the outcome. Additionally, the timeframe between protoplast purification and the experiment needs to be kept as short as possible (<3hours) and the size of protoplasts as uniform as possible and within the range of the microfluidic channels. To adjust cell size and concentration (approx. 350000 - 400000 cells) and to greatly reduce debris that can clock the channels of the device, the existing purification protocol needed to be modified as described above.

Finally, the viability of protoplasts that passed the microfluidic device was recorded to verify which of the studied approaches was best suited to reproduce callus.

TEST

DROPLET MICROFLUIDICS (PROOF OF CONCEPT):

A flow-focusing microfluidic droplet generator device was used to encapsulate single protoplasts into individual monodisperse droplets. Here, the injected dispersed aqueous phase (buffer containing protoplasts) is sheared by the continuous oil phase to form water-in-oil droplets.

MECHANOPORATION:

A microfluidic mechanoporation device was used to introduce a DNA plasmid for Agrobacterium transformation (binary vector) into Merlot protoplasts. The experiment was conducted using 0.4 ml of cell solution (cell concentration approx. 350000 cells/ml) together with 20 μg of pKGWFS7 vector (Karimi, 2002) but without PEG. After passage through the

microfluidic device transfected protoplasts were embedded in alginate disks as previously described (Cankar, 2021). Fluorescence was checked after 24h in a Leica DMi8 laser scanning confocal microscope at a magnification of 10x and 20x. GFP was excited at 488 nm and detected in the 510-560 nm range.

After the successful introduction of the plasmid into Merlot protoplasts using mechanoporation we tested this novel single-cell approach for DNA-free transfection of CRISPR-Ca9 RNPs. To this end protoplasts isolated form Merlot and Chardonnay were mixed with 40 mg of Cas9-GFP protein (Thermo Fisher Scientific, Waltham, MA, USA) and subsequently run on the microfluidic device. Fluorescence of protoplasts was checked after 48 hours in a Nikon Ti2-E fluorescence microscope at a magnification of 10X and 20x. Pictures were taken using an ORCA Flash 4,0 V3 camera (Hamamatsu Photonics).

PROTOPLAST PROPAGATION AND EMBRYO REGENERATION

Protoplasts purified from Merlot callus were used to test if they can propagate and regenerate an embryo after mechanical deformation inside the microfluidic device.

TEST

Therefore, protoplasts that passed the microfluidic device were embedded in alginate disks as described above and monitored every 24h under a binocular microscope. Embedded protoplasts were kept in liquid solution (see figure 8) till aggregation reached head stage and were subsequently transferred on C1p terrain.

RESULTS

EMBRIOGENIC CALLUS OBTAINED FROM VINE WOODY CANE

Propagation Results are showed in table 3 on second table column. Media used were: GR1, B-medium and P4. Then, the following table (table 4) depicts which woody canes were able to produce callus. The results show that four grapevine varieties and clones of interest, namely Chardonnay, Teroldego, Marzemino and EcoIASMA1 were able to produce callus form woody canes. However, other varieties and clones were unable to generate callus such as Traminer (cl.Iasma1) and Pinot-Gris (cl. Iasma-Avit 513). In contrast, some varieties, such as Lagrein, (Isma 216), Teroldego (SMA 133) and Glera were able to generate callus from flowers sampled in the field but not from woody canes. Consequently, there are some positive results, but further studies are needed to understand why only some varieties can generate callus from woody canes.

CULTIVAR/ CLONE	**DATE OF BURST**	**GR1** (2,4 D/BAP=2)	**B-medium** (2,4 D/BAP=1)	**P4 (2,4** D/BAP=0.5)	**TOTAL NUMBER OF TRANSFERRED INFLORESCENCES**
Chardonnay SMA 130	3rd May	30	15	30	75
	5th May	75	60	45	180
	8th May	25	5	0	30
Chardonnay SMA 108	3rd May	10	20	30	60
	5th May	25	10	15	60
	8th May	20			20
Lagrein Isma 216	11th May	50	10	35	95
Teroldego SMA 133	11th May	45	35	40	120
Teroldego SMA 145	11th May	45		40	85

Marzemino MR14	11th May	45	20	45	110
Glera	9th May	15	45	55	110
	11th May	lost for contamination			
	13th May	35	30	55	120
EcoIASMA1	14th May			55	55
	16th May	55		20	75
	18th May	105		20	125
Traminer Iasma1	11th May	40		50	90
Pinot-Gris Iasma-Avit 513	11th May	30	10	50	
Merlot ISV FV2	only fruiting cuttings				

Table 2 Number of woody cane flowers transferred onto solid medium to produce embryogenic callus

CULTIVAR/CLONE	**RESULT**
Chardonnay SMA 130	Positive
Chardonnay SMA 108	Positive
Lagrein Isma 216	Regeneration only from plant cutting
Teroldego SMA 133	Regeneration only from plant cutting
Teroldego SMA 145	Positive
Marzemino MR14	Positive
Glera	Regeneration only from plant cutting
EcoIASMA1	Positive
Traminer Iasma1	No regeneration
Pinot-Gris Iasma-Avit 513	No regeneration
Merlot ISV FV2	Only fruiting cuttings

Table 3 Summary of embryogenic callus production from the woody canes test

PROTOPLAST PURIFICATION FROM CALLUS

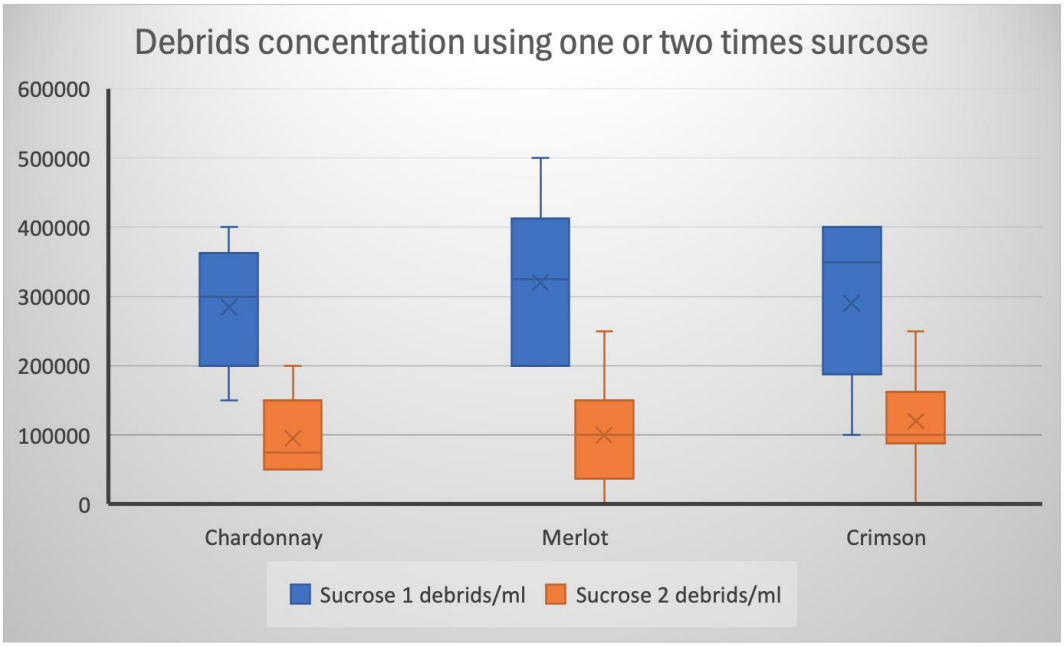
	Sucrose 1 debris/ml	Sucrose 2 debris/ml
Chardonnay	400000	50000

Chardonnay	200000	100000
Chardonnay	400000	50000
Chardonnay	200000	50000
Chardonnay	350000	50000
Chardonnay	350000	150000
Chardonnay	350000	150000
Chardonnay	250000	200000
Chardonnay	200000	100000
Chardonnay	150000	50000
Merlot	500000	0
Merlot	400000	100000
Merlot	350000	150000
Merlot	250000	100000
Merlot	200000	250000
Merlot	300000	50000
Merlot	350000	150000
Merlot	200000	100000
Merlot	200000	0
Merlot	450000	100000
Crimson	200000	150000
Crimson	100000	0
Crimson	350000	100000
Crimson	150000	100000
Crimson	350000	50000
Crimson	200000	100000
Crimson	350000	150000
Crimson	400000	100000
Crimson	400000	250000
Crimson	400000	200000

Table 4 Debris concentrations in diverts analyzed samples.

Debris concentrations were calculated using a student's t-test (P= 0,05) and are given in Figure 6 and table 5 while protoplast viability is given in table 7. Firstly, it was posed H0=0 to demonstrate that there were no difference between the means. Then it was calculated mean square a standard deviation which were used to calculate standard error. Finally, it was verified if there were difference between t value and t0 (Livingston, 2004) A significant difference between one or two washing steps was observed. Moreover, table 7 clearly demonstrates that the number of washing steps did not influence cell viability as confirmed by one-factor ANOVA

Figure 6 Debris concentration. Box chart showing difference between 1 sucrose wash and 2 sucrose wash.



(P=0,05, F = 4,9646), suggesting that the use of two washing steps with sucrose is advisable and sufficient to reduce debris and to obtain clean cells.

GRAPEVINE CULTIVAR		MEAN	STANDARD DEVIATION	T -test RESULT	T- test CRITICAL

MERLOT	SUCROSE 1	320000	108525	4,7142	2,2621
	SUCROSE 2	100000	74536		
CHARDONNAY	SUCROSE 1	285000	94428	5,4596	2,2621
	SUCROSE 2	95000	55025		
CRIMSON	SUCROSE 1	290000	114988	5,6667	2,2621
	SUCROSE 2	120000	71492		

Table 5 Results of t-statistics. H0=0, calculated means standard deviations and verifying if T0 is minor than T critical

	Sucrose1 cells vitality	Sucrose 2 cells vitality
Chardonnay	100	100
Chardonnay	100	100
Merlot	80	100
Merlot	100	66,67
Crimson	100	100
Crimson	100	75

Table 6 FDA staining of protoplasts following debris removal with two different methods as described in M&M (1 sucrose wash and 2 sucrose wash.)

MICROFLUIDICS RESULTS

DROPLET MICROFLUIDICS

Because of Poisson distribution droplets contained single protoplasts, however, as expected, the great part (approx. 91%) of droplets were empty. This proof-of-concept test demonstrated that it is possible to apply droplet microfluidics to grapevine protoplasts, thus, opening the possibility to perform a wide variety of single-cell experiments.

MECHANOPORATION:

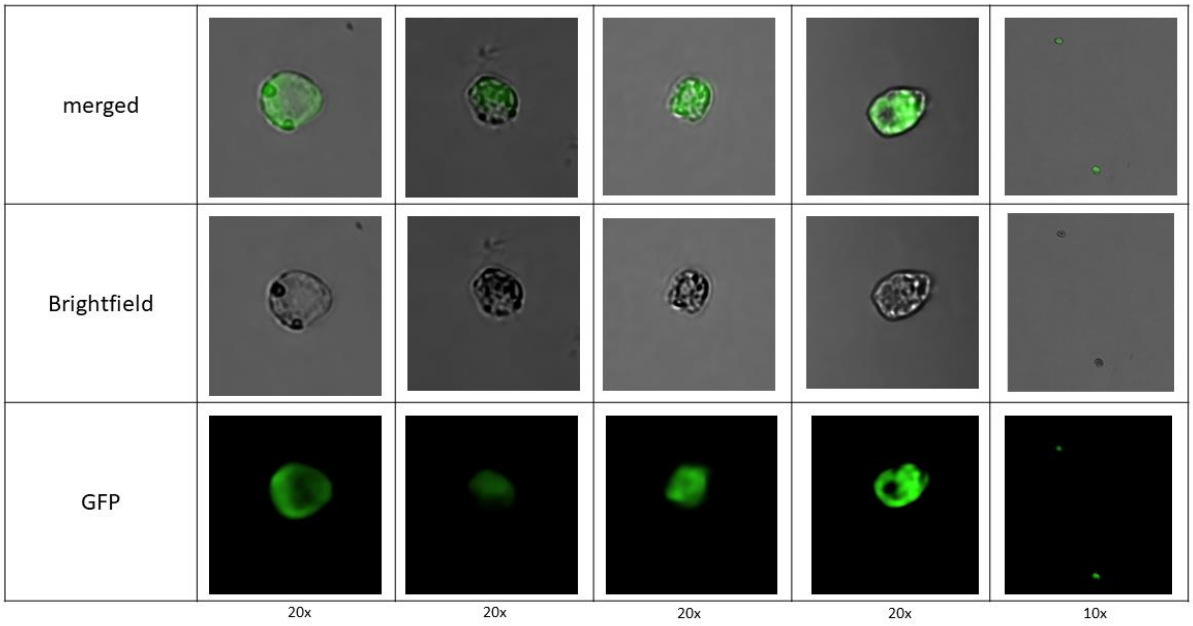


Figure 7 Chardonnay protoplasts 48h after delivery of Cas9-GF

All microfluidic approaches were successful, yet the most promising result was the highly efficient delivery of Cas9-GFP protein into Chardonnay protoplasts and the confirmation of fluorescence in the cytosol and nuclei 48h after transfection (Figure 7). Roughly 10000 cells/ml passed the device in very good shape and approx. 96% of these cells were fluorescent and vital

for >2weeks after transfection. This clearly demonstrates that microfluidics can be successfully applied to study grapevine protoplasts at single-cell level and that it is well suited for a highly efficient and gentle intracellular delivery of membrane-impermeable cargo molecules such as Cas9 protein complexes.

PROTOPLAST REGENERATIO AFTER MECHANOPORATION

After 4 days microcolonies formed and after 18 days embryo like structures were visible(figure 8). The formation of microcolonies was 4 times faster than previously described (Simone

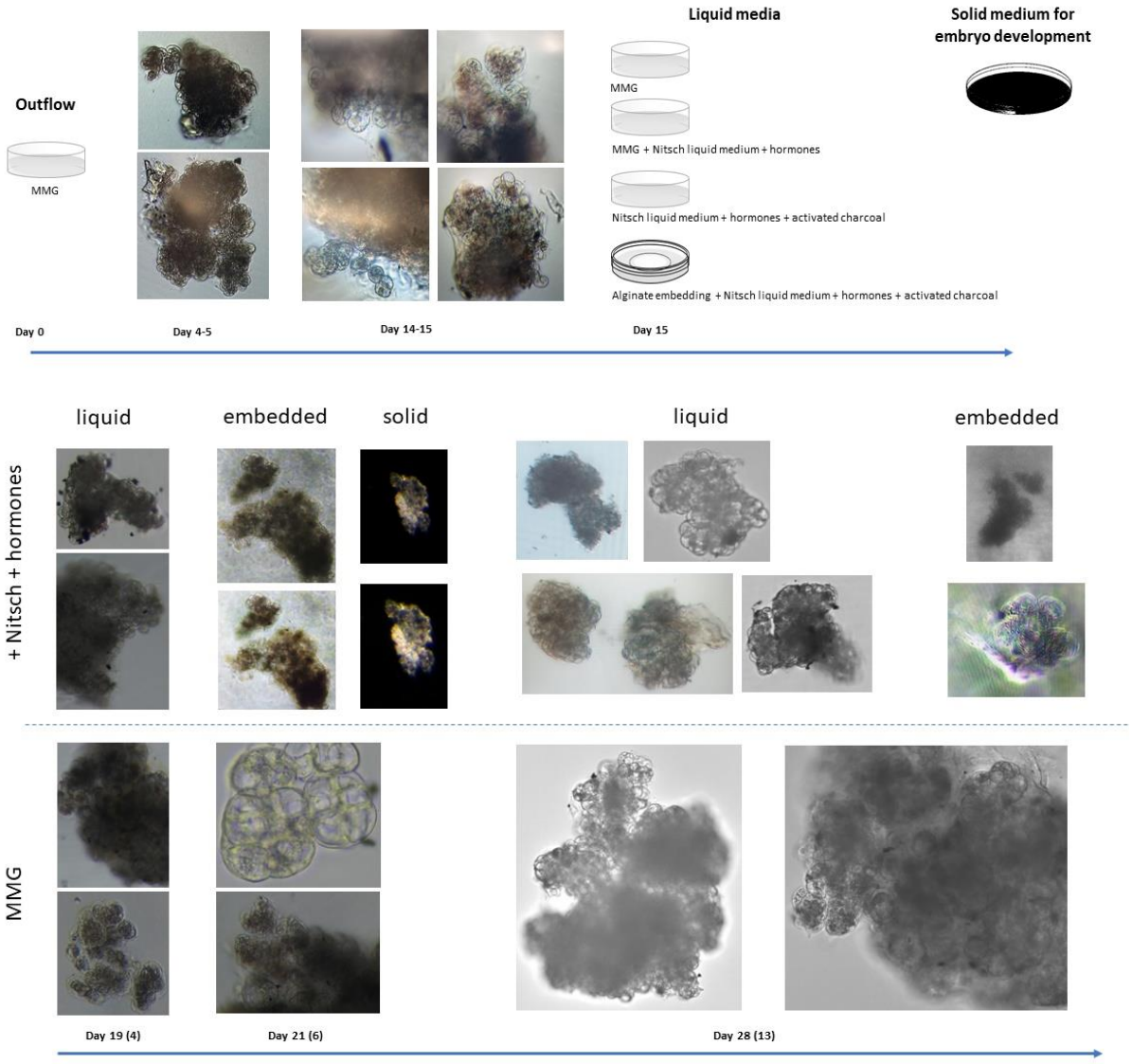


Figure 8 Regeneration of Merlot protoplasts following microfluidic mechanoporation.

Scintilla, 2022), which is a promising result (Figure 8). Further experiments are necessary to test if such microcolonies and embryos can produce plants and to test if there is no chimerism during plant formation.

DISCUSSION

The fascinating result that Cas9-GFP was present in approximately 96% of the protoplast 48h, after delivery by microfluidic mechanoporation, while only a minor fraction of protoplasts were edited in previews transformations (20%) (Simone Scintilla, 2022). This allows the assumption that this innovative technology has a great potential to improve considerably DNA-free gene-editing in plants. Moreover, the regeneration of embryogenetic callus from protoplasts that passed the microfluidic device and their good viability show great promise as well as the method reported in (Simone Scintilla, 2022) where 99% of cells were vital. However, so far this technology has been only tested on three grapevine varieties of global importance, hence, to account for the significant genetic differences between grapevine varieties (Pelsy, 2010), it is important to confirm the results in more varieties. Furthermore, the regeneration of plants from edited protoplasts would be another important step that should be studied in the future. Because grapevine is often resistant to transformation and regeneration, and there are numerous chimeric mutants only a few studies on grapevine regeneration have been published so far. (Edoardo Bertini, 2019)

A preliminary test using mechanoporation for the delivery of CRISPR-Cas9 RNP complex into protoplasts of the varieties Merlot, Chardonnay, and Crimson was done at the very end of this thesis, therefore, the missing steps including DNA extraction, amplification and sequencing could not be performed. The results of this experiment will be important to determine the efficiency of target gene modification inside the living protoplast. Moreover, chimeric formation during callus and plant regeneration needs to be verified, because chimerism is one of the most challenging issues of plants obtained by NGT (Simone Scintilla, 2022).

The promising results obtained during this thesis may be the basis for future studies to develop a new procedure for gene-editing and breeding of grapevine.

CONCLUSION

The urgent need for plants that are both more tolerant to climate change and more resistant to diseases requires novel breeding technologies. The international community has understood this necessity and released a new regulation for NGTs to facilitate their implementation for genome editing in plants. Here, gene-editing technologies that do not introduce foreign DNA into plant protoplasts may mitigate public concerns against genetically modified plants.

In this thesis a new microfluidic method was developed to deliver macromolecules, such as the Cas9 protein, into grapevine protoplasts. The modification of the protoplast purification protocol and the development of the microfluidic method resulted in a successful and efficient delivery of Cas9-GFP protein into Chardonnay protoplasts without the addition of PEG. Future research should be directed to verify if also CRISPR-Cas9 RNPs can be delivered into protoplasts using this innovative technology and to subsequently confirm the effective modification of genome. The work presented here demonstrates that this new DNA-free approach, which relies on mechanical cell-deformation, has great potential to be an alternative to existing gene-editing technologies in grapevine.

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