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TITLE OF THE DOCTORAL THESIS

DNA-free Gene editing in *Vitis vinifera* L.; Knockout green fluorescent protein gene in Sultana grape by direct delivery of RNPs into protoplast

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DNA-free Gene editing in *Vitis vinifera* L.

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Contents

Abstract

Global agricultural productivity, farm incomes, and food security will all be impacted by climate change. Grape (*Vitis vinifera* L.) is one of the world's most commercially significant fruit crops, and it is extensively cultivated for fruits, juice, and, most importantly, wine. Scientific evidence sharply states that climate change represents a dominant challenge for viticulture in the upcoming decades. Agriculture and farming around the world are highly depended on crops that produce food and fiber for humans, either directly or indirectly through livestock. Modern technology has improved agricultural operations over the last two centuries, complementing traditional plant breeding approaches to improve crop productivity and quality. Combining synthetic tools and traditional breeding into genomics-based breeding is a novel way to get over the limitations of traditional breeding. Genome editing offers the potential to speed up basic research and plant breeding by allowing for quick, accurate, and targeted genome editing.

The revolutionary CRISPR-Cas system offers enormous potential for editing gene expression for crop improvement and food production.

Genome editing is a powerful way to find and precisely locate a specific region inside a genome, then edit the targeted regions for a variety of applications. Unlike traditional transgenic technology, which randomly introduces genetic components into a genome, genomic editing operates on a specific genome sequence inside the genome.

The risks of altering genomes via genome-editing technologies are considerably less than those associated with genetically modified (GM) crops since most edits typically affect a few nucleotides leading to changes similar to those found in naturally occurring populations. There is no way to tell the difference between a 'naturally occurring' mutation and a gene edit after the genomic-editing agents have separated. This DNA-free RNP delivery approach is promising for plant breeding since the resulting edited crops are likely falling outside of GMO regulation. Consequently,

DNA-free genome editing is a game-changing technique that allow for faster and more precise crop development.

In this study, we described a successful knockout of a green fluorescent protein (GFP) reporter gene, that is already integrated into the grape genome with a single copy, in *V. vinifera* "Sultana" by direct delivery of RNPs into protoplast. We demonstrated the use of this powerful new tool in targeted knockout of a gene settled in the grape genome. By following the loss of the GFP fluorescence signal, we were able to observe the cells that had endured targeted mutations as a result of CRISPR/ Cas9 activity. In addition, we presented examples of the various types of indels obtained by Cas9-mediated cleavage of the GFP gene, guided by two independent sgRNAs. The application of the CRISPR/Cas9 RNP system enables the generation of grape plants engineered by DNA-free gene editing. Eventually, we provided an optimized protocol to target important native genes in the grape plant in the future.

In this study for the first time, we managed to achieve whole plants regenerated from DNA-free genome edited protoplasts. Monitoring the protoplast to whole plant developmental stages demonstrated that regenerated plantlets derived from gene-edited protoplasts exhibited a normal phenotype concerning leaf shape, color and growth habits compared to wild-type plants. Here an efficient protocol has been presented for foreign DNA-free CRISPR/Cas9 mediated gene editing in *Vitis vinifera* Sultana including detailed protoplast-to-plant steps.

Introduction

Over the last few decades, global climatic trends in many agricultural regions have been quickly changing, in the face of these new problems, considerable advancements in global food systems are required to assure food security. The productivity of global agriculture will continue to be damaged as climatic instability grows as a result of rising CO2 levels and warmer temperatures. To address these mounting problems, innovative techniques will be needed, incorporating all available resources to develop more resilient and tolerant crops with higher quality and yields under more harsh circumstances. One viable strategy for accelerating genetic advances through targeted genetic modification, generating crops that can withstand changing climate conditions, is the integration of genome editing and transgenics into present breeding procedures [1]. In this project, we discussed how revolutionary genome editing techniques may be directly integrated into grapevine breeding programs to quickly address many of the concerns that will influence agriculture productivity in the future.

Global agricultural output, farm incomes, and food security will all be impacted by climate change. Global food security will undoubtedly be endangered as a result of climatic trends such as rising mean temperatures, climate variability, and an increase in extreme weather events [2].

Grape (*Vitis vinifera* L.) is one of the most important commercial fruit crops of the world and is one of the most extensively cultivated temperate fruit crop. Grapes are used to make wine, jam, jelly, pies, raisins, juices, table condiments and medicines. Once established, well-tended grapevines can be productive up to 40 years. The genus *Vitis* has a wide range of distribution, mainly between 25° and 50° north latitude. Grapes' global distribution is combined with the crop's high genetic plasticity, allowing it to adapt to temperate, subtropical, and tropical climates. Grapes are classified into two taxonomic groups: *Euvitis* (2n=38) and *Muscadinia* (2n=40)**.** The majority of today's commercial grape cultivars are *Vitis vinifera* L. Grape is a member of the

Vitaceae family and belongs to the genus *Vitis*. There are 12 genera and 600 species in the Vitaceae family. Remarkably, commercial grape varieties have a narrow genetic base, making them susceptible to diseases and pests, particularly in the tropics and subtropics. Since it can be propagated both by seed and by vegetative means, it has a wider range of genetic manipulation options [3-7].

In many places of the world, its usage in winemaking has played an important cultural role. Grapevines are currently grown in over 90 countries for the production of wine, liquors, juice, table grapes, and raisins (FAO-OIV, 2016). Grapevine has evolved as a model perennial fruit crop species due to its global economic importance, climatic diversity of the producing areas, and a great number of studies (from genomes to production practices) [8, 9]. Climate change will be a major problem for viticulture in the future decades, according to scientific research. Global warming is expected to have a direct impact on ecosystems due to many factors such as greenhouse gas emissions, temperature, precipitation, and human activities. This will make the increase in the growing season mean temperatures, the incidence of pest and disease, oxidative damage, growth inhibition, and change in quality and yield, this will result in shifts in viticulture production pattern [9-11].

Conventional breeding is now the most extensively used strategy in crop development; nevertheless, it is labor demanding, and progressing from the early phases of screening phenotypes and genotypes to the first crosses into marketable varieties can take several years. The transfer of genes (transgenes) or gene elements with known functions into elite crop types produces genetically modified (GM) crops with desirable features. Despite the promise of genetically modified crops for global food security, their deployment is hampered by mostly unconfirmed health and environmental safety concerns. Government regulatory systems aimed at ensuring human and environmental biosafety have resulted in considerable financial hurdles to the quick adoption of novel GM characteristics. As a result, the benefits of genetically modified features have been limited to a small number of farmed crops [12]. Combining synthetic tools and traditional breeding into genomics-based breeding is a

novel way to get over the limitations of traditional breeding. Genome editing offers the potential to speed up basic research and plant breeding by allowing for quick, accurate, and targeted genome alterations[1]. Several gene knockout mutants, as well as some gene replacement and insertion mutants, have been created in a range of plants using genome-editing tools, and many of these mutants have been found to be valuable for crop improvement [12].

Traditional genome editing entails the transport and integration of DNA cassettes encoding editing components into the host genome. Because integration happens at random, it might result in unfavorable genetic alterations [12]. When using transient vector delivery, the editors must be transcribed and the complex must assemble, resulting in brief pause inactivity [14]. Even if the DNA cassettes are degraded, the fragments that arise may be incorporated and have unwanted consequences, however, because nucleases are numerous in plants, the continuous production of genome-editing tools leads to increased off-target consequences. Furthermore, the insertion of foreign DNA into plant genomes presents regulatory difficulties in the context of genetically modified organisms (GMOs) (3.5). While employing RNPs, the complex is already preassembled and active when delivered; This DNA-free RNP delivery approach is promising for plant breeding since the resulting edited crops are likely falling outside of GMO regulation [15]. Consequently, DNA-free genome editing is a game-changing technique that produces genetically modified crops with a lower chance of unwanted off-target mutations while still addressing present and future agricultural demands from a scientific and regulatory perspective [12, 16].

Genome editing is a dream method for detecting and precisely locating a certain region within a genome, then editing the targeted sequences for various purposes. The genomic editing technique works on a specific genome sequence inside the genome, unlike traditional transgenic technology, which randomly introduces genetic elements into a genome [17-19].

RNP delivery of CRISPR/ Cas9 has been demonstrated for genome editing in plant cells. The delivery of DNA through the plant cell wall, and the regeneration of plants from tissue or cell-wall free cells are two fundamental obstacles for DNA-free transformation. Most modifications employ isolated protoplasts, single plant cells with an enzymatically digested cell wall, to get beyond the plant cell wall barrier. Since protoplasts are easily targeted by polyethylene glycol (PEG) mediated fusion, they were the first tissue to be employed for DNA-free Genome Editing. The RNP complex is therefore encased in PEG vesicles and joined to protoplasts [14-16].

The protoplasts have become a powerful and very convenient source for the development and establishment of many techniques in modern plant cell biology due to their high yield of uniform protoplasts, totipotency, and the ability to obtain entire plants or cell lines from single cells [20, 21]. During the last decade, optimizing plant regeneration has been a critical goal underpinning various parts of plant biotechnology. Despite several research, protoplast cultivation remains the most difficult of all in vitro plant regeneration procedures, particularly in perennial woody plant species [22].

Grape is an important target for crop improvement by genetic engineering, and the development of efficient protoplast culture is one of the methods allowing to achieve this aim. (2).

The use of any crop species in plant biotechnology and fundamental research is impossible without development of effective, reproducible, and routine methods for regeneration and genetic transformation. A successful application of methods for gene transfer depends on the possibility to transform a cell and tissue which can be regenerated into a plant afterwards. While for some species these problems have already been solved, for others the methods have not been established or if available, they are suited just for some genotypes [21]. In the first chapter, an optimized protocol has been described for protoplast isolation, shoot germination and whole plant regeneration in four grapes including Sultana (Thompson Seedless) as one of the most

marketable table grapes, Syrah, Corvina and Cabernet sauvignon cultivars of grapevines with a reputation for producing high-quality wine [23].

In the next step to test the efficacy of the CRISPR/Cas9 system in targeting a gene within the grape genome, we have generated a transgenic grape line carrying a T-DNA insert containing a GFP expression cassette. We conducted the study described herein to understand the process and to learn the limitations of the gene editing technology for grape improvement.

Among the various ways of plant genetic transformation, *Agrobacterium*-mediated technologies are specified as powerful tools and effective techniques for delivering genes of interest into a host plant. Even though it is technically challenging, these *Agrobacterium*- mediated approaches are still preferred for transgenic plant production, as they present several advantages, include the ability to transfer large intact segments of DNA, simple transgene insertions with defined ends and low copy number, stable integration and inheritance, and consistent gene expression over the generations [24, 25]. In order to understand the efficacy of the CRISPR/Cas9 system to target a gene within the genome of grape, we made use of a transgenic grape line that had a green fluorescent protein (GFP) gene integrated into its genome.

In the second chapter transform GFP gene into the genome and provided a stable transgenic line caring GFP gene, followed by protoplasts isolation and plant regeneration in Sultana has been explained.

A successful delivery of a complex into the plant cells always is challenging. To address this issue, first we have optimized the conditions for cell wall digestion. Second, to evaluate the efficiency of in difficult-to-transfect cell lines, we have employed transient expression vector carrying YFP gene. Finally, we investigated the feasibility of improving CRISPR/Cas9 transformation; a cutting-edge technology has been utilized to visualize CRISPR complex transfection. In this approach GFP was used as a visual reporter to facilitate Cas9/sgRNA-transfection monitoring [26].

In the last chapter, we described a successful knockout of a GFP reporter gene, that is already integrated into the grape genome with a single copy number, in the Sultana variety by direct delivery of RNPs into protoplast. We demonstrated the use of this powerful new tool in targeted knockout of a gene settled in the grape genome. By following the loss of the GFP fluorescence signal, we were able to observe the cells that had endured targeted mutations as a result of CRISPR/ Cas9 activity. In addition, we presented examples of the various types of indels obtained by Cas9-mediated cleavage of the GFP gene, guided by two independent sgRNAs. The application of the CRISPR/Cas9 RNP system enables the generation of grape plants engineered by DNA-free gene editing. Eventually, we provided an optimized protocol to target important native genes in the grape plant in the future.

In this study for the first time, we managed to achieve whole plants regenerated from DNA-free genome edited protoplasts. Monitoring the protoplast to whole plant developmental stages demonstrated that regenerated plantlets derived from gene-edited protoplasts exhibited a normal phenotype concerning leaf shape and color and growth habits compared to wild-type plants.

In closing, for the consumer, it is critical to guarantee that regulations are clear and that products are safe. It is easy for activist groups to spread data-free ideas and anecdotal reasoning to promote dread of any new technology, particularly when it includes food production, as was the scenario with GM crops. Additionally, market access for gene editing goods must be considered, which poses a risk to business investment in the technology. Many jurisdictions have yet to rule on gene-edited crops and foods, which continues to be a challenge for plant breeding firms and researchers. The European Union continues to be a significant hurdle to the development of new markets for sustainable food production technologies [19].

Aims and objectives

CRISPR/Cas9 mediated transgene-free genome editing in grapevine has not been widely reported. Precisely, whole plant regeneration from the edited protoplast is the main bottleneck in the protocols. The aim of this thesis was to improve the protocols of the protoplast-to-plant system and establish an efficient method for transgene-free genome editing in *Vitis vinifera* as one of the recalcitrant crop plants. The influence of a range of factors on plant regeneration, direct delivery of RNP complex to protoplasts, and effective gene editing was investigated to provide an optimized protocol to target important native genes in the grape plant in the future.

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Chapter 1

Protoplast isolation and plant regeneration in *Vitis vinifera* L.

Optimizing a protocol for protoplast isolation, shoot germination and whole plant regeneration in four grapes including Sultana, Syrah, Corvina, and Cabernet sauvignon cultivars

1.1. Introduction

1.1.1. The importance of grape

Grape (Vitaceae, *Vitis* spp.) is a deciduous temperate fruit crop of ancient origin. Grape is one of the most important commercial fruit crops of the world and is one of the most extensively cultivated temperate fruit crop. Grapes are used to make wine, jam, jelly, pies, raisins, juices, table condiments, and medicines. Once established, well-tended grapevines can be productive up to 40 years.

Vitis contains two subgenera, *Euvitis* Planch., the bunch grape species that all contain 38 somatic chromosomes, and *Muscadinia* Planch., the musca- dine grapes with 40 somatic chromosomes. With the exception of muscadine grapes, which are morphologically, and genetically distinct, most cultivated grapes are either pure strains or hybrids of *V. vinifera* and account for the vast majority of world production. There are 12 genera and 600 species in the Vitaceae family. Commercial grape varieties, in particular in the tropics and subtropics, have a narrow genetic base, making them susceptible to diseases and pests. It has a wider range of genetic manipulation options since it can be propagated both by seed and by vegetative means. Conventional breeding, on the other hand, is hampered in producing novel cultivars due to a high degree of heterozygosity, polygenic inheritance of many desired characters, and a long juvenile growth phase. As a result, non-traditional grapevine improvement methods are required. Biotechnology provides a powerful alternative to supplementing ongoing efforts to develop genetically enhanced germplasm for disease resistance and increased yield. Genetic transformation to introduce novel genes into plants for quality production, in addition to traditional and molecular breeding approaches, is an appealing option. However, a good plant regeneration system is required for genetic transformation to be successful [1-5].

Protoplasts were used as a very convenient source for the development and establishment of many techniques in modern plant cell biology due to their high yield

of uniform protoplasts, totipotency, and the ability to obtain entire plants or cell lines from single cells. [6, 7]. Optimizing plant regeneration has been a fundamental goal underpinning numerous aspects of plant biotechnology during the last decade. Despite several studies, protoplast cultivation remains the most challenging of all in vitro plant regeneration processes, especially in perennial woody plant species. [8]. Grape is an important target for genetic engineering crop improvements, and one of the strategies for achieving this goal is the creation of efficient protoplast culture [7].

In this chapter, we described a protocol for protoplast isolation, shoot germination and whole plant regeneration in four grapes including Sultana as one of the most marketable table grapes, Syrah, Corvina and Cabernet sauvignon cultivars of grapevines with a reputation for producing high-quality wine [9].

Shiraz is Australia's most important red grape variety, historically, the name refers to the wine produced around the city of Shiraz in Persia/Iran. It's used to make a wide range of red wines, including some of the country's "icon" wines. Anecdotal information claims that some high-quality Australian Shiraz wines have a spicy, "pepper" aroma. Shiraz (the name given to the grapevine variety known as Syrah in France by several New World growers) is an old variety that is estimated to have originated around 100 AD in the northern Rhône Valley from Mondeuse blanche and Dureza [10].

The world's most known grape variety for the creation of excellent red wines is Cabernet Sauvignon. Although it is now planted in numerous countries, Cabernet Sauvignon has a long history in the Bordeaux region of France, where it has been grown since at least the 17th century [11].

Corvina grape is main part of the Amarone (a dry wine produced exclusively in the Italian region of Valpolicella (Verona) by the combination of *Vitis vinifera* L. cv. Corvina and *V. vinifera* L. cv. Rondinella withered red grapes (45–95% Corvina, 5–30% Rondinella) and it has been shown to hold a fundamental role in conferring the organoleptic characteristics to the wine [12].

“Thompson Seedless” is thought to have originated in Persia in Asia Minor, in an area that now makes up parts of Iran and Turkey. The variety was introduced into California in 1872. a derivation of ‘Sultanieh,’ believed by some to be a recognition of a sultan’s appreciation for or ownership of the grape, or of its possible origination in or near the town Soultanieh, which is situated in Persia not far from the Caspian Sea. The Sultana is a "white" (pale green), oval seedless grape variety also called the Sultanina, Thompson Seedless (United States), Lady de Coverly (England), and oval fruited Kishmish (Iraq, Iran, Israel, Palestine, India). is one of the most widely planted light-skinned grape types in the world. The great majority of Sultana vineyards are used to grow table grapes or dried grapes (raisin), with only a few wines made from this *Vitis Vinifera* seedless grape variety. In compared to more noble varietals, white wines made from Sultana are frequently described as being rather sugary and lacking in quality [13].

1.1.2. What is Protoplast

A protoplast is a plant cell that has had its cell wall removed in whole or in part, either mechanically or enzymatically [2]. Plant protoplasts (also known as "naked" cells) are a single-cell system that underpins many aspects of current biotechnology [7].

Protoplasts are naked cells that, in general, are the same as cultured animal cells. However, unlike the latter, protoplasts exhibit the unique property of totipotency. Consequently, protoplasts provide a cell system that can be easily manipulated with physiological and pharmacological perturbations, and this experimentation can be carried out all the way to the whole (plant) organism and following generations. Protoplast isolation from a wide range of species is increasingly commonplace, and healthy protoplasts have the potential to be totipotent. Therefore, each protoplast can theoretically regenerate a new wall and undergo recurrent mitotic division to produce daughter cells from which fertile plants can be regenerated via the tissue culture process

when given the right chemical and physical stimuli. Plant-to-protoplast systems are available for many species, with extensive literature on their utilization. It is noteworthy that the basic procedures of protoplast preparation have not changed much since the first report. However, significant progress has been made in the number of species for which protoplast-to-plant systems exist. Furthermore, plant genetic manipulation through fusion and protoplast transformation has changed dramatically in recent decades [14, 15]. Protoplasts must be successfully separated from plant tissues before they may be used as model systems for physiological, biochemical, and virological research. In addition, if plants can be regenerated from manipulated protoplasts, protoplast technology can bring methods to improve plants [8].

Isolated protoplasts often began cell wall renewal shortly after being introduced into culture (usually minutes). However, until their new main walls can counterbalance the cytoplasm's turgor pressure, they require osmotic protection. In some circumstances, gradually lowering the osmotic pressure by diluting the culture medium with a solution with a comparable composition but lower osmotic pressure is required for mitotic division to continue, resulting in the creation of daughter cells and tissues [7, 14].

Significant efforts to produce crops with beneficial characteristics, including disease resistance, herbicide resistance, drought and salt tolerance, biomass growth and wall structure Modified cell is performed. With the introduction of a new generation of molecular breeding tools, such as CRISPR-Cas9 and TALENs, as well as more classic gene silencing techniques like dsRNA, miRNA, and siRNA, a bottleneck has been formed in which more plants may be produced than can be screened.

Plant protoplasts have emerged as plant responses to single-cell biology. Protoplast platforms are particularly useful for measuring high-performance gene expression, for genome editing, gene silencing, and other types of molecular modification methods. When compared to the utilization of entire plant tissue or cell suspension cultures, protoplasts offer significant benefits.

The lack of a cell wall, which is a fundamental barrier that contributes to plant cells' normally low transformation efficiency [6], is the primary benefit. Furthermore, protoplasts are a real single-cell culture, as opposed to multicellular plant cell suspension cultures and plant tissue. The ability to extract protoplasts from practically any organs and tissues from entire plants, therefore representing the developmental and spatial aspects inherent in those organs and tissues, is the last benefit of protoplasts. This allows for the detection of tissue-specific expression, the evaluation of functioning chloroplasts, and the examination of gene expression in various tissues [16, 17].

1.1.3. Protoplast isolation

Plant protoplast isolation was initially described almost 40 years ago [18]. Klercker produced protoplasts using a mechanical approach as early as 1892, but it yielded low yields and was difficult to operate and apply. Cocking isolated tomato root tip protoplasts for the first time using enzymatic hydrolysis in 1960. Because to its high yield, high activity, ease of operation, and versatility, this approach was widely adopted [15]. Protoplasts have been used to study biological processes and activities such as cell division, embryogenesis, and cell wall formation. Differentiation during regeneration, photosynthetic activity, calcium signaling and control, ion channel modulation by light, stress and hormone responses in many plant species have all been studied. To explore cell type–specific responses, protoplasts were separated from several cell types [18].

Grape (*Vitis* spp.) is a widely grown perennial plant. Many of the difficulties in the modern grape industry can be addressed because to recent breakthroughs in plant biotechnology. Grape protoplast isolation and culture, on the other hand, has been rather limited [19].

Grape protoplasts are recalcitrant to plant regeneration and were first isolated by Benbadis and Baumann (1973). Grape mesophilic protoplasts have a high rate of

survival but do not reproduce. Protoplasts have been isolated from a variety of explants, including leaves, shoots, stems, roots, calluses, and embryonic tissue, using efficient procedures. In somaclonal variation, in vitro selection, somatic hybridization, and genetic alterations, protoplast technology offers a wide range of uses. Due to the lack of a cell wall, this approach makes it straightforward to transfer genes to the plant genome [1].

Because protoplasts are easily targeted by polyethylene glycol (PEG) mediated fusion, they were the first tissue to be employed for DNA-free Genome Editing [20]. Therefore, optimizing a protocol is critical to having a healthy protoplast. The extent of thickness of cell walls, temperature, period of enzyme incubation, pH optima of the enzyme solution, quiet agitation, and type of the osmoticum are all parameters that influence protoplast release [14].

1.1.4. Embryogenic calli induction

Morel's work on grapevine callus induction was the pioneering, and numerous researchers have since acquired callus from various explants i.e. stem, petiole, tendril, node, internode, flower, fruit and immature berries. It has been observed that growth regulators are added to obtain callus and that specific vitamins, such as myo-inositol, are added to maintain callus. The majority of grapevine organ culture studies focused on inflorescence culture since it could be a useful tool for examining the mechanisms of floral induction [1].

High levels of heterozygosity and inbreeding depression worsen grapevine genetic improvement; it is also a time-consuming procedure because to the 2–3-year generation cycle. The use of biotechnology might be a viable solution. Both shoot organogenesis and somatic embryogenesis can be used to induce adventitious plant regeneration in grapes from a variety of explant types.

As a first step, a successful use of gene technology necessitates is a reliable regeneration approach that allows for both transformation and regeneration into plantlets.

Embryo tissue, in particular, has been found to be an ideal cell source for applying genetic transformation procedures to grapevines. Somatic embryogenesis, on the other hand, appears to be influenced by the interplay of genotype, explant source, and culture medium, necessitating the development of specialized regeneration techniques for each *Vitis* species and V. vinifera cultivar [6]. For the insertion of desirable characteristics into elite varieties, genetic engineering of *Vitis* has emerged as a viable alternative to traditional breeding. Because embryogenic cultures are commonly used for grapevine transformation, techniques for culture initiation and maintenance must be optimized. Although somatic embryogenesis from *Vitis* had previously been documented, only a few varieties showed embryogenic competence, and there was substantial variability among responding kinds. Factors that influence somatic embryogenesis, such as explant type and developmental stage, macro- and microelement composition of the culture medium, and growth regulator concentration, should be investigated to improve embryogenic competence and produce cultures that result in genetically stable regenerants. In several investigations of *Vitis* somatic embryogenesis, inflorescence tissues were employed as explants to start embryogenic cultures. In most parts of the world, grapevine flowering happens just once a year for a few weeks, leaving only a tiny window of opportunity to start embryogenic cultures. To maximize embryogenic competence, it is critical to determine the right developmental stage of explants. For grapevine culture initiation, a variety of medium compositions are now employed. It would be easier to start embryogenic cultures for *Vitis* species and variants if one or a few good culture media could be identified [21].

The grapevine's once-a-year crop cycle limits the number of reproductive and physiological investigations that may be undertaken on this crop species. Mullins and Rajasekaran (1981) [22], devised a technique for producing small fruiting plants from

dormant canes under specified environmental situations, providing year-round production of experimental plants.

1.1.5. Somatic embryogenesis

Many years ago, somatic embryogenesis in the grapevine was developed. Isolation of natural somatic mutations arising during grapevine vegetative multiplication, improvement through exploitation of somaclonal variation, germplasm cryopreservation, and viral disease elimination are some of the key uses. In addition to using somatic embryos for plant improvement, large-scale mutant production from somatic embryos via insertional mutagenesis has become a fascinating goal for genomic programs devoted to gene function assignment, due to the recent presence of drafts of the grapevine genome sequence. Somatic embryos could be produced from a variety of explants, primarily reproductive organs such as ovaries, stigmas, anthers and whole flowers. Somatic embryos have also been created from tissues generated from vegetative structures like as leaves and petioles, tendrils, or stem nodal explants, however this is less usual.

Even though the number of *Vitis* species and cultivars for which somatic embryogenesis procedures are accessible is growing all the time, it is still not a common practice, especially for the most valuable genotypes. The main bottlenecks include large experimental variations due to genotype, as well as differential responses due to the interaction of several physiological factors involving the chosen explant, its developmental stage, and the chemical factors present in the culture medium. Other drawbacks of this technology include the difficulty in producing mature, properly developed somatic embryos that can be converted to normal plants at high rates from embryogenic callus [23].

1.1.6. Callus maintenance

The regeneration of grape vines has been extensively investigated since the first report of somatic embryogenesis and adventitious organogenesis. The regeneration of entire plants from somatic cells or tissues is a necessary condition for grapevine genetic engineering. Organogenesis has been shown to be inappropriate for genetic transformation and regeneration of non-chimaeric plants, whereas embryogenesis has been utilized successfully for transformation multiple times since the original report of Mullins et al. (1990) [24]. Despite the considerable work done on embryogenesis, *Vitis vinifera*, like many other woody species, appears to be a recalcitrant plant for embryogenic tissue culture start and maintenance. Torregrosa (1998) [24], improved the overall process of embryogenic callus formation from anthers and the long-term maintenance of stable undifferentiated embryogenic cultures.

1.1.7. Research aims

The use of any crop species in plant biotechnology and fundamental research is impossible without development of effective, reproducible, and routine methods for regeneration and genetic transformation. A successful application of methods for gene transfer depends on the possibility to transform a cell and tissue which can be regenerated into a plant afterwards. While for some species these problems have already been solved, for others the methods have not been established or if available, they are suited just for some genotypes [7]. In this chapter, we described a protocol for protoplast isolation, shoot germination and whole plant regeneration in four grapes including Sultana as one of the most marketable table grapes, Syrah, Corvina and Cabernet sauvignon cultivars of grapevines with a reputation for producing high-quality wine [9].

1.2. Materials and methods

1.2.1. Embryogenic calli induction

In Sultana, unopened leaves, and fully opened leaves from micropropagation cultures were utilized as the explants to investigate embryogenic responses.

In the case of fully opened leaves, explants were excised into 3 pieces longitudinally before transfer to induction medium NB2 [25] consisting of Nitsch and Nitsch (1969) salts, Murashige and Skoog vitamins, 0.1 g/l Myo-inositol, 50 mg/ml folic acid, 20.0 g/l sucrose, 5.0 μM 2,4- Dichlorophenoxyacetic acid, (2,4-D) and 1.0 μM Benzyl- adenine (BA). Medium pH was adjusted to 6.0 before the addition of 7.0 g/l TC agar. Unopened leaves were placed adaxial side down on the medium. Five leaves were placed in each petri dish and incubated in darkness at 28 °C for 8 to 10 weeks [21].

In Corvina, Cabernet and Syrah; Inflorescences of dormant vine cuttings were induced after surface sterilization. Cuttings were forced to flower by treated with 1.5 g/L of the rooting hormone indole butyric acid (IBA) (Clonex, Growth Technology, Perth, Australia) on the basal cut end and were planted, for prerooting, in washed river sand in a heat-bed and maintained in a dark cold room at 4°C [26].

After five weeks, rooted cuttings with more than five roots, at least 5 cm in length (as illustrated by Mullins and Rajasekaran (1981) [22], were planted in pots filled with perlite, vermiculite, and ground mixture. Pots were transferred to a growth room (27°C day and 22°C night, 16 h photoperiod, humidity of 40% and 350 μE of light intensity at the plant level) Standard leaf and shoot tip removal to promote inflorescence development was performed as described in Mullins and Rajasekaran (1981) [22]. Initially, 150 mL of half-strength Hoagland's solution was used on alternate days until the plants developed five leaves on the first lateral shoot. At later stages (when the second shoot had developed four or more leaves), 200 mL of solution was used every

2 days [27]. After 2 weeks of planting in the growth room Inflorescence collection has started at different stages of development. Inflorescences were excised and samples of individual flowers were dissected and observed with a stereomicroscope to determine the developmental stage of stamens. The flowers were surface sterilized by immersing them in 100 mL of 7% $Ca(ClO)_2$ containing one/two drops of Tween-20 for 10-min with continuous shaking, followed by three 5-min washes in sterile distilled water [21].

Stamens (anthers with intact filaments) were carefully separated from the calyptra and the pistil under a stereomicroscope and placed on the adaxial side in contact with the medium. Each petri dish contained ∼50 stamens and was placed in darkness at 28°C. (18) Explants were initially cultured on callus induction medium (PIV) containing Nitsch and Nitsch (1969) mineral salts, Murashige and Skoog (1962) vitamins, 6% sucrose, supplemented with 4.5 mM 2,4-D and 8.9 mM BA and the pH adjusted to 5.8 with 0.5 N NaOH, 0.3% Phytagel (Sigma-Aldrich) added before autoclaving [21, 27, 28].

1.2.2. Callus maintenance

Calli inducted from explants of NB2 and PIV after 3 months and transferred to $C1^p$ consisting of MS macroelements, MS microelements (Murashige and Skoog 1962) [17], vitamins and amino acids as described by Torregrosa (1994) [24], casein hydrolysate (1 g/L), 1 μM BAP,5 μM 2,4-D, Fe-EDTA, and 30g/L sucrose. The pH values of the media were adjusted to 5.8 with KOH after incorporating 5g/L Sigma' Phytagel.

1.2.3. Protoplast isolation

In Corvina and Cabernet embryogenic calli induced from PIV were directly used for protoplast isolation. Protoplasts were isolated from Sultana and Syrah embryogenic calli 7-10 days after subculture in $C1^p$. Embryogenic calli (1 g FW) was incubated in 10 ml of filter-sterilized (Millipore, 20 μm pore size) enzyme solution containing 2% (w/v) Cellulase R-10 Onozuka and 1% (w/v) Macerozyme R-10 (both Duchefa-

Biochemie), 0.05% (w/v) Pectolyase Y-23 (Duchefa-Biochemie), 10 mM $CaCl_2$.2H2O, 5 mM MES and 0.5 M mannitol, on a gyratory shaker (~30 cycles/min) at RT and darkness. The pH of the enzyme solution was adjusted to 5.7 before filter sterilization. After 1 h of incubation, the embryogenic calli were made to liberate into small cell clusters with a needle and a Pasteur pipette. After further incubation for 5 h for Sultana and Syrah, and 4 h for Corvina and Cabernet, the mixture was passed through a nylon sieve (60 μm) and the protoplasts were collected by centrifugation (100 x g, 5 min). Protoplasts were washed twice with a washing solution containing 0.5 M mannitol and 10 mM $CaCl_2$.2H2O by resuspension and centrifugation (100 x g, 4 min). Viability of protoplasts was assessed with 0.5 mg/mL FDA (fluorescein diacetate) staining [29]. Fluorescein diacetate remains the standard and most reliable fluorochrome for assessing protoplast/cell viability [14] and the cell wall digestion controlled by Calcofluor White stains 2 μM [30].

1.2.4. Protoplast culture for somatic embryogenesis

The protoplast was counted using a hemocytometer and [31, 32]. In our study, protoplasts were cultured in the range of 1×10^5, 5×10^5 and 1×10^6 protoplasts per ml plating density, in a plastic Petri dish by embedding in 2 g/l gellan gum solidified Nitsch's medium containing 2 mg/l NAA, 0.5 mg/l BAP, 0.3 M glucose, and 0.09 M sucrose. The method for embedding the protoplasts in the gellan gum medium was the same as that reported previously. In most of the experiments, protoplasts were cultured in the disc culture method. In this method, five small droplets (each 0.8 ml) containing protoplasts in a culture medium were quickly poured with a Pasteur pipette into a plastic Petri dish. After solidified, 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 and supplemented with 0.3% activated charcoal was added as a source [29, 33].

Every two weeks, the liquid medium was changed with the same fresh media as described, but without the glucose. The pH of culture media was adjusted to 5.7 before

autoclaving. All dishes had been closed with Parafilm and maintained at 27°C in the dark. Protoplast was monitored first 3 days after culturing for cell division then weekly for colonies and somatic embryogenesis stages. The number of somatic embryos produced was recorded after 2 to 3 months of culture [29, 33].

1.2.5. Mature embryos

Cotyledonary somatic embryos were placed in darkness in Nitsh's medium supplemented with 30 g/L sucrose and 2 g/L gellan gum for about 1 month to enable the full development of the embryo and avoid browning [33].

1.2.6. Shoot germination:

To identify the best shoot germination medium for these cultivars, two different media by two different growth regulators consistency were tested including C2D and C2D plus 4µM BAP, also MG1 and MG1 plus 10 µM BAP medium. Well-developed germinated somatic embryos were then transferred to mentioned media under 16h\8 h photoperiod conditions for 1 month for shoot induction [33-36].

Culture medium for the germination of embryos (MG1) consists of NN macroelements, MS microelements, Fe-EDTA, vitamins B5, 30 g/L sucrose, and 7 g/L Agar TC and 2.5 g/L activated charcoal and MG2 which consist of MG1 with 10 µM BAP [9, 35].

C2D medium include of C2D macro (NH_4NO_3, 1650mg/l, KNO_3 1900mg/l, $MgSO_4.7H_2O$, 370mg/l, KH_2PO_4 170mg/l, Ca $(NO_3)_2.4H_2O$ 709mg/l, $FeSO_4.7H_2O$ 27.8mg/l, $Na_2EDTA.2H_2O$ 37.3mg/l), C2D micro (H_3BO_3 6.2mg/l , $CoCl_2 \cdot 6H_2O$ 0.025mg/l, $MnSO_4 \cdot 4H2O$ 0.845 mg/l, $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg/l, $ZnSO_4 \cdot 7H_2O$ 8.6mg/l, $CuSO_4.5H_2O$ 0.025mg/l), C2D vitamins including Thiamine HCL 1mg/l, Myoinositol 10mg/l, Pyridoxine 1mg/l and Nicotinic acid 1mg/l. (30) supplemented with 30 g/L sucrose and 7 g/L TC agar, pH 5.8 , (C2DBAP is C2D plus 4 µM 6-BAP).

1.2.7. Whole plants regeneration

Once shoots were developed, they were placed in a Sterivent high container containing root induction media including MSN (MS with 0.5 μM NAA) and RIM (root-inducing medium) [9]. Shoots germinated from MG1, MG1BAP moved to RIM medium, and shoots regenerated from C2D and C2DBAP media transferred to MSN medium, to induce rooting and whole plant development.

Medium to induce and stimulate the rooting of shoots (RIM): MS macroelements, MS microelements, Fe-EDTA, vitamins T (50 g/L of myoinositol, 1 g/L of nicotinic acid, 1 g/L of thiamine HCl, 1 g/L of pyridoxine HCl, 1 g/L of calcium pantothenate, and 0.01 g/L of biotin), 0.5 μM NAA, 30 g/L sucrose, and 7 g/L Agar TC; adjust pH to 6.0 with 1 M KOH [35].

And medium to induce and stimulate the roots of shoots, MSN, containing Murashige and Skoog (MS) medium, 30 g/L sucrose, 0.5 μM NAA (naphthaleneacetic acid.) and 7 g/L TC agar, pH 5.8 [9, 33, 34].

Rooted plantlets were potted into the soil and placed in a greenhouse after adaptation. Rooted shoots from in vitro rooting experiments were taken out of the high container and their roots were gently washed with tap water to remove adhering medium [14], then transferred to ground soil in the growth chamber for 1 month. Finally, acclimated plants were moved to the bigger pot and in the glasshouse [9]. Acclimatized plants exhibited a normal phenotype concerning leaf shape and color and growth habits compared to the original plant [29].

1.3. Results

1.3.1. Calli induction

Embryogenic calli (EC) in Sultana were initiated from unopened leaves of in-vitro shoot tip cultured in NB2 medium. Leaf explants cultured on NB2 medium after 10 weeks produced fragments of compact, cream-colored embryogenic calli, and loose non-embryonic callus that were brown in color (**Fig.1 a, b**).

While in Syrah, Corvina and Cabernet sauvignon stamens were collected from flowers and cultured in PIV medium, EC were observed after about 10 weeks. Stamens cultured on PIV produced a sector of compact, cream-colored embryogenic calli, and flaky and crispy non-embryogenic calli white and red in color.

EC induction in Syrah was not significantly correlated with different developmental stages of stamens. As the EC induction was similar in the tetrad and mature development stage of stamens. EC induction in Corvina and Cabernet sauvignon were initiated from mature (IV) and completely mature (V) respectively (**Fig.2 a,b; Fig.3 a,b,c; Fig.4 a,b,c**).

Since the long-term maintenance of embryogenic calli has been under debate in similar studies, $C1^{P}$ is verified as an efficient medium for long-term maintenance of embryogenic cultures for Sultana and, Syrah. In the case of Corvina and Cabernet sauvignon, embryogenic calli were loos and brown in C1. Therefore, embryogenic calli were used directly from the PIV medium for protoplast isolation and regeneration.

1.3.2. Supplementary information:

Corvina and Cabernet Sauvignon: An attempt was made to preserve the embryonic calluses of Corvina and Cabernet sauvignon cultivars for long-term

maintenance of embryogenic lines. long-term maintenance medium (LTMM) [37] consisted of Nitsch and Nitsch (1969) salts supplemented with 30 g/L sucrose, 100 mg/L Myo-inositol, 0.8 g/L casein hydrolysate, and 2,4-D, 2 μM, the medium pH was adjusted to 5.6 before autoclaving and 2.5 g/L Phytagel were added as the gelling agent) showed that could be suitable for these cultivars, However, further studies are needed to confirm the good performance of this medium.

Unopened, young leaves were cultured on NB2 medium, and the results showed that the leaves had the potency to induce embryonic calli in NB2 and the calli were successfully maintained on $C1^{P}$ medium. Callus embryogenesis was assessed by X6, which confirmed their ability for embryogenesis.

1.3.3. Protoplast isolation

The yield of isolated protoplasts per gram for every variety is described in **Table 1**. The viability of isolated protoplasts was estimated by FDA staining immediately after isolation. The isolation of a sufficient number of high-quality protoplasts is a prerequisite for using protoplasts for biotechnological applications. Fluorescein diacetate remains the standard and most reliable fluorochrome for assessing protoplast/cell viability. (**Fig. 1 d,e,f; Fig.2 c,d,e; Fig.3,e,f,g; Fig.4 e,f,g**).

1.3.4. Regeneration of protoplasts

Isolated protoplasts were then cultivated to regenerate the whole plant. The final density of protoplasts in the culture medium (plating density) is crucial for maximizing wall regeneration and concomitant daughter cell formation. To investigate the effect of protoplast culture density on plating efficiency 10^5 and 5 x 10^5 and 1x 10^6 protoplast/ml concentrations for Sultana and Syrah, and 1x10^5 and 5 x 10^5 for Corvina and Cabernet sauvignon was plated by the disc-culture method. In this method, isolated protoplasts resuspend in a warm solid culture medium are placed on a dish in separate droplets before solidification. Droplets were surrounded by a liquid culture medium

supplemented with activated charcoal, which prevents the browning of the culture and thus promotes cell division and colony formation.

1.3.5. Sultana and Syrah

We observed that the concentration of $1x10^5$ protoplasts/ml was the most efficient density with the highest mature somatic embryo recovery in both Sultana and Syrah cultivars. Therefore, we have reported the result of this density in the following sections. Results of other concentrations are mentioned in **Table 1**.

In the concentration of $1x10^5$ protoplasts/ml, the first cell division was observed after 4 days in both cultivars. Further cellular divisions occurred during the next three weeks and microcolonies were formed after ~25 days from protoplast isolation and developed to proembryo and globular stages respectively during the fifth week after cultivation. Heart and torpedo stages appeared during the 7^{th} and 8^{th} weeks which was followed by the development of the cotyledonary embryo. No significant difference did not observe between the two cultivars, while by increasing the density of protoplasts, developmental stages were delayed so that the cotyledonary stage developed after three months post cultivation in both 5×10^5 and 1×10^6 protoplast/ml.

Well-developed somatic embryos plated on shooting medium after incubation on Nitsch's medium supplemented with sucrose in the dark for 3-4 weeks to avoid browning and further development. Among all 170 embryos developed from Syrah protoplasts, 110 somatic embryos were selected based on the normal morphology and especially the balance in the development of different organs. (**Fig.1. g-r; Fig.2. f-m**). In the case of Sultana 36 of 75 somatic embryos were competent for shoot regeneration while remaining abnormal somatic embryos were discarded. To identify the best shoot regeneration medium for these cultivars, two different shooting media by two different growth regulators consistency was tested including C2D and C2D plus 4µM BAP, also MG1 and MG1 plus 10 µM BAP media were used, under 16h light\8 h dark condition for one month.

Shoot development normally occurred within 3 to 5 weeks after starting the photoperiod in both varieties. While the shooting time was varying so that in some cases 8 weeks was needed to achieve the highest shoot regeneration rate. Results showed that MG1 plus 10 μM BAP was the most efficient medium for shooting in both Syrah with 57% and Sultana with 44% shoot regeneration.

Shoots germinated from MG1 and C2D medium transferred to RIM and MSN medium respectively, to induce root and plant development. The germinated shoots developed into whole plants with expanded leaves and roots after 4 weeks for Syrah was 15 (32%) and for Sultana was 11 (30%) plants. The regenerated plants were transferred to the greenhouse following by acclimation. In both cultivars' macroscopic features and morphology of plants were normal. (**Fig.1. s-z; Fig.2. n-r**).

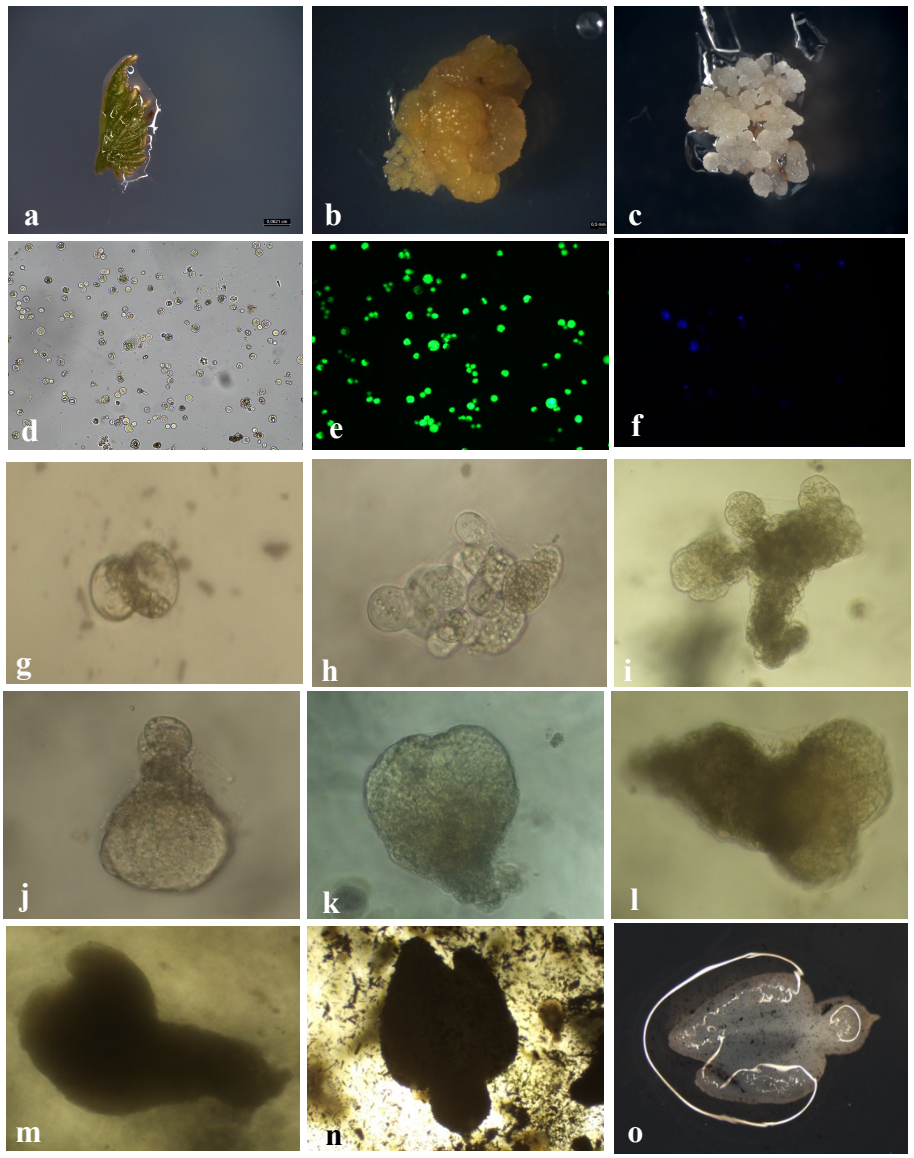

Figure 1. Plant regenerated from protoplast in Sultana. **a**. Unopened leaves on induction medium, **b**. Cultured on initiation medium produce sectors of embryogenic and non-embryogenic callus, **c**. Embryogenic calli in C1p, **d**. Isolated protoplasts (white light), **e**. Protoplasts FDA staining, **f**. Protoplasts calcofluor staining, **g**. First cell division, **h**. Micro calli, **i**. Pre-globular stage, **j**.

Globular embryo, **k**. Heart stage, **l**. Transient stage between heart and Torpedo, **m**. Torpedo stage, **n** and **o**. Cotyledonary stage.

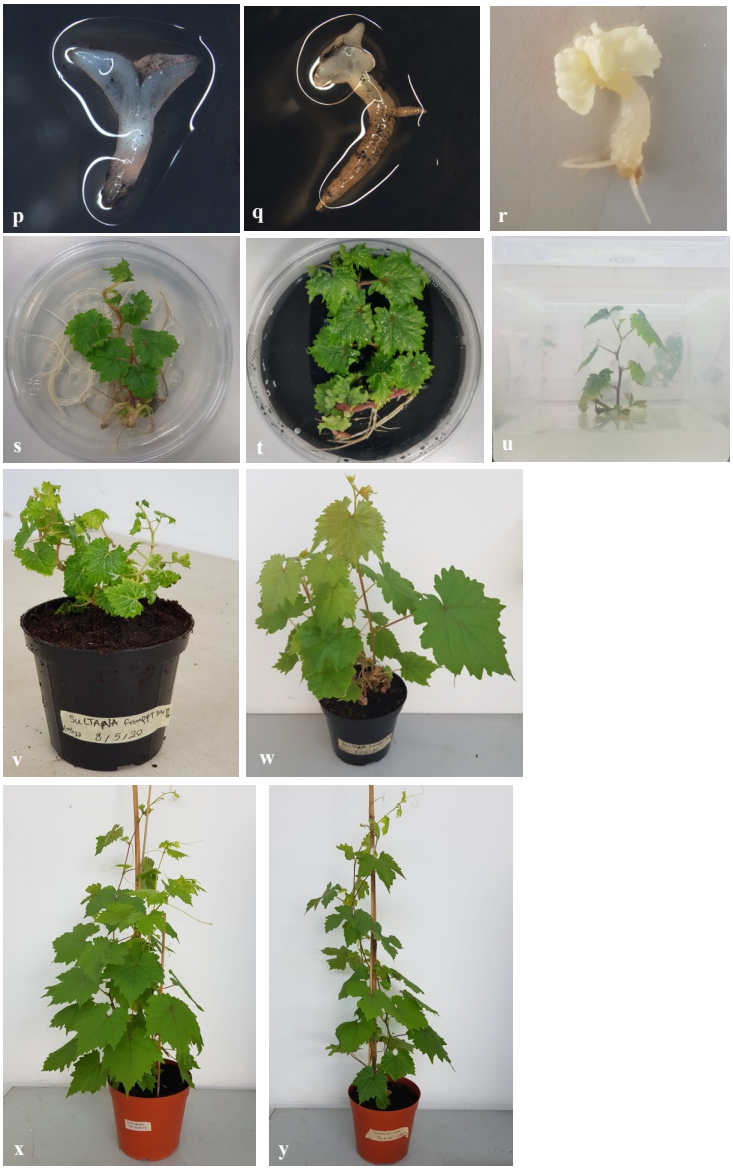

Figure 1. **p** and **q**. mature somatic embryos, **r**. well-developed germinated somatic, **s**. Shoot regenerated in C2DBAP, **t**. Shoot regenerated in MG1BAP, **u**. Whole plant in the Steri Vent Containers in the phytotron, **v** and **w**. Plant in the soil in growth chamber, **x**. and **y**. Plants in the green house.

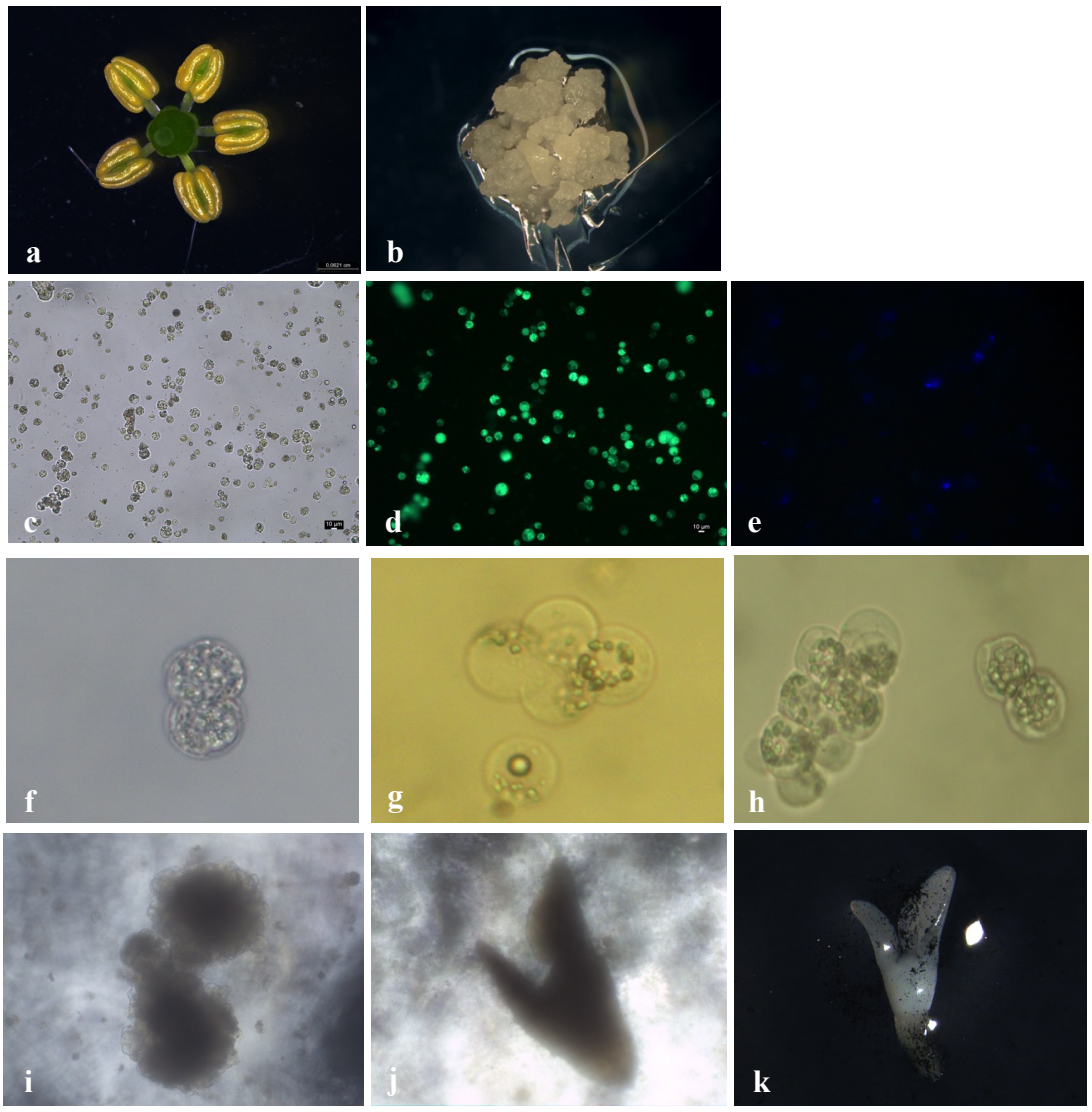

Figure 2. Plant regenerated from protoplast in Syrah; **a**. Stamens in PIV, **b**. Embryogenic calli in $C1^{p}$, **c**. Isolated protoplasts (white light), **d**. Protoplasts FDA staining, **e**. Protoplasts calcofluor staining, **f**. First cell division, **g** and **h**. Micro calli, **i**. Globular embryo, **j**. Torpedo stage, **k**. Cotyledonary stage.

Figure 2. **l**. Mature somatic embryos, **m**. Well-developed germinated somatic, **n**. Shoot regenerated in MG1BAP, **o**. Whole plant in the Steri Vent Containers in the phytotron, **p** and **q**. Plant in the soil in growth chamber, **r**. Plants in the green house.

1.3.6. Corvina 48

In Corvina first cell division was observed after the first week of cultivation days in both concentrations. Further cellular divisions occurred during the next two weeks, and microcolonies formed after ~20 days from protoplast isolation and developed to proembryo and globular stages respectively during the fifth week after cultivation. Heart and torpedo stages appeared during the 6th to 8th weeks which was followed by the development of the cotyledonary embryo. No significant difference did not observe between the two concentrations.

There was not any significant delay of embryogenesis step in the density of 5×10^5 concentration compared to $1x10^5$ protoplast density. well-developed somatic embryos plated on shooting medium after incubation on Nitsch's medium supplemented with sucrose in the dark for 3-4 weeks to avoid browning and further development. Among 121 embryos developed from protoplasts in the concentration of 1×10^5, 85 and among 96 embryos developed from 5×10^5 density, 72 somatic embryos were selected to transfer in shoot regeneration medium. To identify the best shooting medium for this cultivar, two different shooting media by two different growth regulators consistency was tested including C2D and C2D plus 4μM BAP, also MG1 and MG1 plus 10 μM BAP medium under 16h light\8 h dark condition for 1 month. (**Fig.3 h-r**).

Shoot development normally occurred within 3 to 5 weeks after starting the photoperiod. Results showed that both MG1 and MG1 plus 10 μM BAP were the most efficient media for the shooting of Corvina with 38% and 33%. Shoot regeneration and density of 1×10^5 protoplasts/ml is more efficient to have more whole plants regenerated from protoplasts. The germinated embryos developed into whole plants with expanded leaves and roots after 4 weeks was 23 (27%) plants. (**Fig.3 s-w**).

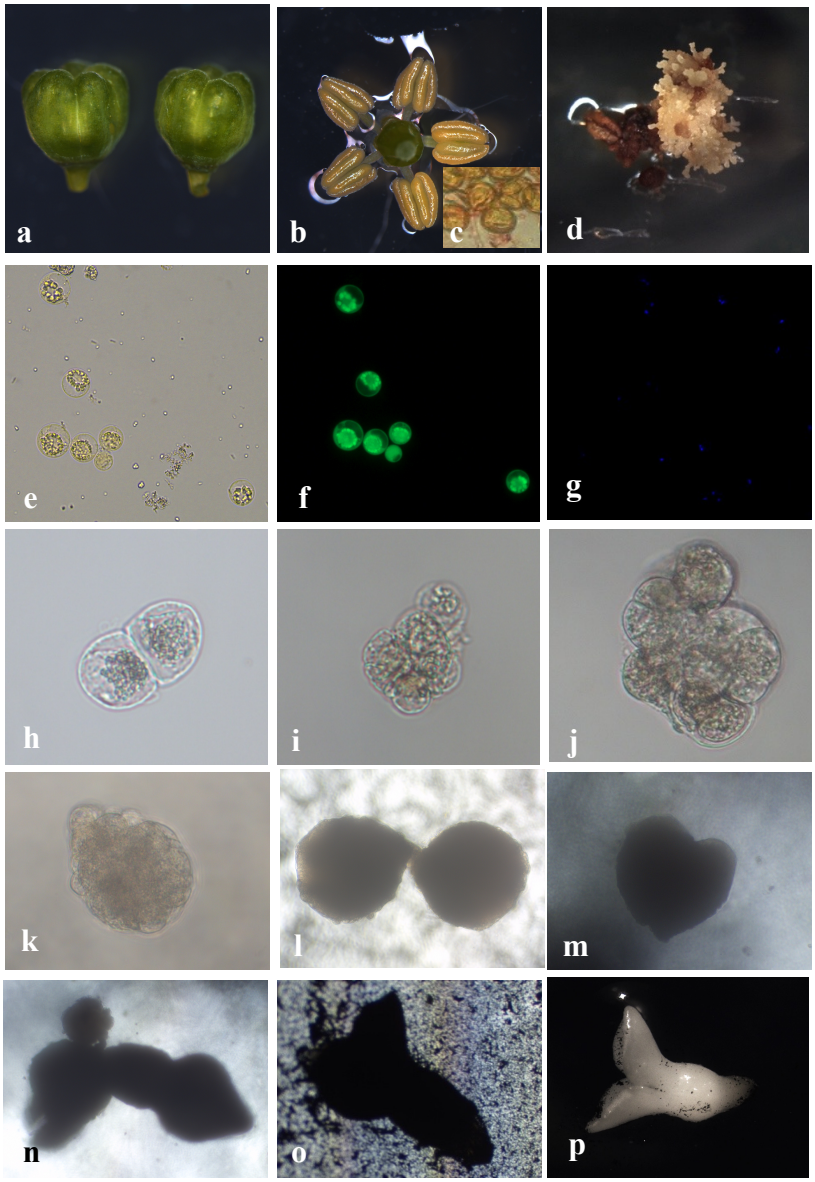

Figure 3. Plant regenerated from protoplast in Corvina, **a**. grapevine inflorescence, **b**. Stamen and pistil explants from unopened grapevine inflorescence, **c**. Microspore, **d**. Callus production from filament tip or connective tissue of stamens, **e**. Protoplasts isolated protoplasts FDA staining, g. Protoplasts calcofluor staining, **h**. First cell division, **i** and **j**. Micro calli, **k**. pre-globular, **l**. globular stage, **m**. Heart, **n**. Torpedo and cotyledonary stage, **o** and **p**. Cotyledonary stage.

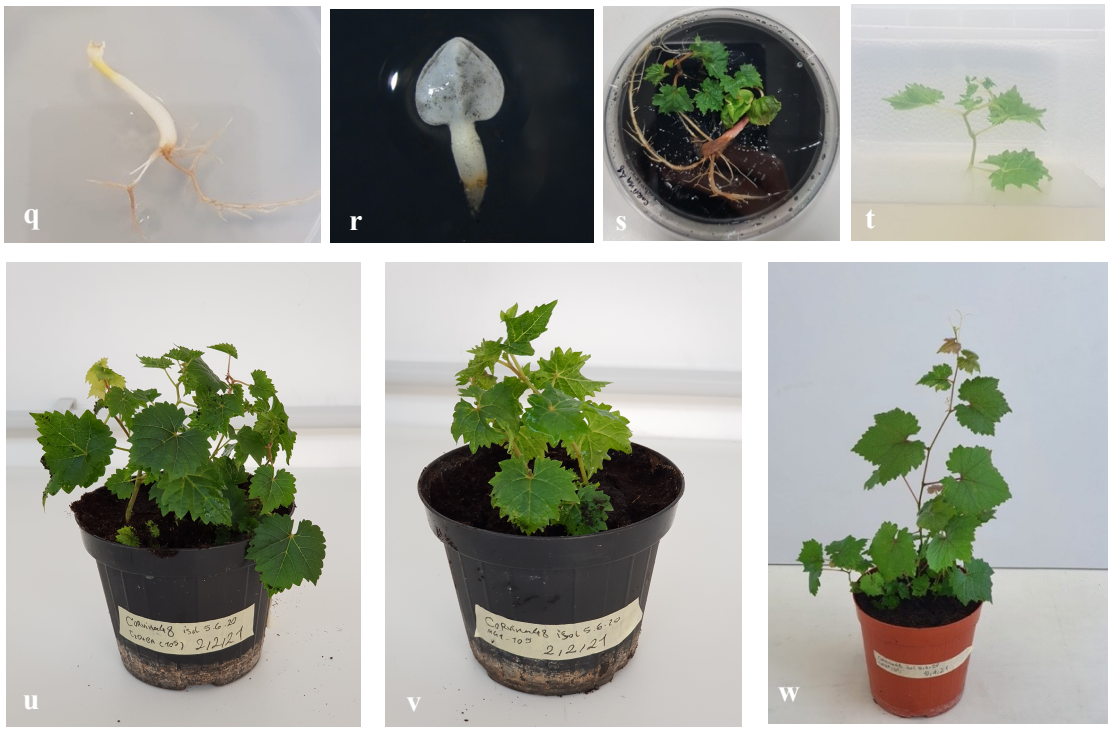

Figure 3. **q**. Mature somatic embryos, **r**. well-developed germinated somatic, **s**. Shoot regenerated in MG1BAP, **t**. Whole plant in the Steri Vent Containers in the phytotron, **u** and **v**. Plant in the soil in growth chamber, **w**. Plants in the green house.

1.3.7. Cabernet sauvignon

In Cabernet sauvignon timetable of cell division, micro calli formation and proembryo and globular stages are the same to Corvina and there was no significant difference between the two concentrations. From the concentration of 1×10^5 and 5×10^5 developed 15 and 22 somatic embryos respectively. And among them 3 and 4 somatic were selected to transfer in the shoot induction media including C2D and C2D plus 4μM BAP, also MG1 and MG1 plus 10 μM BAP medium. Germinated embryos efficiency in Cabernet was very low and only four well shaped embryos germinated out of 22 mature cotyledonary embryos; they regenerated two shoots in C2DBAP in the density of 5×10^5 protoplasts/ml. (**Fig.4 h-u**).

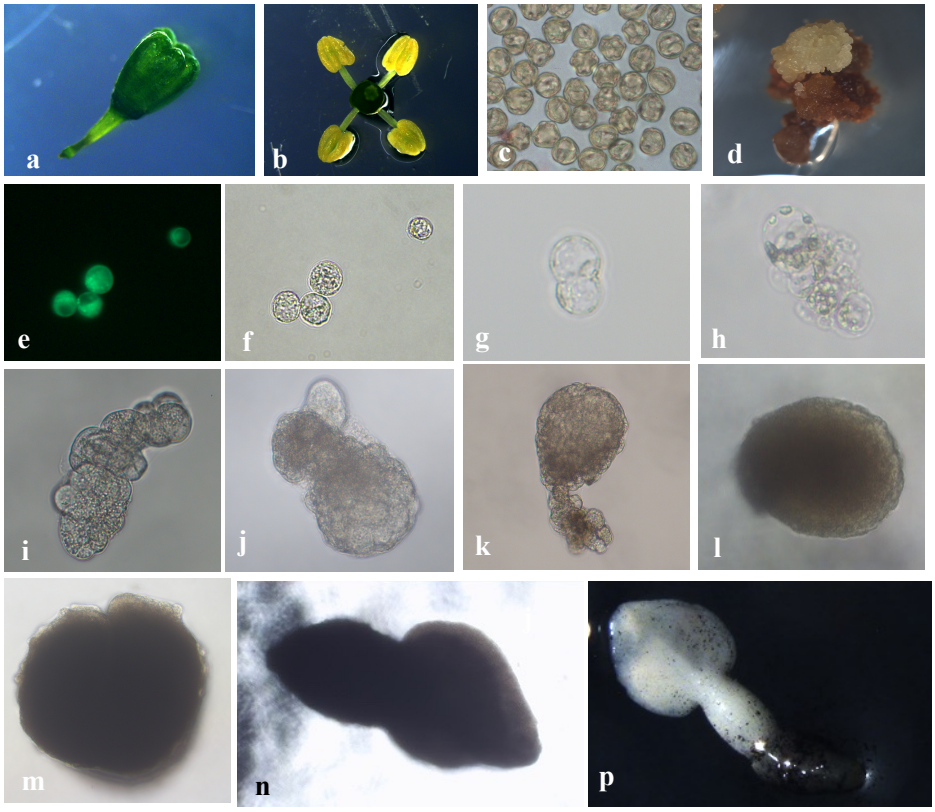

Figure 4. Plant regenerated from protoplast in Cabernet sauvignon, **a**. grapevine inflorescence, **b**. Stamen and pistil explants from unopened grapevine inflorescence, **c**. Microspore, **d**. Callus production from filament tip or connective tissue of stamens, **e**. Protoplasts isolated protoplasts FDA staining, **g**. Protoplasts calcofluor staining, **g**. First cell division, **h**, and **i**. Micro calli, **j** and **k**. pre-globular, **l**. globular stage, **m**. Heart, **n** and **p**. Cotyledonary stage.

Figure 4. **q**. Mature somatic embryos, **r**. well-developed germinated somatic, **s**. Whole plant in the Steri Vent Containers in the phytotron, **t**. Plant in the soil in growth chamber, **u**. Plants in the green house.

Table 1. The effect of different conditions in protoplast to plant system efficiency.

Variety	PPT/gr	PPT/ml	PPT cultivated	N° of Mature cotyledonary embryos	Germinated embryos		N° of shoot regenerated								N° of regenerated plant			
					N	%	MG1		MG1BAP		C2D		C2DBAP		RIM		MSN	
							N	%	N	%	N	%	N	%	N	%	N	%
Sultana	21×10^6	1×10^5	8×10^5	75	36	48	3	33	4	44	2	22	2	22	7	39	4	22
		5×10^5	40×10^5	50	24	48	2	25	3	37	2	25	1	12.5	5	41	3	25
		1×10^6	8×10^6	45	24	53	2	16	4	30	7	54	0	0	6	50	7	58
Shiraz	6.8×10^7	1×10^5	12×10^5	170	112	66	8	28	16	57	7	25	5	17	24	43	12	21
		5×10^5	60×10^5	98	88	89	5	23	13	60	4	18	7	32	18	81	11	50
		1×10^6	12×10^6	90	68	75	0	0	10	59	4	24	2	12	10	59	6	35
Corvina	5.7×10^7	1×10^5	16×10^5	121	84	70	8	38	7	33	3	14	5	23	15	35	8	19
		5×10^5	20×10^5	96	72	75	2	11	6	33	1	5	5	27	8	22	6	16
Cabernet	4.2×10^7	1×10^5	4×10^5	15	3	20	-	-	-	-	-	-	-	-	-		-	-
		5×10^5	5×10^5	22	4	18	-	-	-	-	-	-	2	50	-	-	2	22

1.4. Discussion

Grapevine (*Vitis vinifera* L.) is a woody perennial vine that is grown all over the world. The traditional technique of propagating grape vines takes a long time and permits illnesses to spread. It takes four to five years for a planted grapevine to yield cuttings for propagation. The tissue culture approach allows for the bulk generation of genetically uniform populations as well as healthy plants. As a result, is a critical method for the grape vine culture program [38].

Plant cells have the remarkable trait of totipotency, which allows somatic cells or protoplasts from fully differentiated, non-dividing cells to dedifferentiate, re-enter the cell cycle, and proliferate, eventually regenerating the entire plant. (Papadakis et al. 2009) [41]. The capacity to isolated large numbers of healthy protoplasts is critical for effective protoplast culture and the creation of a reliable protoplast-to-plant system [39]. Many factors, including genotype, physiological status and growth circumstances of the protoplast source tissue, protoplast isolation, and medium composition, impact its establishment [14, 40]. Therefore, when given the correct chemical and physical stimuli, each protoplast is theoretically capable of regenerating a new wall and of undergoing repeated mitotic division to produce daughter cells and finally a whole plant. The key growth regulators of prolonged protoplast growth are auxins and cytokinins [41].

In this chapter we described the results for the improvement of protoplast to plant system as the main platform for genome editing application in grape. Since protoplasts are easily targeted by polyethylene glycol (PEG) mediated fusion, they were the first choice to be employed for DNA-free Genome Editing [20].

EC induction was successfully performed according to Dhekney et al., 2009 [21], in Sultana and Dhekney and Li., 2011 [25] in Syrah, Corvina and Cabernet sauvignon. The establishment and maintenance of embryogenic cultures in *Vitis* sp. is highly genotype- dependent. Embryonic calli are maintained for a long time in the C1 medium

for Sultana and Syrah, although this medium is not suitable for the Corvina and Cabernet varieties long maintenance, LTMM medium was adequate to maintain long-term embryogenic cultures of both cultivars. The roles of growth regulators in somatic embryogenesis have previously been studied. In grapevine, auxins, especially 2,4-D, have been very effective for inducing somatic embryogenesis, but can inhibit subsequent embryo development. They have been reported to arrest the development of embryos at the globular proembryonic stage and induce indefinite proliferation of embryonic cells [37].

In line with the previous studies on two recalcitrant grape cultivars 'Niagara' and 'Fredonia' our results on Corvina and Cabernet also showed that 2,4-D alone was the most important growth regulator for maintenance of long-term embryogenic cultures and essential for proliferation. In case of Sultana and Syrah the synergistic effect of auxins (2,4-D) in combination with cytokinins (BAP) is essential.

It was observed that the plating density $1x10^5$ protoplasts/ml was the most efficient density with the highest mature somatic embryo recovery in all the cultivars. We employed medium enriched with combinations of NAA and BAP [42] for plant regeneration from protoplast as described by Zhu et al.,1998 [29] and Bertini et al., 2019 [33]. According to Davey et al. 2005 [14]; the overall density of protoplasts in the culture medium (plating density) is crucial for maximizing wall regeneration and concomitant daughter cell formation. As they reported, the optimum plating density is in the range 5×10^4 to 1×10^6 protoplasts ml^{-1}. A very high plating density could cause to consume nutrients quickly and protoplast-derived cells can fail to undergo sustained division. Minimum protoplasts inoculum density can lead to fail to undergo sustained division as well. This failing can explain by the effect of medium conditioning. Many studies emphasizing that cells stimulate mitotic division of adjacent cells by releasing growth factors, including amino acids, into the surrounding medium, a process commonly known as medium conditioning or nurse culture [14].

In shoot regeneration medium study, two different media, i.e., MG1 followed by RIM, and C2D followed by MSN were compared in the present or absent of cytokinin for their effect on shoot production. **Table 1** illustrates that C2D medium is not suitable for micropropagation of neither Sultana, Syrah, nor Corvina, while MG1 medium gave the best results for shoot regeneration. The efficiency of shoot regeneration noticeably increased in present of BAP which suggest the impact of BAP in the shoot regeneration in these cultivars. Similar results in different grape verities showed that BA is the most effective among other cytokinin for inducing shoot development and enhancement of bud multiplication in *Vitis* [38, 43]. In case of Cabernet C2D medium supplemented by BAP was the only condition showed shoot regeneration as previously reported in other grape varieties [34, 38]. However, as a subsequent result of high rate of abnormal embryo development and low number of well-shaped germinated embryos, plant recovery frequencies remained low.

The effect of cytokinins (BA) on shoot development of micropropagation of grape vine cultivar was studied using the number of shoots formed. Both RIM and MSN were shown good efficiency for root induction as previously reported for other different cultivars. Although the difference between two medium is not huge but using RIM should be more recommendable [21, 38].

As a recap, in this chapter we demonstrated the significant improvement of plant recovery from protoplast derived SE in 4 *Vitis* vinifera cultivars based on timed application of optimized media. In addition, the resulting plants developed robust and vigorous shoot and root systems and were easily established in the greenhouse. Further, three of four cultivars responded positively to this culture procedure.

Enhanced plant recovery from genetically modified protoplasts facilitates improvement for a wide range of *Vitis* cultivars. This culture procedure thereby removes a significant obstacle to creating the large numbers of genetically modified lines of *Vitis* that are required for the efficient selection of improved cultivars [34].

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Chapter two

Plant transformation, protoplast isolation and plant regeneration in Sultana

Created GFP stable line of grapevine in aims of knock out the GFP to understand the efficacy of the CRISPR/Cas9 system to target a gene within the genome of grape

2.1. Introduction

Agriculture and farming around the world are highly depended on crops that produce food and fiber for humans, either directly or indirectly through livestock. Modern technology has improved agricultural operations over the last two centuries, complementing traditional plant breeding approaches to improve crop productivity and quality. However, a number of issues, including population expansion, environmental stress, ecological concerns, and the demand for renewable energy, have increased the demand for improved crop quality and quantity. Plant genetic engineering has become one of the most essential molecular techniques in modern molecular breeding of crops, opening new options in this area [1]. As a result of advances in plant genetic engineering, it is now possible to transfer genes into crop plants from unrelated plants and even nonplant organisms; as a result, many crop species are being genetically modified for better agronomical traits such as disease resistance, insect tolerance, nutritional value, and other desirable qualities [2]. Foreign genes from various sources, as well as the creation of products in transgenic plants, are currently a new facet of the molecular agriculture revolution. Furthermore, transgenic plants have a wide range of non-agricultural applications and can be used to produce medically valuable and recombinant proteins and vaccines [3].

The grapevine (*Vitis vinifera* L.) is one of the world's most commercially significant fruit crops, and it is extensively cultivated for fruits, juice, and, most importantly, wine. The completion of the grapevine genome sequencing project a few years ago paved the way for more in-depth genetic research [4], it is now possible to use molecular tools like the CRISPR/Cas9 system to research gene function and use fundamental biological knowledge to enhance agronomic performance and quality attributes. Therefore, to test the efficacy of the CRISPR/Cas9 system in targeting a gene within the grape genome, we have generated a transgenic grape line carrying a T-DNA

insert containing a GFP expression cassette. We conducted the study described herein to understand the process and to learn the limitations of the gene editing technology for grape improvement.

Among the various ways of plant genetic transformation, *Agrobacterium*-mediated technologies are specified as powerful tools and effective techniques for delivering genes of interest into a host plant. Even though it is technically challenging, these *Agrobacterium*- mediated approaches are still preferred for transgenic plant production, as they present several advantages, include the ability to transfer large intact segments of DNA, simple transgene insertions with defined ends and low copy number, stable integration and inheritance, and consistent gene expression over the generations [5].

Genetic improvement of grapevine is based on traditional breeding and genetic engineering, with the availability of germplasm resources and the identification of agronomically relevant genes being key factors. Grapevine genetic engineering is a promising method for increasing economic value and productivity [6]. For grape wine improvement, the modification by genetic transformation is an ideal approach, since the essential characters and identity of the cultivar remain unaltered, which is impossible by conventional means [7]. Gene transfer methods allow foreign sequences to be expressed in target plant tissues while interfering with native genetic expression. As a result, they are ideal for determining the function and regulation of newly identified genes. Stable transformation makes it possible to study stable gene expression at the whole plant level [4].

Genetic transformation of grapevine (*Vitis* sp.) provides a means to incorporate important traits, including disease resistance, into existing elite varieties without altering their desirable characteristics [8]. One of the most significant advancements in current agriculture is the capacity to introduce foreign DNA into plants. The elegant and traditional studies on *Agrobacterium* tumefaciens laid the groundwork for *Agrobacterium* becoming the primary mode of plant transformation [9].

Mullins et al., (1990) [10] produced the first transgenic vines by combining Agrobacterium-disarmed vector transformation with rootstock regeneration by somatic embryogenesis. However, the first publication to point to a successful gene transfer in grapes was reported by [11]. Although *A. tumefaciens* is the most common *Agrobacterium* strain used for grape transformation, the use of *A. rhizogenes* strains to convert roots (hairy roots) also provides an intriguing environment for functional investigations. Stable transformation in grapevines is a lengthy and often challenging process. Its effectiveness is greatly influenced by the genotype, explant donor, medium composition, and transformation method utilized [5, 12]. Also, the bacterial strain, bacterial suspension cell density, and selection methods all have an impact on transformation efficiency [6].

The most popular way for stably transforming grapevine is to co-cultivate somatic embryos with *A. tumefaciens*, because embryogenic culture transformation of *Vitis vinifera* is difficult and limited to a few genotypes [12]. The mostly *A. tumefaciens* strains used for grapevine transformation are EHA105, GV3101 and C58C1. Co-cultivation of somatic embryos with *A. tumefaciens* was largely exploited to achieve functional characterization and evidence of putative key genes. There are two types of *Agrobacterium*-mediated techniques: stable and transient transformation. The first happens when DNA is transported within the nucleus of a plant, it becomes inserted into the genome of the plant for future generations to inherit. Alternatively, transient transformation refers to the situation in which the foreign DNA transiently remains in the nucleus without being integrated into the plant genome but is transcribed to express the genes of interest [5].

With the advancement of genome editing technology, genetic modification techniques have progressed dramatically in recent years. Endonucleases are used in these technologies, particularly those based on CRISPR/Cas9, to modify gene structure and expression in a more specific and targeted manner than earlier technologies. Ren et al. presented the first evidence of for grapevine who modified the metabolism of tartaric acid in Chardonnay 2016 [13].

Gene transfer technology has developed and been used to a variety of *Vitis* species during the time [14]. However, despite many improvements, the regeneration of non-chimeric transgenic plants remains a long and complex process in many grapevine genotypes.

Therefore, in this study in order to circumvent the chimeric problem and to prepare initial material for generating site-specific mutations in the GFP gene by direct delivery of RNP into protoplast, somatic embryos were transformed by *Agrobacterium* caring green fluorescent gene (strain EHA105 harboring the transformation vector pEGB3α1-TNOS::NPTII::PNOS-SF-35S::GFP::TNOS-SF) and placed in a medium to induct embryonic calli, to introduce a platform for gene editing application and following that protoplast isolated from GFP positive embryogenic calli. Plant regenerated from these protoplasts are non-chimeric plant since derived from a single cell. The versatility of a protoplast-based platform helps biologists to explore new approaches in plant crop improvement programs.

In the procedure of *Agrobacterium*-mediated transformation, target materials (mid- cotyledonary stage somatic embryos) were the suitable target [15] precultured in the suitable medium. Overall, enhancing plant regeneration and genetic transformation in the grapevine requires both developmental genes and innovative delivery mechanisms. In the near future, we believe that combining CRISPR/Cas-mediated genome editing with highly efficient plant regeneration and genetic transformation would result in major changes in grapevine genetic improvement [6].

2.1.1. Research aim

To understand the efficacy of the CRISPR/Cas9 system to target a gene within the genome of grape, we made use of a transgenic grape line that had a green fluorescent protein (GFP) gene integrated into its genome.

In this chapter the main aim is to transform GFP gene into the genome and provided a stable transgenic line caring GFP gene, followed by protoplasts isolation and plant regeneration in Sultana.

2.2. Material and method

Plant transformation by GFP and preparation of Agrobacterium culture were performed as described by [8] and [15].

2.2.1. Preparation of Agrobacterium culture:

A single colony of Agrobacterium (strain EHA105 harboring the transformation vector pEGB3α1-TNOS::NPTII::PNOS-SF-35S::GFP::TNOS-SF), was inoculated into 25 mL of selective MG/L liquid medium(MG/L medium ([16]) containing 5.0 g/l mannitol, 1.0 g/l glutamate, 5.0 g/l tryptone, 2.5 g/l yeast extract, 5.0 g/l NaCl, 0.15 g/l KH_2PO_4, 0.10 g/l $MgSO_4$.$7H_2O$, 2.5 ml Fe-EDTA solution (7.44 g/l $Na_2EDTA.2H_2O$ and 1.86 g/l $FeSO_4.7H_2O$) without biotin was used for initiation of Agrobacterium cultures) to create the Agrobacterium culture. The suspension culture was cultivated overnight at 28 °C and 160 rpm. When the bacterial suspension attained an optical density at 600 nm (OD600) of 0.8–1.0, it was centrifuged at 5000 rpm for 10 minutes and resuspended in 25 mL of modified liquid culture medium X2, (X2 is a liquid medium is an X6 modified which is consist of 20.0 g/l sucrose without TC agar and activated charcoal was utilized for Agrobacterium induction culture), supplemented with acetosyringone 100 mM. The bacterial suspension was then transferred to a 125 ml flask and cultivated for a further 3 hours at 28 °C before being used in transformation.

2.2.2. Plant materials

EC of Sultana (were initiated from young leaves of in vitro shoot tip cultures) transferred to fresh X6 medium (consists of MS, [17]) lacking glycine, and modified to contain 3.033 g/l KNO3 and 0.364 g/l NH4Cl as nitrogen sources, 60.0 g/l sucrose, 1.0 g/l Myo-inositol, 7.0 g/l TC agar and 0.5 g/l washed activated charcoal) for somatic

embryogenesis regeneration. SE at the mid-cotyledonary stage of development were used for transformation.

2.2.3. Inoculation of SE explants with Agrobacterium

For inoculation with *Agrobacterium*, the somatic embryos at mid-cotyledonary stage of development in Sultana were collected in a Petri plate and submerged for 10 minutes in 3 ml of the bacterial suspension. The bacterial suspension was extracted with a transfer pipette, and any residual moisture was blotted with sterile Whatman 3MM filter paper. After that, SEs were placed on Petri dishes with three layers of sterilized filter paper moistened with liquid DM medium (a DM medium was modified based on the DKW medium developed by Driver and Kuniyuki in (1984) [18], was used in callus induction. This medium contains DKW salts supplemented with 0.3 g /l KNO_3, 1.0 g /l Myo-inositol, 2.0 g /l each thiamine–HCl and glycine, 1.0 mg /l nicotinic acid, 30.0 g/l sucrose, 5.0 μM BA, 2.5 μM each of NOA and 2,4-D, 7.0 g /l agar TC and a pH value adjusted to 5.7 with 1 M KOH. For liquid DM medium, TC agar was omitted.).

SEs were placed into a 125 ml flask with 25 ml liquid DMcc medium (DM medium supplemented with 200 mg/L each of cefotaxime and carbenicillin) after 72 hours of co-cultivation and kept at 26°C for 24 hours on a rotary shaker (110 rpm). Liquid medium was withdrawn and replaced with the same amount of fresh DMcck50 medium (DMcc + 50 mg/l kanamycin) for the next 48 hours.

2.2.4. Selection of transgenic calli and SE

SEs were then recovered and placed on solid DMcck100 medium for calli induction (containing 200 mg/L each of cefotaxime, carbenicillin and 100 mg/L kanamycin). There were 30 – 35 SE on each culture plate. To generate transgenic calli, cultures were kept in dark at 26°C. Afterward, GFP positive-embryogenic calli induced in DMcck100, divided and transferred into a different medium:

1. $C1^{P}$cck70 for long maintenance and subsequent uses.

2. X6cck70 for somatic embryogenesis and plant regeneration.

Stereomicroscope was used to pick GFP positive-embryogenic calli and transferred in C1pcck70 medium for long maintenance and protoplast isolation (C1p supplemented with 200 mg/l each of cefotaxime and carbenicillin and 70 mg/l kanamycin). For the first month, GFP-embryogenic calli was subcultured to C1P, then subcultured every 4 weeks to fresh C1Pk70 medium. Simultaneously, SEs were moved to X6cck70medium for embryo induction from transgenic embryogenic calli. Cultures were maintained under the same conditions for two months. Transgenic SEs were identified by the expression of GFP-specific green fluorescence. Transgenic SEs were transferred onto a fresh shoot regeneration medium for further development.

2.2.5. Protoplast isolation and cultivation of GFP-embryogenic calli

Protoplast isolation and cultivation of GFP-embryogenic calli have done the same as described in the first chapter with a little bit of modification. GFP-embryogenic calli subcultured to C1^{P}k70 media 7-10 days before isolation and GFP positive-calli have selected at the stereomicroscope before isolation. Embryogenic calli (1 g FW) was incubated in 10 ml of filter-sterilized enzyme solution on a gyratory shaker (30 cycles/min) at RT and darkness for 5 h. After 1 h of incubation, the embryogenic calli were made to liberate into small cell clumps with a sterile plastic pipette. Cell wall digestion of protoplast observed by calcofluor staining. GFP- protoplasts were cultured at 1 $\times 10^5$ protoplasts/ml by disc culture method and were monitored during first three-days by Olympus invert microscope after culturing for cell division then every week for further division, microcolonies, somatic embryogenesis step, and GFP expression.

2.2.6. Plant regeneration

Well-developed cotyledonary somatic embryos transferred to Nitsh's medium for 3 – 4 weeks in the dark. In parallel, the GFP expression in this level was controlled using a stereomicroscope by GFP filter. To identify the best shoot regeneration medium for transgenic cultivars, both derived-protoplast and from X6, two different media with

two different growth regulators consistency was tested as described for wild type in first chapter, including C2D and C2D plus 4μM BAP, also MG1 and MG1 plus 10 μM BAP medium. Well-developed germinated somatic embryos were then transferred to shoot regeneration media under the 16h light\8h dark photoperiod for one month. Once shoots were developed, they were placed in a root induction medium including MSN and RIM [19-22].

2.2.7. Analysis of transformants

- **Visual observation**
- **Polymerase Chain Reaction (PCR)**

Stable integration of the GFP gene and its expression was observed visually using a fluorescent microscope fitted with a blue filter. PCR and RT-PCR have been done to confirm the stable transformation of GFP in the host gene.

2.2.8. Plant tissue genomic DNA extraction

A small piece of young leaves was taken and placed in a sterile 1.5 ml Eppendorf tube and, after adding 100 ul of extraction buffer (including Tris-HCl pH 8.0 200 mM, NaCl 250 mM, SDS 1% (w/v), EDTA 25 mM and β-mercaptoethanol 10 mM), the plant tissue was crushed with a pestle. Another 300 μL extraction buffer was added and the mixture obtained, was centrifuged for 10 minutes at 13000 rpm at room temperature.

300 μL of supernatant were taken and transferred to a new Eppendorf and 300 μL of isopropanol were added. The tube was left for 15 minutes at room temperature and then centrifuged for 15 minutes at 13000 rpm. After eliminating the supernatant, the pellet was allowed to dry overnight. Then resuspended in 100 μL of sterile double-distilled water. Put in the fridge for overnight or some hours, centrifuge for 2 minutes at 13000 rpm, and the supernatant transferred to a new Eppendorf. The concentration of the DNA extracted was determine using the Nanodrop spectrophotometer.

2.2.9. GFP amplification

Detection of the genes of interest by PCR was performed in a 50 µL reaction mixture containing primers that cover GFP sequence (717 bp), high Fidelity Taq polymerase enzyme (PCRBIO HiFi Polymerase-Biosystems), and 1-2 µL DNA template. DNA amplifications were performed in a thermal cycler using the program: initial denaturation at 95 ℃ for 1 min, followed by 29 cycles of denaturation at 95℃ for 15 sec, annealing at 65℃ 15 sec and extension at 72℃ for 30 sec. An additional extension was performed for 5 min at 72℃ (**Table 1**). The amplification products were visualized on 2% w/v agarose gel stained with Syber Safe. The PCR product was then purified by a purification PCR kit (Wizard® SV Gel and PCR Clean-Up System. Promega) and send to Custom DNA Sequencing service of Eurofins for sequencing using Mix2Seq Kit.

Table 1. GFP primers sequences and thermal cycle program used for GFP amplification.

Primer	*Sequence 5'-3'*	
Forward	ATGGTGAGCAAGGGCGAGGAGCTGT	
Reverse	CTTGTACAGCTCGTCCATGCCGAGA	
Reaction preparation, Final volume of 50 µL		
DNA template	1 – 2 µL	22 – 150 ng/µL
Hi-fi buffer	10 µL	5x
Forward	1 µL	20 pmol
Reverse	1 µL	20 pmol
Hi-fi polymerase enzyme	0.5 µL	
H_2O	35.5 - 36.5 µL	
PCR Cycle		
1	95 ℃	1 minute (01:00)
2	95 ℃	15 second (00:15)
3	65 ℃	15 second (00:15)
4	72 ℃	30 second (00:30)
5	Go to step 2	29 times
6	72 ℃	5 minutes (05:00)

2.2.10. RT- PCR for GFP protein expression

Total RNA of transgenic plants was extracted by the Spectrum TM Plant Total RNA kit (Sigma-Aldrich) and RNA quantity was determined using a Nanodrop spectrophotometer.

RNA of GFP- Sultana was extracted by the Spectrum Plant Total RNA Kit. Young leaves were ground to a fine powder in liquid nitrogen and lysed in a lysis solution that releases RNA and at the same time inactivates ribonucleases and interfering secondary metabolites, such as polyphenolic compounds. After the removal of cellular debris, RNA was captured onto a binding column using a unique binding solution, which effectively prevents polysaccharides as well as genomic DNA from clogging the column. Residual impurities and most residual genomic DNA were removed by wash solutions, and purified RNA was eluted in RNase-free water. Up to 100 µg of total RNA can be purified from 100 mg of plant material in 30 minutes after the tissue has been ground. After that, it's necessary to remove the trace amount of genomic DNA from RNA by Turbo DNase (TURBO DNA-free kit - Ambion). DNase- treated RNA was then used for cDNA synthesis using the SuperScriptIII Reverse Transcriptase kit (Invitrogen). The cDNA was correctly synthesized based on the amplification which has been done on the Ubiquitin as a house keeping gene. Then cDNA was used for the RT-PCR analysis to assess the transgene expression, using Go Taq DNA Polymerase (Promega) (**Table 2**).

Table 2. UBQ and GFP primers sequences and thermal cycle program used for RT-PCR.

Primer		*Sequence 5'-3'*
UBQ forward		TCTGAGGCTTCGTGGTGGTA
UBQ reverse		AGGCGTGCATAACATTTGCG
RT-PCR forward		GAAGTTCGAGGGCGACAC
RT-PCR reverse		CCGTCCTCCTTGAAGTCG
Component		1x – final volume 20 *µl*
cDNA		1 *µl*
Go Taq green buffer 5x		4 *µl*
dNTP (50 µM)		0.4 *µl*
UBQ forward (20 µM)		0.4 *µl*
UBQ reverse (20 µM)		0.4 *µl*
Go Taq		0.1 *µl*
H2O		13.7 *µl*
Cycle		
1	94 °C	5 minutes (05:00)
2	94 °C	30 second (00:30)
3	55 °C	30 second (00:30)
4	72 °C	0 second (00:20)
5	Go to step 2	30 times
6	72 °C	5 minutes (05:00)
Component		1x final volume 50 *µl*
cDNA		2 *µl*
Go Taq green buffer 5x		10 *µl*
dNTP (10 mM)		1 *µl*
GFP forward (20 µM)		1 *µl*
GFP reverse (20 µM)		1 *µl*
Go Taq		0.4 *µl*
H2O		34.6 *µl*
Cycle		
1	94 °C	5 minutes (05:00)
2	94 °C	30 second (00:30)
3	55 °C	30 second (00:30)
4	72 °C	20 second (00:20)
5	Go to step 2	35 times
6	72 °C	5 minutes (05:00)

2.3. RESULTS

2.3.1. Stable genetic transformation

To achieve a stable line of Sultana carrying green fluorescence reporter gene integrated to the genome as a tool for further genome editing application,150 somatic embryos of Sultana in the mid-cotyledonary development stage were lightly placed on three layers of sterile filter paper after inoculation with *Agrobacterium* and co-cultured in the dark. After the co-cultivation treatment, the explants were washed several times with cefotaxime and carbenicillin at matching concentrations to inhibit the growth of *Agrobacterium* and finally transferred to the embryogenic calli induction medium with a selection agent to screen the positive transgenic calli. 12% of SEs inducted EC after 10 weeks and ~83% of these ECs showed GFP expression. **Fig. 1, a-e**.

2.3.2. Long maintenance

GFP positive embryogenic calli as maintenance for a long time in $C1^{P}$ medium, plus antibiotic kanamycin, with success and in some months without kanamycin, which embryogenic calli received less stressful conditions for recovery and faster proliferation. **Fig. 1, f-g**.

2.3.3. Germination of transgenic SE and plant recovery

GFP-embryogenic calli moved to X6 for embryo induction from transgenic embryogenic calli. Somatic then transferred to regeneration medium for 1 month. well-developed and good expressed GFP somatic moved to MG1BAP and C2DBAP for shoot regeneration under the light and follow that into rooting medium. this part aimed to control the quality of transgenic embryogenic calli for plant regeneration. **Fig. 1, h-k**.

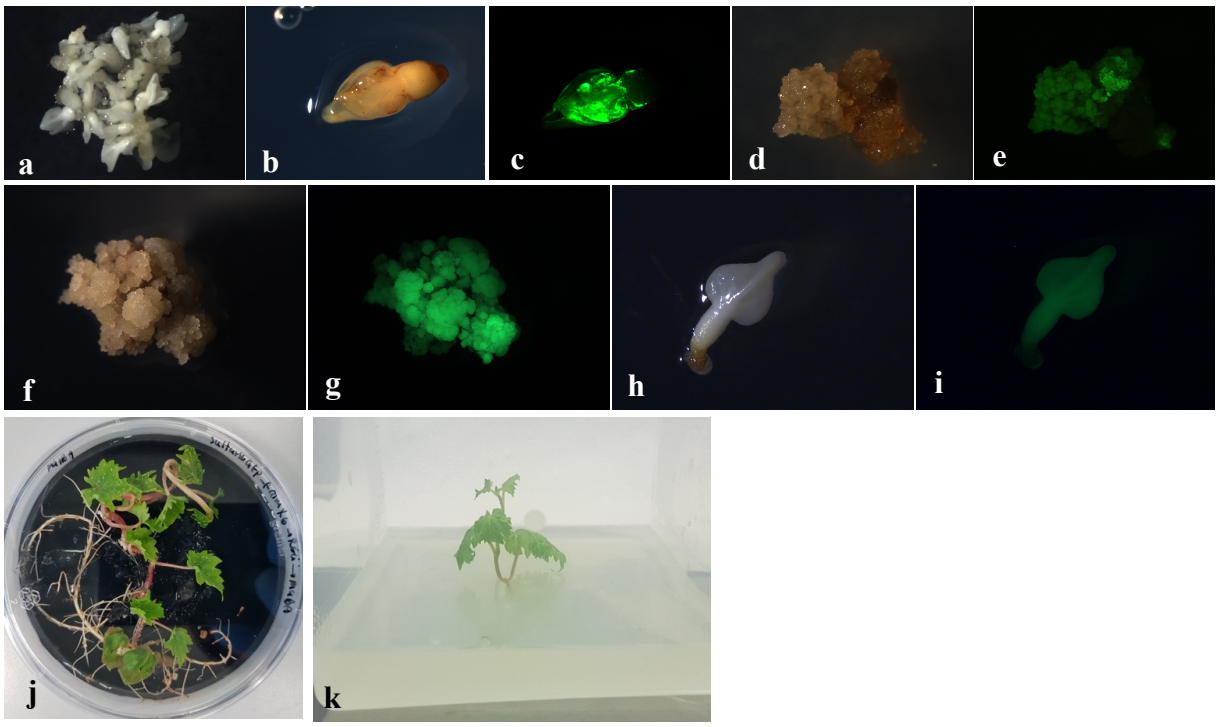

Figure 1. **a**. Somatic embryos inducted in X6 medium and ready for transformation, after 72h co-cultivation by *Agrobacterium* WL*(**b**) and GFP (**c**), EC inducted from transformed somatic embryo in DMcck WL(**d**) and GFP (**e**), GFP-EC in C1cck70 WL (**f**) and GFP (**g**), Somatic inducted from GFP- EC WL (**h**) and GFP (**i**), **j**. Shoot regenerated in MG1BAP medium, **k**. Whole plant regenerated. WL* white light.

2.3.4. Protoplast isolation and cultivation of GFP-embryogenic calli

Protoplasts were isolated from the embryogenic calli 7-10 days after subculture, which yielded 4×10^7 protoplast /g fresh callus. The disk culture method was practical to cultivate the protoplast and AC added to the liquid medium to prevent the browning of somatic. The first cell division happened during the first 3 days, formation of micro

calli and pre globular embryoids formed after 10-14 days of cultivation. Within the next 30-35 days produced heart shape, torpedo, and cotyledonary embryoids (**Fig. 2**).

74 mature somatic of Sultana transferred to regeneration medium for 3-5 weeks and follow that 48 well developed somatic moved in the shoot regeneration media (MG, MG1BAP, C2D, and C2DBAP) and then root induce medium (RIM and MSN). Results showed that MG1 and C2DBAP media are more efficient for shoot regeneration **(Table 3).** The germinated shoots developed into whole plants with expanded leaves and roots after four weeks with the efficiency of 41% in RIM and 50% in MSN media.

Plantlets with four to five completely developed leaves that had been grown on RIM and MSN were then transferred to the soil. They acclimatized quickly and were successfully transported to the greenhouse. Plantlets generated from protoplasts were planted in the greenhouse. When compared to wild-type plants, acclimated plants had a typical phenotype in terms of leaf shape, color, and growth behavior (**Fig. 3**).

Table 3. The effect of different culture mediums on whole plant regeneration.

	ppt cultivated	**ppt/ml**	**Mature embryos**	**Germinated embryos**	**N° of shoot regenerated**								**Whole plant regenerated**			
			N°	N°	**MG1**		**MG1+BAP**		**C2D**		**C2D+BAP**		**RIM**		**MSN**	
Sultana-GFP	12×10^5	$\mathbf{10^5}$	74	46	8	(%) 66	3	(%) 25	4	(%) 33	9	(%) 75	10	(%) 41	12	(%) 50

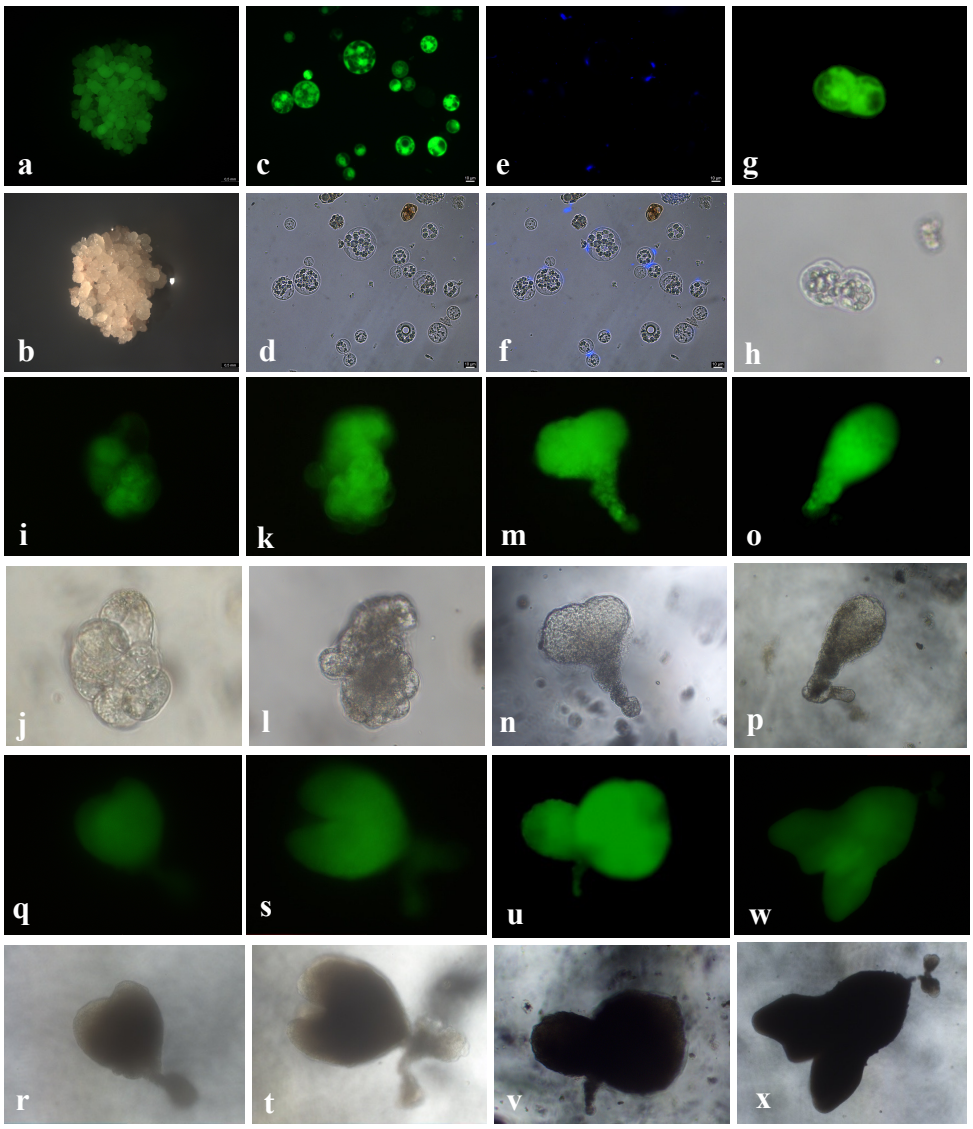

Figure 2. Protoplast's derived somatic embryogenesis steps in GFP-transformed Sultana. Embryogenic calli GFP(**a**) and WL*(**b**), Protoplast isolated GFP (**c**) and WL(**d**), Calcofluor staining Blue Light(**e**) and WL (**f**), First cell division GFP(**g**) and WL(**h**), Further cell division GFP(**i**) and WL (**j**), Microcalli GFP (k) and WL(l), Proembryo GFP(m) and WL(**n**), Globular GFP (**o**) and WL (**p**), Triangular GFP (**q**) and WL(**r**), Heart stage GFP(**s**) and WL(**t**), Torpedo GFP (**u**) and WL (**v**) cotyledonary stages GFP(**w**) and WL(**x**). WL* white light.

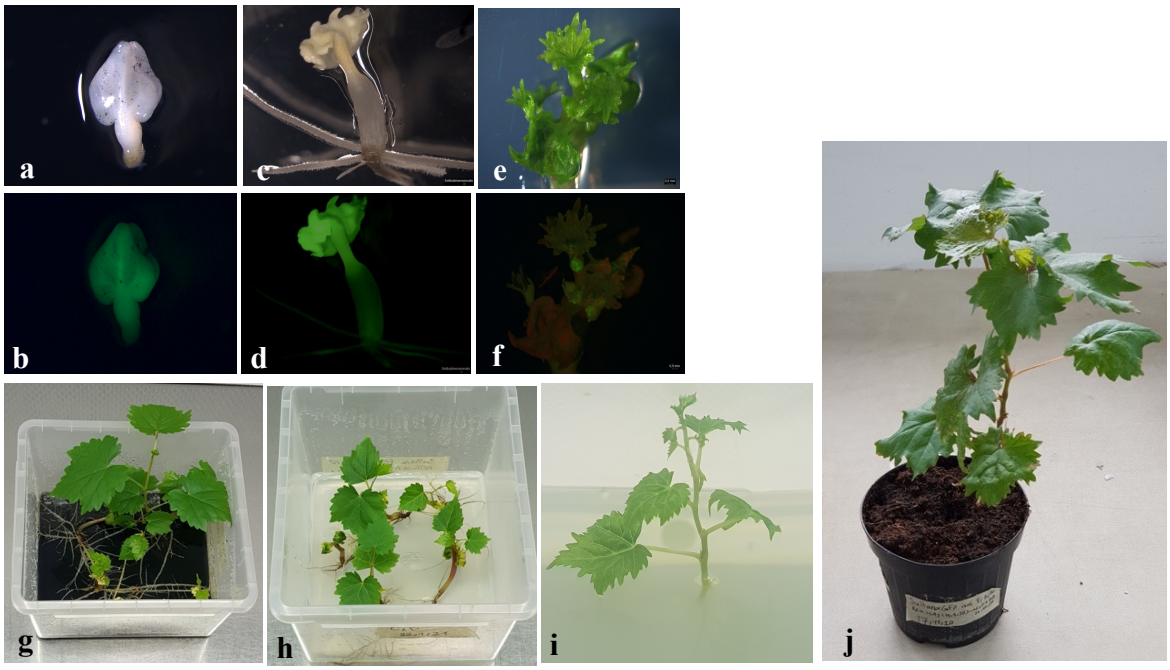

Figure 3. Mature SE WL (**a**) and GFP filter (**b**), Well-developed SE (**c**) and WL (**d**) GFP filter, GFP expression in shoot apical young leaves WL (**e**) and GFP filter (**f**), Shoot regenerated in different regeneration media: **g**.MG1BAP and **h**.C2D, **i**. Regenerated plantlet in root regeneration medium, **j.** Potted plant in green house.

2.3.5. Molecular analysis of transgenic plants; PCR analysis using genomic DNA of transgenic grapevines containing GFP

The confirmation of transgene expression was carried out by PCR, RT-PCR and sequencing analysis. Genomic DNA was extracted from transgenic plants lines and used for amplification of the GFP gene. Amplification of a 717 bp fragment corresponding to the GFP gene following PCR was observed in transgenic plants. Following that presence and stable integration of transgenes in the plant genome were confirmed by sequencing of 717 bp GFP gene fragment (**Fig. 4, a**).

2.3.6. RT-PCR

RNA was extracted from Sultana-GFP derived from protoplast; the quantity of RNA measured by Nanodrop was 326 ng/µl. Amplification of Ubiquitin showed that cDNA has been synthesized correctly (**Fig. 4, b**). RT-PCR using GFP primers confirmed the expression of GFP protein in the transgenic plants (**Fig. 4, c**).

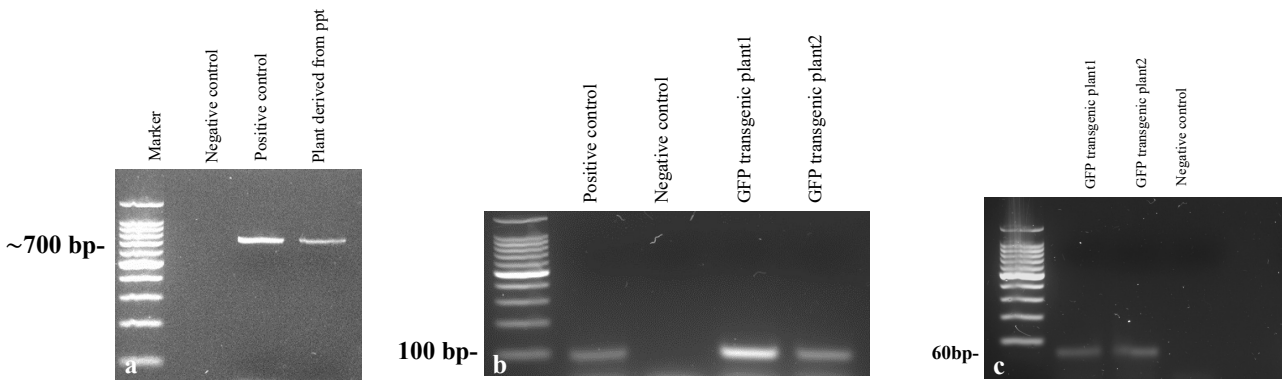

Figure 4. **a.** PCR amplification of transformants plants showing *gfp* gene. **b.** Amplification of Ubiquitin as a house keeping gene to confirm cDNA synthesis correctly, **c.** RT-PCR using GFP primers confirmed the expression of GFP in the transgenic plant**.**

2.1. Discussion

Grapevine (*Vitis* spp.) is a globally important perennial fruit crop that is grown for fresh fruit, raisins, and a variety of processed goods such as jam, jellies, juice, vinegar, wine and grape seed oil in many parts of the world [6, 23]. Grapevine biotechnology is one of the most promising developments in the global wine industry, which is increasingly faced with conflicting demands from markets, consumers and environmentalists [24]. Throughout the history of grape growing, efforts to improve grape production and quality have been made; traditional breeding methods, such as interspecific hybridization are, however, extremely time-consuming due to the grape's long-life cycle and heterozygous genome. In recent years, transgenic technologies have successfully improved many other crops through the introduction of insect or disease resistance. Similarly, using gene insertion technologies to introduce specific desirable features into present grapevine varieties could be a viable option for grapevine improvement [6, 12].

As stated earlier, the DNA-free genome editing technologies is problematic in *Vitis vinifera* species and has not been done in Sultana yet. This is largely due to the recalcitrant nature of *Vitis* spc. transformation, and its large genome size [5].

As a first step towards addressing these difficulties, we conducted the study described herein to understand the process and to learn the limitations of the technology for grape improvement. We made use of a transgenic grape line that has a T-DNA insert containing a GFP expression cassette. The use of GFP gene as the target allowed us to follow the putative mutations easily. Although earliest loss of GFP fluorescence was observed at 15 days post infection, it is possible that the mutation responsible for the phenotype had occurred much earlier. In many woody perennial plant species somatic embryogenesis have been used for micropropagation and genetic transformation. In general, the efficacy of somatic embryo induction has been extremely poor, and it is highly dependent on the explants' developmental stage [25].

This study specifically details the methodology to produce transgenic SE lines of Sultana. Somatic embryos of Sultana in mid-cotyledonary stage were used for transformation followed by embryogenic calli induction.

Li et al., 2006 [26] in a study aimed on develop procedures to facilitate transformation of grapevine have reported the SEs are ideal targets for transformation because the regenerative cells are accessible to *Agrobacterium*, and the single cell origin of secondary SEs results in non-chimeric transformants. Most previous reports on successful recovery of transgenic grapevines also utilized somatic embryos as explants [26]. In this study, to decrease the frequent of chimeric transformants to the minimum rate, we used protoplast isolation which initiated from callus inducted of transformed SEs in DM medium. In this way the produced line will be originated from a single cell and will be pure. In grapevine, as in many plant species, SE induction generally begins with callus formation on a medium supplemented with moderate concentrations of auxin and cytokinin, mostly 2,4-D and BAP as described before [8, 25]. In this study we found that X6 medium free of growth regulators is the best condition for SE induction, while 5.0 μM BA, 2.5 μM each of NOA and 2,4-D are required for EC induction from SEs in Sultana cultivar.

In addition, long-term maintenance of regenerated embryogenic callus (EC), proembryonic masses (PEM), and SE is critical, and a circulatory system for embryogenic culture maintenance and transformation of Sultana was recently published in this regard by [25]. We managed the maintenance of regenerated transformed embryogenic callus in C1^{p}k for long time. The totipotency of transformed EC has confirmed by regeneration of SEs and whole plants. These ECs subsequently has been used for protoplast isolation followed by whole plant regeneration with a considerable efficiency.

As a result, we have established an efficient plant regeneration system which is the key to successful genetic transformation and the consequent generation of transgenic plants.

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Chapter three

Set up an efficient platform for DNA-free gene editing

Investigation the feasibility of improving CRISPR/Cas9 transformation in grape protoplasts as a difficult-to-transfect cell line

3.1. Introduction

CRISPR-associated nuclease9 (Cas9) system has recently been announced as an emerging genome editing tool for plant breeding to improve plant varieties with novel traits using artificial nuclease enzymes such as clustered regularly interspaced short palindromic repeat (CRISPR)—CRISPR associated nuclease 9 (Cas9) system (Details in Chapter 4) [1, 2].

A successful delivery of a complex into the plant cells always is challenging. To address this issue, first we have optimized the conditions for cell wall digestion. Second, to evaluate the efficiency of in difficult-to-transfect cell lines; we have employed transient expression vector carrying YFP gene. Finally, we investigated the feasibility of improving CRISPR/Cas9 transformation; a cutting-edge technology has been utilized to visualize CRISPR complex transfection. In this approach GFP was used as a visual reporter to facilitate Cas9/sgRNA-transfection monitoring.

Reporter genes have long been used to identify transformed from untransformed cell tissues and to improve transformation processes by measuring expression. They can be used to investigate the transcriptional activity of a particular gene under various environmental or physiological conditions and to localize gene products [3].

3.1.1. Cell wall digestion

The efficacy of genome editing is largely dependent on nuclease protein transfection using proper delivery techniques. In the majority of animal investigations, Cas9 protein is delivered through lipofection transfection reagent or electroporation. Tissue culture-dependent transient expression techniques such as callus culture, protoplast transfection, and *Agrobacterium*-mediated agroinfiltration are used in plants [2, 4].

Delivery through the plant cell wall is one of the problems for DNA-free transformation. Most edits employ isolated protoplasts, single plant cells which cell wall has been enzymatically digested, to circumvent the plant cell wall barrier [4].

Plant protoplasts without cell walls provide a unique single-cell system that offers a versatile cell-based experimental system. Various techniques, such as PEG–calcium fusion, electroporation and microinjection, can be used to transfer macromolecules like DNA, RNA, and proteins into protoplasts [5].

Cells of primary plant tissues are made up of cellulose walls with a pectin-rich matrix, the middle lamella, which connects adjacent cells. The cytoplasm of each cell is surrounded by the plasma membrane, which constitutes the protoplast. Normally contact between the plasma membrane and the wall is maintained, because the plasma membrane is engaged in wall formation. On the other hand, hypertonic solutions, cause cell plasma membranes to contract away from their walls. Subsequent removal of the latter structures releases large populations of spherical, osmotically fragile protoplasts (naked cells), where the plasma membrane is the only barrier between the cytoplasm and its immediate external environment. Protoplast isolation from a wide range of species is increasingly commonplace; viable protoplasts are potentially totipotent. As a result, each protoplast is capable when given the right chemical and physical stimulation, theoretically, regeneration of a new wall and recurrent mitotic division to produce daughter cells from which viable plants can be grown via tissue culture [6].

Plant regeneration from protoplasts is a multi-step phenomenon that protoplasts must go through cell wall neo-formation, de-differentiation, cell cycle activation, and cell proliferation. Furthermore, enzymatic isolation is also known to cause a variety of metabolic alterations to produce stressors such as the generation of activated oxygen species (AOS) and therefore, to activate de novo synthesis of stress-related compounds. Recalcitrance that has been seen at various stages of protoplast isolation and culture, has been attributed to the lack of recovering from the stress of isolation. Interesting observations have been made about the differences between non-regenerating

protoplasts and regenerating protoplasts: It has been demonstrated that during isolation and culture, grapevine recalcitrant protoplasts create activated oxygen species (AOS), however, the antioxidant machinery is not effectively stimulated, thus reducing their ability to regenerate.

The synthesis of stilbene phytoalexins in *Vitis* spp. protoplasts were discovered by Commun et al. (2003) [7], Trans-resveratrol was discovered as early as 4 hours after the start of enzyme digestion, due to the activation of the *vst1* gene, which codes for stilbene synthase. The presence of resveratrol and its phytoalexins, $\varepsilon -$ vinifera and pterostilbene, which are generated from it, could explain protoplast viability reduction. Cell wall digestion assay by calcofluor staining shows the majority of the most cell wall has been digested after 5h, although digestion of all the cell wall in the woody plants is difficult, on the other hand, a long-time treatment with the digestion enzymes could decrease the totipotency and viability of protoplasts [6, 7].

In this chapter we have shown that 5h is optimum for cell wall digestion of protoplasts to keep both totipotency and viability of protoplast.

3.1.2. The transient transformation technique

Expression of fluorescent proteins (FPs) has been used in many systems to investigate protein interactions, trafficking, turnover, organelle biogenesis, movement, and inheritance. During the last decade we have seen the development of GFP variants allowing the imaging of several FPs in the same cell, and recently photoactivatable and photo switchable forms have making FP technology an extremely powerful tool. The transient transformation technique of plant protoplasts, lacking specific cell wall features is widely employed in genetic research including gene function identification, subcellular localization, and gene editing [8-10].

Plants can theoretically be engineered to overproduce basically any product, endogenous or foreign, using new molecular tools, so long as the plant species is amenable to the manipulation procedures demanded. For a plant to express any gene in such a way, the first step is to introduce it into the plant cells. This could be achieved by stable transformation, usually with agrobacterium-delivered T-DNAs, some- times through bombardment or by other means. However, stable plant transformation has limits. To give only a few examples, first, proven approaches for regenerating transgenic plants from transformed cells in calli obtained from plant tissues or cell cultures are confined to only a few plant species. Second, obtaining homozygous transgenic lines might take a long time, possibly more than a year. Third, if the product to be expressed is deleterious or harmful to the plant, regeneration of full size, healthy-looking plants may not be possible, or require the use of for example inducible promoters or other specialized approaches. Fourth, in the field of transgenic plants, licensing imposes restrictions in those countries/economic regions where they are permitted, as well as long safety and regulatory procedures that would delay their availability for non-research use by many years. An alternative to planting stable transformation is the use of transient expression systems to express the desired products on already grown, non-transgenic plants [11].

Transient expression experiments are a quick and easy way to perform primary plant biology research. They were created for gene function research and have also proven useful for evaluating the activity of gene constructs prior to undergoing stable transformation. Many sequencing data sets have recently been published in the grapevine community, sparking interest in developing effective transient expression methods in these species [9, 12].

Transient expression assays are the most effective technique to analyze a large number of genes in a short period of time. They are based on the transcribing of DNA sequences at a high level that does not necessarily integrate into the plant genome. Methods for transient gene expression in plants were developed alongside stable transformation processes in the 1980s. These mainly involve *Agrobacterium*

tumefaciens-mediated transformation or direct gene transfer by chemical (polyethylene glycol, i.e. PEG treatment) or physical (particle bombardment) techniques.

Indeed, numerous copies of the transgene are actively translated in plant cells for a brief period after incubation with A. tumefaciens, allowing for expression up to 1000-fold greater than in stably transformed tissues. Direct transformation techniques, on the other hand, result in quick and high-level expression of the inserted DNA.

Protoplasts, cell suspension cultures, single organs or entire plants are exposed to the gene transfer procedure in transient expression research. No photosynthetic tissues, such as onion epidermal cells or petals, are ideally suited for fluorescence- or color-based reporter gene localization or quantitative expression investigations. Due to the current lack of mutant collections in grapevine, transient expression assays constitute an appropriate approach to decipher the huge amount of genetic information becoming available. As evidenced by recent research, heterologous systems can be employed and have proven to be beneficial. Agroinfiltration of *Nicotiana Benthamian* leaves, for example, revealed the participation of the grapevine enzyme anthocyanin O-methyltransferase (AOMT) and its cytosolic location. Likewise, the ATP-binding cassette protein ABCC1 was localized to the tonoplast. Particle bombardment of onion cells can also help investigate the localization of grapevine proteins, as shown for the zinc transporter ZIP3 in the plasma membrane. However, gene expression in heterologous systems may exhibit aberrant traits, presumably due to a foreign genetic background. Grapevine is a woody perennial species, characterized by unique features whose study preferentially requires a homologous gene transfer system [3, 13-15].

PEG-mediated transformation, electroporation-mediated transformation and microinjection-based transformation are all typical strategies for plant protoplast transformation. Among these, the PEG-mediated approach is the most popular because of its ease of use, low cost, lack of equipment requirements and generation of stable results [3, 16].

PEG-mediated transformation makes use of an inert ethylene oxide hydrophilic polymer that aids in the transport of DNA into protoplasts. In this approach, DNA molecules are directly incubated with protoplasts, and the transfer is triggered by adding divalent cations to the mixture. The addition of PEG to the protoplast mixture destabilizes the permeability of the plant membrane and allows free DNA to enter the plant cytoplasm. On the one hand, the application of PEG-mediated delivery systems for mature plant transformations is limited due to the lack of an efficient approach and methodology for the regeneration of complete plants from protoplasts. On the other hand, it has been widely utilized in plants for transient experiments to verify gene function.

In grapevine, PEG-treated protoplasts from Cabernet sauvignon cell suspension cultures were used to study protein subcellular localization, promoter analysis, protein/protein and DNA/protein interactions. More recently, PEG-mediated transformation of Chardonnay protoplasts was exploited as a preferential method for the direct delivery of purified CRISPR/Cas9 RNPs [4, 16]. The protoplast transient expression system is a flexible technique for genome editing in plants using CRISPR/Cas9. The main advantages of CRISPR are its simplicity of usage and protoplast system, which may give a high degree of transgenic expression. Many plants have been successfully edited with CRISPR-Cas9 employing a transient transfection technique, resulting in the functional evaluation of certain prospective genes as well as genetic enhancement of various agricultural crops [2].

Transient production of plasmid-based CRISPR-DNA or stable integration with subsequent backcrossing has both been used to start mutations in several circumstances. Integration of DNA into the host genome is still conceivable with both procedures since plasmids decay in the cells and might merge into cut locations [4, 17].

3.1.3. DNA-free genome editing systems

Cleavage efficiencies by genome editing applications are unmatched by any other technology. However, researchers are always looking for ways to overcome experimental hurdles. One of the challenges is the absence of visual cues during CRISPR experiments. Scientists need a way to evaluate a successful delivery of Cas9 that can take several days before knowing if an experiment was successful. Uncertainty is removed by having a visualization checkpoint providing affirmation by a Cas9 fused a GFP. Cas9- GFP visibly show the presence of CRISPR reagents once they have been transfected, giving us peace of mind that the components have been delivered [18].

Traditional genome editing involves the delivery and integration into the host genome of DNA cassettes encoding editing components. Integration occurs at randomly and therefore can generate undesirable genetic changes. Even if the DNA cassettes are degraded, the resulting fragments may be integrated and could produce undesirable effects. Prolonged expression of genome-editing tools increases off-target effects in plants since nucleases are abundant in these organisms. Moreover, the introduction of foreign DNA into plant genomes raises regulatory concerns in relation to GM organisms. Therefore, DNA-free genome editing is an innovative technology, producing genetically edited crops with a reduced risk of undesirable off-target mutations, and meeting current and future agriculture demands from both a scientific and regulatory standpoint [4, 17, 19, 20].

Next, tools have been developed using solely RNA, preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) for the purpose of mutation induction, which is completely free of foreign DNA. This is one of the most significant advantages of employing direct Cas9 protein delivery is the ability to induce mutations fast and precisely. So that the potential of DNA integration into the genome may be ruled out and edited plant obtained could potentially bypass current GM regulations and may create a product not considered a GMO. Moreover, off-targets perform a small role in DNA-free approaches: compared with stable and transient expression, the

CRISPR/Cas9 complex is degraded within the cell within hours and no longer available, thus the CRISPR/Cas9 complex mode of action is only present in the original cells (protoplasts) of the edited plant [2, 4]. These DNA-free genome editing tools have gained more than the earlier plasmid-mediated delivery approach, which necessitates tissue-specific delivery equipment [2].

Cas9 proteins tagged with GFP (**Fig. 1**) were utilized to see if Cas9 nucleases could be transfected into protoplasts by PEG-mediated transformation. The presence of GFP fluorescence in transfected protoplasts suggests that Cas9 nucleases can undergo PEG-mediated transformation [21, 22].

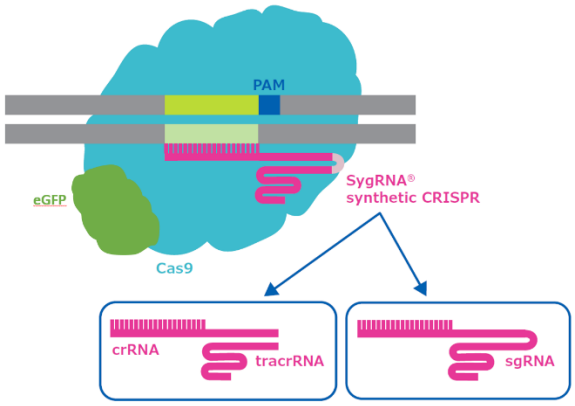


Figure 1. Ribonucleoprotein (RNP) consist of gRNA and SpCas9 fused to enhanced GFP (SpCas9-EGFP).

3.1.4. Research aim

The main aim of this chapter is to investigate the ability of protoplasts for transformation and evaluate a successful direct delivery of a RNP complex into the protoplasts.

Further objectives are including:

- Using a transient vector carrying YFP to confirm the transfectability of protoplasts via PEG-mediated transformation method.
- To validate successful delivery of RNP complex into protoplasts using GFP tagged Cass9 protein.
- To study the cell wall digestion efficacy and optimize the protocol.

3.2. Material and methods

Protoplast isolation have been done as described in chapter one (see **1.2.3. Protoplast isolation**) for both Sultana and Syrah. After cell walls are removed using enzymatic cocktail, protoplasts are released from EC of Sultana and Syrah, washed, and collected from washing solution by centrifugation (4 min at 100 g) and used for PEG mediated transfection by vector carrying YFP gene. The same process has been done for preparation of protoplasts for transfection of Cas9 tagged by GFP, only the time of incubation in digestion solution has been decreased to 5h.

3.2.1. Protoplast transfection by vector caring YFP gene

PEG-mediated transfection of protoplast was performed as described by [5] and (2015) [23].

Protoplasts were divided into different density of 10^5 and 5×10^5 cell/ml, washing solution removed and protoplast resuspended in 200 μl MMg solution (0.2 M Mannitol, 15mM MgCl2.6H2O, 4mM MES (PH 5.7). 10 µg pEGB3Ω1-35S::YFP::Tnos vector carrying out the YFP marker gene [24] were added to the protoplast's suspension, mixed well and an equal volume of freshly prepared PEG solution (40% (w/v) PEG 4000 , 0.2 M Mannitol and 0.1 M CaCl2.2H2O) was immediately added and the suspension was carefully mixed by pipetting to a homogeneous phase. The mixture was incubated for 20 min in the dark at room temperature. After incubation, 950 ml of W5 (2 mM MES (pH 5.7), 154 mM NaCl, 125 mM CaCl2.2H2O and 5 mM KCl) solution was added carefully to stop the reaction and washed the protoplasts. The protoplasts were collected (2 min at 100 g) then resuspended in 1 ml W1 (0.5 M Mannitol, 20 mM KCl and 4 mM MES (pH 5.7)). Transfected protoplasts were transferred into 6-well flat-bottomed plates and incubated in the dark at RT. The efficiency of transfection was evaluated 24,48 and 72 h post transfection using stereomicroscope (Leica MZ 16 F) under blue light (Leica CLS 150 X light source) utilizing YFP filter set comprising an excitation filter (500/20 nm) and a barrier filter (535/30 nm).

3.2.2. Protoplasts transfection by Cas9 tagged by GFP

The transfection of protoplast by Cas9-GFP was performed based on the protocol described by [25].

Ready-to-use recombinant fluorescent Cas9 protein purchased from Sigma Aldrich. An Enhanced Green Fluorescent Protein (EGFP) is fused, via a proprietary linker to the N-terminus of either wild type Streptococcus pyogenes Cas9 (Cas9-GFP Protein). This protein contains three varied nuclear localization sequences positioned for optimal activity. The molecular mass of Cas9-GFP Protein is 194 kDa. (26). gRNA design (see **4.1.9.** and **4.2.1. Design gRNA**) and synthesis (see **4.2.2. gRNA synthesis**) describe in detail in chapter four. Molar mass ratio of Cas9-GFP/gRNA; 1:3 = 60µg of Cas9-GFP and 30µg of gRNA used for protoplast transfection. Cas9-GFP and gRNA pre-mixed before transfection and incubated at RT for 10 min in darkness.

2×10^5 protoplasts resuspend in 200 μl MMg solution and transfected by RNPr-GFP, 200 µL of PEG 4000 (40%) added immediately before aggregation occurs, suspension was carefully mixed by pipetting to a homogeneous phase and incubated for 20 min at RT and darkness. 400 µL of W5 washing solution added, mixed well, and incubated at RT for a further 10 min. Then 800 µL of W5 washing solution added again, mixed gently, and incubated at RT for a further 10 min. Protoplast's mixture was centrifuge at 100g for 5 min at RT, supernatant discarded, and protoplasts resuspended in 1 mL of WI (plus 5 mM glucose) washing solution, and incubated overnight in darkness at RT. Protoplast monitored by confocal microscope in 0 and 24h of post-transfection. NucBlue® Live ReadyProbes® ThermoFisher were used for staining the nucleus of protoplasts.

3.2.3. Preparation of calli for confocal imaging

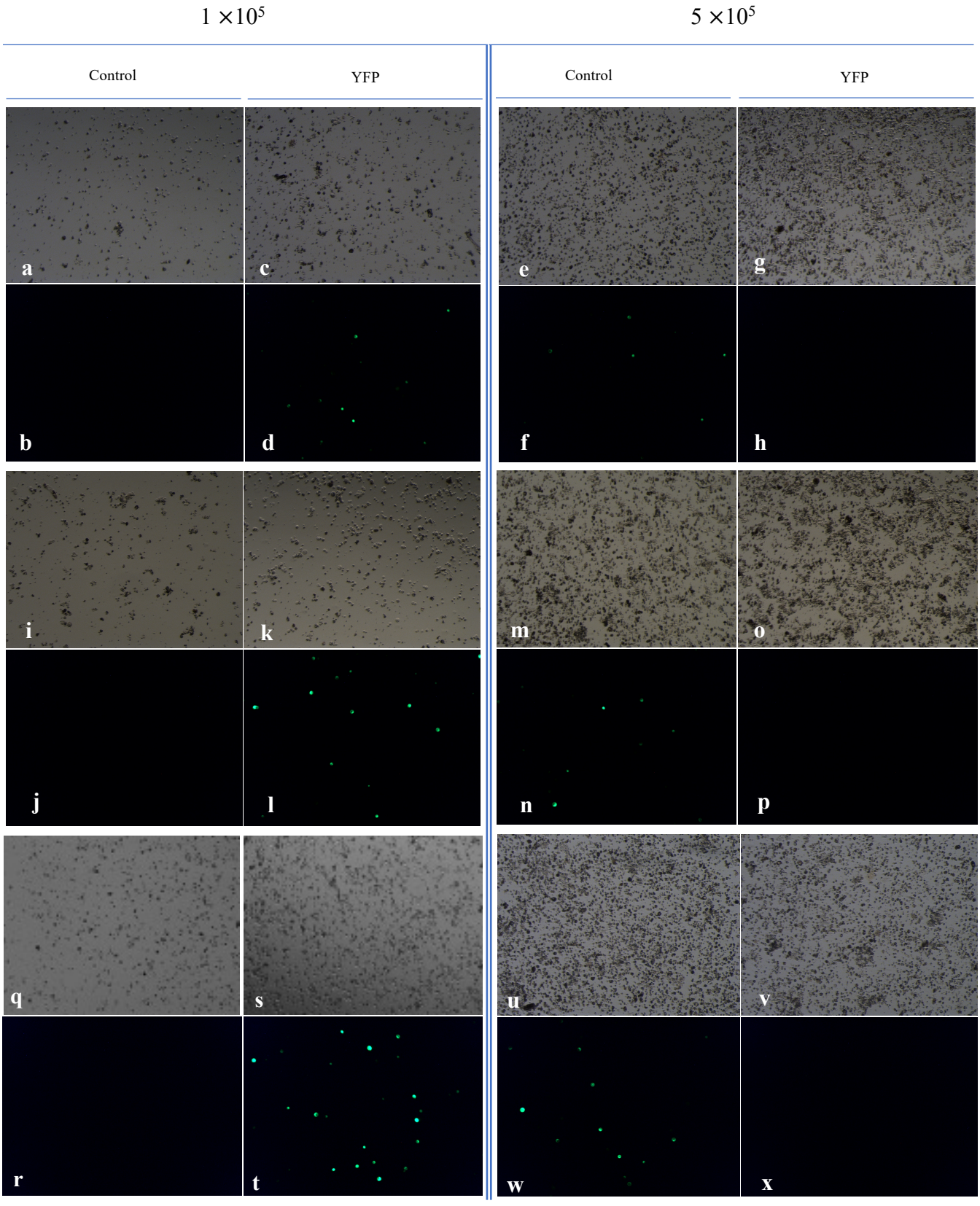
EC (1 g FW) of both Sultana and Syrah were subcultured 7-10 days in C1 medium and incubated in 10 ml of filter-sterilized solution (digestion solution without enzymes)

containing 10 mM CaCl2.2H2O, 5 mM MES and 0.5 M mannitol, on a gyratory shaker (~30 cycles/min) at RT and darkness. The pH of the enzyme solution was adjusted to 5.7 before filter sterilization. After 1 h of incubation, the embryogenic calli were made to liberate into small cell clumps. After further incubation for 4h, then were collected by centrifugation (100 x g, 5 min). cells were washed twice with washing solution containing 0.5 M mannitol and 10 mM CaCl2.2H2O by resuspension and centrifugation (100 x g, 4 min). Afterward, cells were using for confocal imaging, viability of protoplasts was assessed with 0.5 mg/mL FDA staining. Fluorescein diacetate remains the standard and most reliable fluorochrome for assessing protoplast/cell viability and the cell wall digestion controlled by Calcofluor White stain 2μM.

3.3. Results

3.3.1. Protoplast transfection by vector caring YFP gene

The PEG-method of protoplast was used for testing of transformation efficiency in protoplast released from EC. Transformation efficiency of the protoplasts was detected according to the expression of YFP reporter gene using the transient expression vector. The YFP fluorescence was observed, and images were taken in random distribution under Leica MZ 16 F stereomicroscope equipped with a Leica CLS 150 X light source and YFP filter set comprising an excitation filter (500/20 nm) and a barrier filter (535/30 nm). Transfection of protoplast by vector has done successfully and YFP expression was stable at all three times (24 to 72h). Results showed that 1 $\times 10^5$ density is more efficient for transfection of protoplasts in both cultivars. There was not any signal of YFP expression in the negative control (vector without YFP), which confirms the protoplasts transfection by successfully (**Fig. 2** and **3**).


1 ×10^5
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Control
YFP
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Figure 2. Investigation of Sultana protoplasts PEG-mediated transfectability.

Protoplasts transfection by a transient vector carrying YFP gene and monitored via Leica MZ 16 F stereomicroscope by white light and YFP filter, after 24h (**a-h**), 48h (**i-p**), and 72 hours post transfection(**q-x**) in two densities of protoplasts (1×10^5 and 5×10^5).

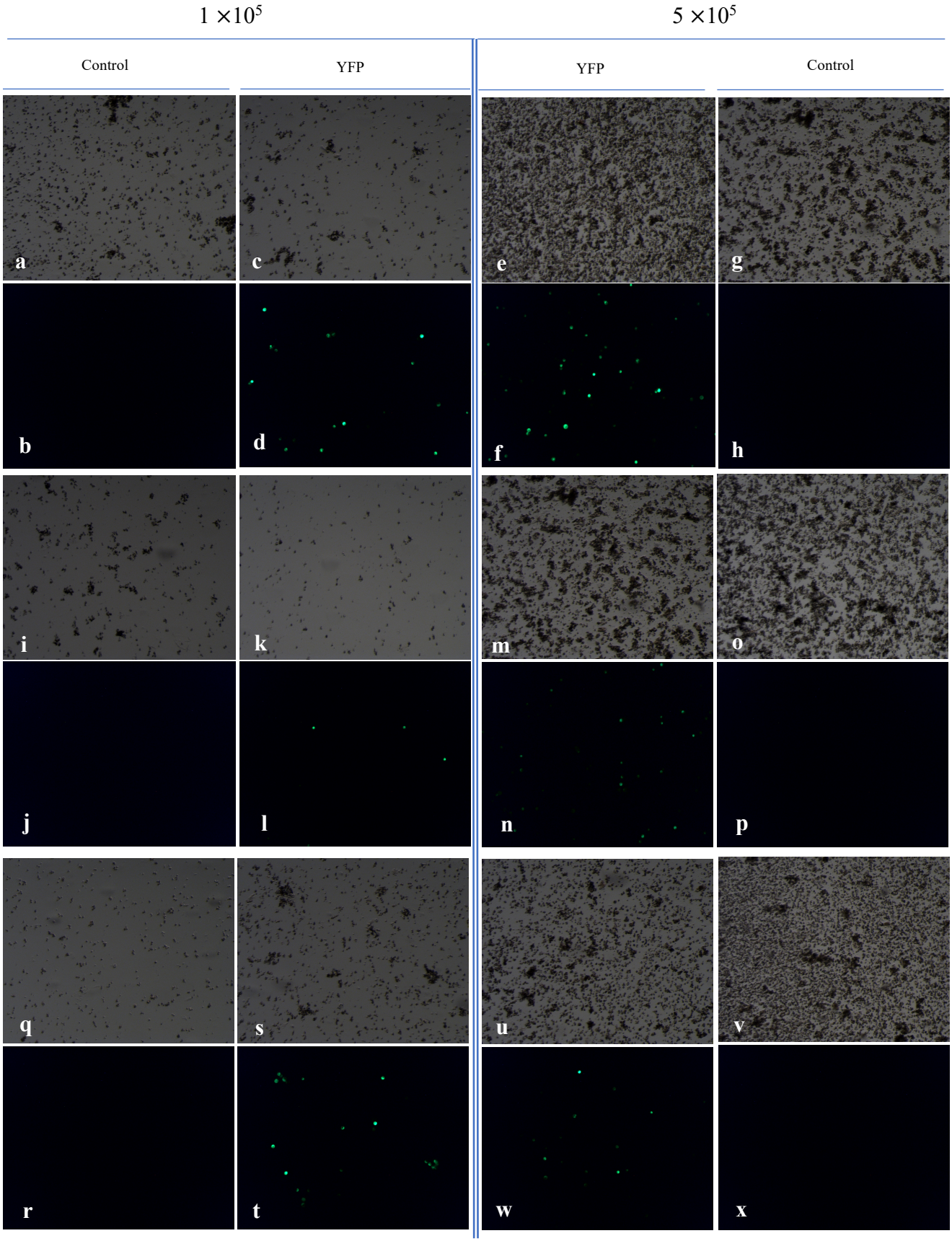
1 ×10^5
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Figure 3. Investigation of Syrah protoplasts PEG-mediated transfectability.

Protoplasts transfection by a transient vector carrying YFP gene and monitored via Leica MZ 16 F stereomicroscope by white light and YFP filter, after 24h (**a-h**), 48h (**i-p**), and 72 hours post transfection(**q-x**) in two densities of protoplasts (1×10^5 and 5×10^5).

3.3.2. Protoplasts transfection by Cas9 tagged by GFP

To investigate whether Cas9 proteins can be transfected into protoplasts via PEG-mediated transformation, Sigma-Aldrich 3×NLS-Cas9-EGFP was used prior to the introduction of the main complex. GFP fluorescence was clearly observed in transfected protoplasts of both cultivars (**Fig. 4** and **5**) and indicating that Cas9 nucleases can be subjected to PEG-mediated transformation.

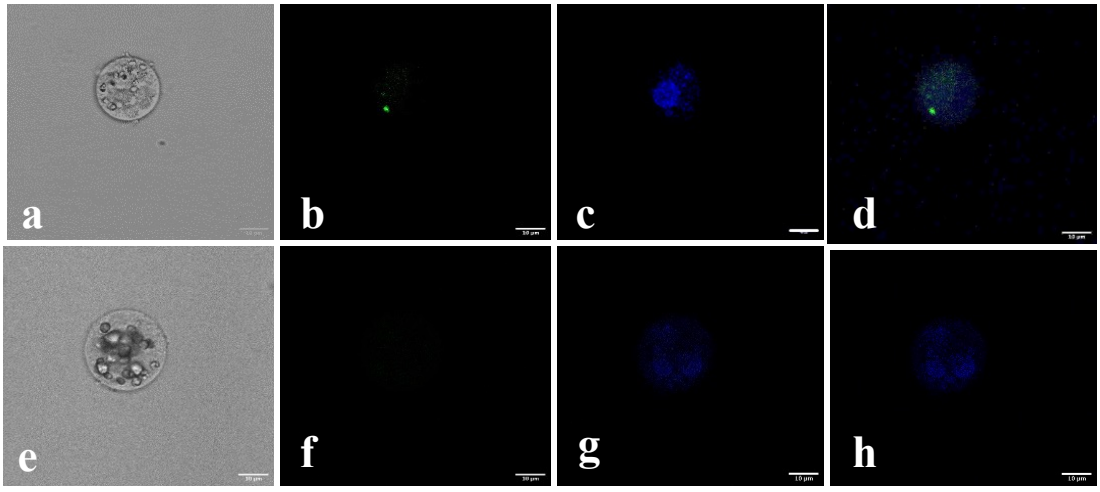

Figure 4. Confocal microscopy images for visual confirmation of RNP complex direct delivery to the protoplasts of Sultana and nuclear localization of 3×NLS-Cas9-EGFP-gRNA complex (**a-d**), control (**e-f**). **a-d** transfected by Cas9-GFP: **a**. WL, **b**. GFP filter, **c**. DAPI filter, **d**. overlay. **e-f** control: e. WL, **f**. GFP filter, **g**. DAPI filter, **h**. overlay.

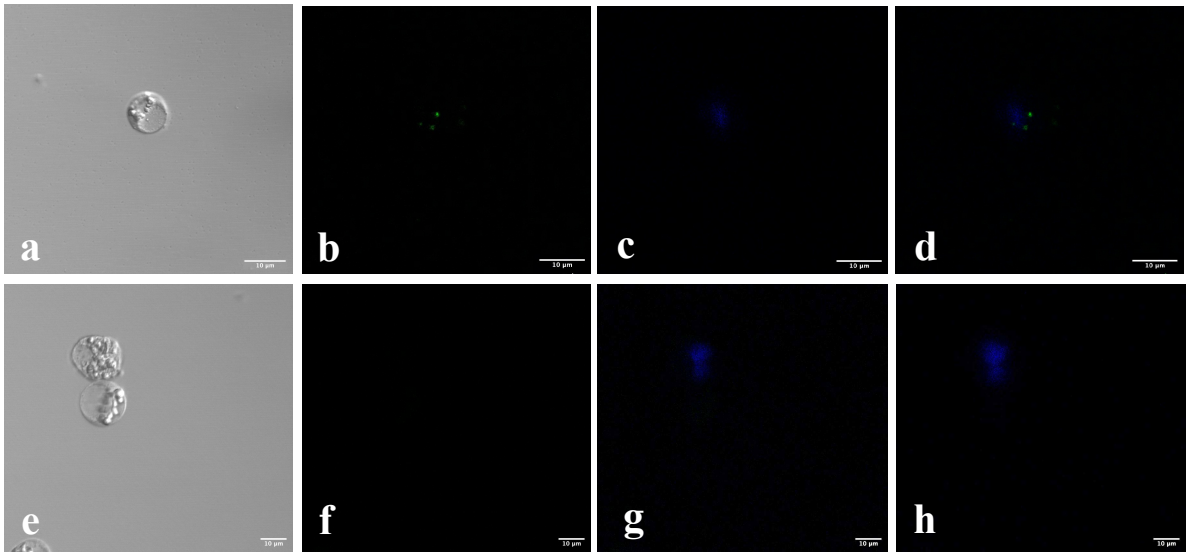

Figure 5. Confocal microscopy images for visual confirmation of RNP complex direct delivery to the protoplasts of Syrah and nuclear localization of 3×NLS-Cas9-EGFP-gRNA complex (**a-d**), control (**e-f**). **a-d** transfected by Cas9-GFP: **a**. WL, **b**. GFP filter, **c**. DAPI filter, **d**. overlay. **e-f** control: **e**. WL, **f**. GFP filter, **g**. DAPI filter, **h**. overlay.

in preliminary experiment, molar mass ratio of Cas9-GFP/gRNA 1:2 (90µg :30 µg) were used for protoplasts transfection, imaging by light microscopy showed that transfection of RNPs-GFP has been done with successfully but there were a lot of free RNPs-GFP in the medium. In the next step amount of Cas9-GFP decreased to 60µg, as previous studies, suggesting that its off-target effects might be low, and results showed that transfection of RNPs-GFP has been done with successfully.

Since PEG is considered as an important factor in chemical mediated transfection in plants, even if 40% PEG is toxic to protoplasts; to avoid a lot of toxicity of PEG for the protoplast, after 20 min of PEG treatment, W5 was added in 2 steps.

3.3.3. Cell wall digestion essay

To investigate cell wall digestion in Sultana and Syrah cultivars while keeping protoplasts viable and totipotent we performed the cell wall digestion based on the protocol described before. The protoplasts stained by Calcofluor and observed in different incubation time (4h, 5h, 6h). Our results show that after 4h incubation in digestion enzyme cocktail cell wall was digested poorly while no difference in cell wall digestion has observed in 5h and 6h. we have selected 5h incubation time to optimize the protocol and minimize the stress to cells.

To validate successful cell wall digestion and viability of protoplasts the 5h incubated cells were used for further calcofluor and FDA co-staining via confocal microscopy (**Fig. 6** and **7**). Our results confirm that the digestion efficacy percentage were very high and the cells with undigested walls were very rare. Considerably, cell wall digestion was more efficient in well separated cells, while in protoplasts aggregated together, the cell wall resulted not digested exactly in conjugation position (**Fig. 8**).

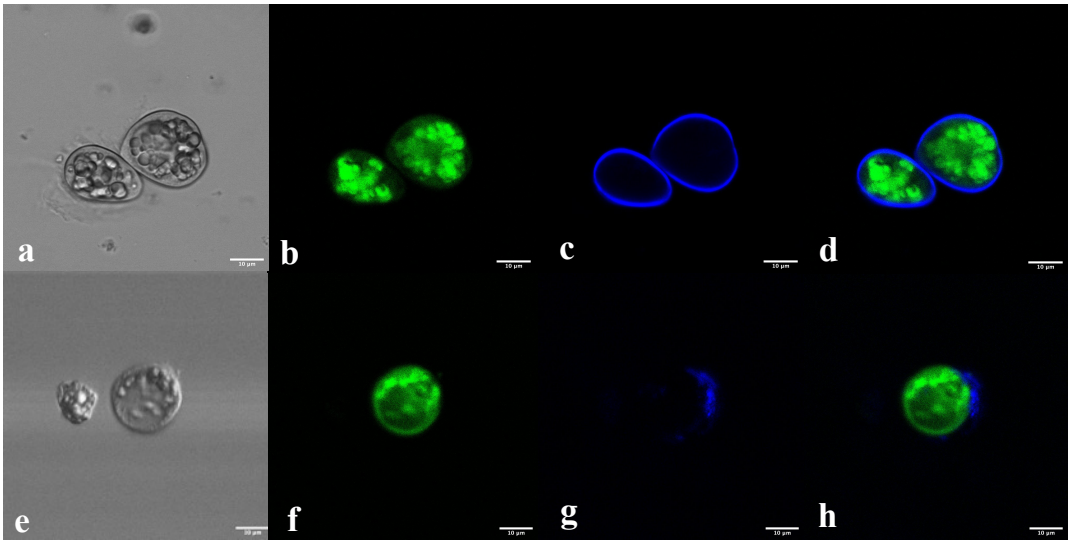

Figure 6. Confocal Microscopy analysis of cell wall digestion in Sultana. FDA staining was used to show the viability of protoplast; and Calcofluor staining to observe the cell wall. **a-d** callus cell and **e-f** protoplast. **a**. WL*, **b**. GFP filter (FDA staining), **c**. DAPI filter (calcofluor staining), **d**. overlay. WL*: white light.

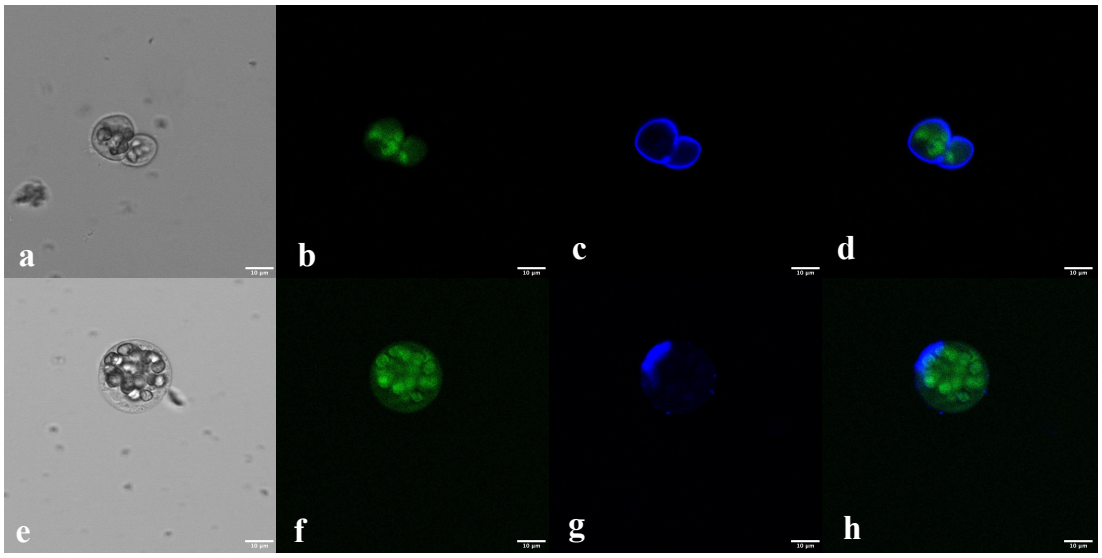

Figure 7. Confocal Microscopy analysis of cell wall digestion in Syrah. FDA staining was used to show the viability of protoplast; and Calcofluor staining to observe the cell wall. **a-d** callus cell and **e-f** protoplast. **a**. WL, **b**. GFP filter (FDA staining), **c**. DAPI filter (calcofluor staining), **d**. overlay.

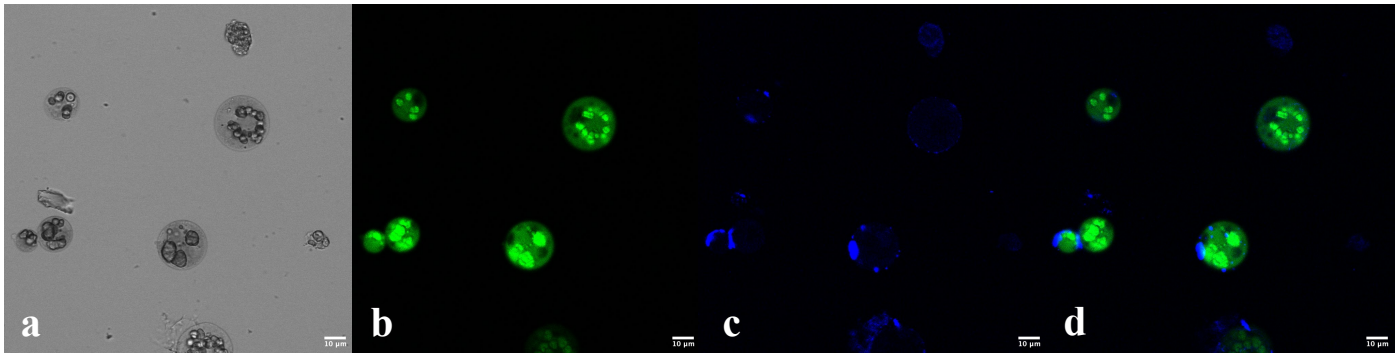

Figure 8. Validation of cell wall digestion and viability by calcofluor and FDA co-staining via confocal microscopy. **a**. WL, **b**. GFP filter (FDA staining), **c**. DAPI filter (calcofluor staining), **d**. overlay.

3.4. Discussion

Enhanced agricultural production through innovative breeding technology is urgently needed to increase access to nutritious foods worldwide. Recent advances in CRISPR/Cas genome editing enable efficient targeted modification in most crops, thus promising to accelerate crop improvement [22].

The efficacy of genome editing is largely dependent on nuclease protein transfection using proper delivery techniques. Tissue culture-dependent transient expression techniques such as callus culture, protoplast transfection and *Agrobacterium*-mediated agroinfiltration are employed in plants [2].

The ability of transformation and way to evaluate a successful delivery of a complex into the protoplasts always are challenging. To address these issues, we have employed two strategies including transient expression of a vector carrying YFP gene and then a direct delivery of Cas9 tagged by GFP. A major advantage of transient expression assays is their rapid nature. Indeed, expression can be detected as little as 2–3 days after gene transfer, avoiding the lengthy process of stable transformation, and allowing large-scale genetic analyses. In the last 20 years, transient expression assays enabled the validation of many plant gene functions, as well as promoter activity and transgene functionality, especially in model species like *Nicotiana benthamiana*. Recently, as described above, transient expression assays have also become a key technology for better understanding grapevine biology [14].

However, when plasmid vectors are used, all endonucleases have been reported to cause off-target effects and unwanted genome integration due to the persistence of plasmids. To overcome these problems, recent studies have been demonstrated that direct delivery of purified recombinant nuclease proteins such as Cas9 [2].

In this study, transformation efficiency of the protoplasts was detected according to the expression of YFP reporter gene using the transient expression vector. the most efficient protoplasts density is 1×10^5 ppt/ml for transfection in both Sultana and Syrah

cultivars. These results are in line with several studies in different species such as *Cymbidium* orchids [9], *Chardonnay* [26] and *Zea mays* L. [27] that 10^5 to 10^6 ppt/ml reported as the best density for transfection. The final (overall) density of protoplasts in the culture medium (plating density) is crucial for maximizing wall regeneration and concomitant daughter cell formation.

The green fluorescence was used for visual confirmation of RNP complex delivery after transfection. Protoplasts were transfected by Cas9 protein fused with GFP via PEG-mediated transformation.

GFP fluorescence was clearly observed in transfected protoplasts in both cultivars using confocal microscopy. This type of imaging is used to examine the performance of direct delivery of RNP complex. recently GFP tagged Cass9 has widely used for imaging techniques. For example, Chen et al. used an EGFP-tagged endonuclease-deficient Cas9 protein and a structurally optimized small guide (sg) RNA for robust imaging of repetitive elements in telomeres and coding genes in living cells [28].

The nuclear localization of RNP complex after 24h post transfection was confirmed. This evidence shows the impact of nuclear localization sequences (NLS) for optimal activity of the complex. Nakamura et al., 2019, also used Cas9 proteins fused with GFP to evaluate Cas9 nucleases transfection into *C. saensevieriae* protoplasts through PEG-mediated transformation. They reported that GFP fluorescence was clearly observed in transfected protoplasts indicating that Cas9 nucleases can be subjected to PEG-mediated transformation [29].

Cell wall digestion has been considered as key point in protoplast isolation based biotechnological approaches [5, 6]. Enzymatic Protoplast isolation is a stress-inducing procedure with accumulation of peroxides and degradation products that induce cell lysis, especially in cereals. Attention has refocused on this phenomenon. Commun et al. (2003) reported the production of stilbene phytoalexins in protoplasts of *Vitis* spp. and detected trans-resveratrol as early as 4 h after the beginning of enzyme digestion, through activation of the vst1 gene encoding stilbene synthase. The presence of

resveratrol and its derived phytoalexins, episilonviniferin and pterostilbene, may account for loss of protoplast viability [6].

In this study we have optimized the protocol for cell wall digestion in Sultana and Syrah and we found 5h is the most appropriate incubation time in digestion solution. Zhu et al., 1997 used 6h incubation time for *Vitis vinifera* L. cv. Koshusanjaku. Malnoy et al., 2016 to facilitate cell wall digestion in grape cultivar Chardonnay performed vacuum infiltration of E.C with cell-wall digestion enzyme for 20 mins before incubating them for 4h. based on our results, 4h incubation time was not enough to obtain a high-rate cell wall digestion. Although there is no difference between 5h and 6h incubation time but, in order to minimize the stress induced by process, 5h is preferable. The effects of stress during isolation may be long-term and may account for the recalcitrance of some protoplast systems in culture [5, 6].

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Chapter 4

DNA-free Gene editing in *Vitis vinifera* L.

Knockout green fluorescent protein (GFP) gene in Sultana grape by direct delivery of RNPs into protoplast

4.1. Introduction

Over the last few decades, global climatic trends in many agricultural regions have been quickly changing, in the face of these new problems, considerable advancements in global food systems are required to assure food security. The productivity of global agriculture will continue to be damaged as climatic instability grows as a result of rising CO2 levels and warmer temperatures. To address these mounting problems, innovative techniques will be needed, incorporating all available resources to develop more resilient and tolerant crops with higher quality and yields under more harsh circumstances. One viable strategy for accelerating genetic advances through targeted genetic modification, generating crops that can withstand changing climate conditions, is the integration of genome editing and transgenics into present breeding procedures [1]. In this chapter, we discussed how revolutionary genome editing techniques may be directly integrated into grapevine breeding programs to quickly address many of the concerns that will influence agriculture productivity in the future.

Global agricultural output, farm incomes, and food security will all be impacted by climate change. Global food security will undoubtedly be endangered as a result of climatic trends such as rising mean temperatures, climate variability, and an increase in extreme weather events [2]. Two of the key issues posed by climate change are rising temperatures and decreasing and unpredictable precipitation, both of which are expected to increase simultaneously in many climate-vulnerable countries [1].

Grapevine (*Vitis vinifera* L.) is one of the most economically important fruit crops worldwide and in many places of the world its usage in winemaking has played an important cultural role. Grapevines are currently grown in over 90 countries for the production of wine, liquors, juice, table grapes, and raisins (FAO-OIV, 2016). Grapevine has evolved as a model perennial fruit crop species due to its global

economic importance, climatic diversity of the producing areas, and a great number of studies (from genomes to production practices). Almost all wine regions in the globe are in temperate climate zones, and many of them have a Mediterranean climate with warm and dry summer [3, 4]. Climate change will be a major problem for viticulture in the future decades, according to scientific research. Global warming is expected to have a direct impact on ecosystems due to many factors such as greenhouse gas emissions, temperature, precipitation, and human activities. This will make the increase in the growing season mean temperatures, the incidence of pest and disease, oxidative damage, growth inhibition, and change in quality and yield, this will result in shifts in viticulture production pattern. Even while the highest wine quality rankings include countries with Mediterranean-like climates, the effects of climate change on viticulture and winemaking extend beyond the industry's economic and cultural dynamics. Future trends hint at the disruption of a variety of plant natural functions, including grapevine development, physiology, and berry ripening, which could result in significant yield and quality losses [4-6].

Plant improvement is a key mechanism for agricultural production adaptation to climate change, with present breeding procedures relying on the long-term selection of occasional, naturally occurring genetic variation to select for favorable combinations [1, 2]. Traditional breeding is presently the most widely employed approach in crop production; nonetheless, it is labor intensive, and it can take several years to go from the early stages of screening phenotypes and genotypes to the first crosses into marketable varieties. Genetically modified (GM) crops with desirable characteristics are generated by transferring genes (transgenes) or gene components with known functions into elite crop kinds. Despite the promise of genetically modified crops for global food security, their widespread use is impeded by generally unsubstantiated health and environmental concerns. Government regulatory procedures designed to ensure human and environmental biosafety have created significant financial barriers to the rapid adoption of innovative GM traits.

As a result, the advantages of genetically modified traits have been restricted to a few agricultural crops.[2]. Combining synthetic technologies with traditional breeding to create genomics-based breeding is an innovative technique to overcome traditional breeding's constraints. Genome editing has the potential to accelerate basic research and plant breeding by allowing for quick, precise, and targeted genome alterations [1]. Using genome-editing technologies, several gene knockout mutants, as well as several gene replacement and insertion mutants, have been generated in a variety of plants, and many of these mutants have been proven to be useful for crop improvement [2].

The risks of altering genomes via genome-editing technologies are considerably less than those associated with genetically modified (GM) crops since most edits typically affect a few nucleotides leading to changes similar to those found in naturally occurring populations [7]. There is no way to tell the difference between a 'naturally occurring' mutation and a gene edit after the genomic-editing agents have separated. As a result, incorporating genome editing into contemporary breeding methods should allow for faster and more precise crop development. Traditional genome editing includes transporting and integrating editing components encoded by DNA cassettes into the host genome. Because integration occurs at random, it may lead to undesirable genetic alterations [2]. The editors must be transcribed, and the complex must assemble when employing transient vector delivery, resulting in a momentary break in activity. [8]. Even if the DNA cassettes are degraded, the resulting fragments may be integrated and have undesirable consequences; nonetheless, because nucleases are abundant in plants, the constant manufacture of genome-editing tools leads to an increase in off-target consequences. Furthermore, in the case of genetically modified organisms, the introduction of foreign DNA into plant genomes creates regulatory challenges (GMOs) [2, 8]. While employing RNPs, the complex is already preassembled and active when delivered; since these edited crops produced by this DNA-free RNP delivery technique are unlikely to be regulated as GMOs, it is promising for plant breeding [9]. As a conclusion, DNA-free genome editing is a game-changing technology for creating genetically modified crops with a lower risk of unwanted off-target mutations while

still meeting current and future agricultural demands from a scientific and regulatory prospective. [2, 10].

The distribution of CRISPR/ Cas9 RNPs for genome editing in plant cells, DNA delivery through the plant cell wall, and plant regeneration from tissue or cell-wall free cells are two major bottlenecks to DNA-free transformation. To get through the plant cell wall barrier, most improvements use isolated protoplasts, which are single plant cells with an enzymatically dissolved cell wall. Protoplasts were the first tissue to be used for DNA-free Genome Editing because they are easily targeted by polyethylene glycol (PEG) induced fusion. Therefore, the RNP complex is enclosed in PEG vesicles and attached to protoplasts.[8-10]. Genome editing is a superb way to find and precisely locate a specific region inside a genome, then edit the targeted regions for a variety of applications. Unlike traditional transgenic technology, which randomly introduces genetic components into a genome, genomic editing operates on a specific genome sequence inside the genome. [11-13].

4.1.1. History of genome editing: From meganucleases to CRISPR

The first examples of genome editing come from random mutagenesis, which is a natural process. Plant and animal breeders use genetic diversity in the form of single nucleotide polymorphisms (SNPs) to boost grain output, improve beef cattle quality, and increase milk production in dairy cows. Other examples of naturally occurring genetic diversity include microorganisms, which result in novel yeast strains utilized in fermentation. This spontaneous genetic variability is influenced by two main factors: the amount of time it takes for genetic variability to arise and the effort necessary to pick superior genetics [14]. The cornerstone to biomedical and biotechnological research is the genetic engineering of organisms. In a straightforward manner, the ultimate instrument would allow exact and infinite change of any nucleic acid sequence. We have never been closer to achieving this aim than we are now, especially with the emergence of genome editing [15]. In eukaryotes, genome editing has revolutionized DNA manipulation, allowing for the precise mutagenesis of single base

pairs, the introduction of insertions and deletions (indels), DNA fragment replacement and nucleotide base conversion. ODM (oligonucleotide-directed mutagenesis), TALENs (transcription activator-like effector nucleases), ZFNs (zinc finger nucleases), and CRISPR-Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins) are the most common genome editing technologies. TALENs and ZFNs are still being used in agriculture and medicine for study [16].

4.1.2. New breeding technology's state-of-the-art

There are four major mechanisms of site-specific genome editing that have paved the way for new agricultural breakthroughs. Meganucleases (MegNs), zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR/Cas-9). However, over the last 15 years, three major classes of programmable nucleases have been used for precision genome editing: ZFNs, TALENs, and CRISPR/Cas9 [15, 17] (**Fig. 1**).

The origin of genome editing technology began with the introduction of zinc finger nucleases (ZFNs) [18]. Zinc fingers are tiny protein motifs that can bind to DNA based on their sequence. They were initially identified in frog oocytes in 1985 as part of a transcription factor, but they are found in numerous species, including humans. Because they are stabilized by a zinc ion to engage their DNA target, they are called zinc fingers. They use a cascade of modules, unlike other DNA binding motifs, in which a few critical residues in the protein mediate recognition. A DNA binding motif unique to three consecutive base pairs of the target sequence is found in each module. They are particularly adaptable in binding specific DNA sequences of varying lengths due to their modular nature.

In 1996, zinc finger modules were joined to the DNA cleavage domain of the restriction enzyme FokI, resulting in zinc finger nucleases, which are programmable nucleases (ZFN). The ease with which ZFN inducted DSB led to a variety of applications in biotechnology and medicine. In 2005, the latter research was the first to use the phrase "genome editing" as a metaphor for word processing. If a repair template

is given, DSB, such as those caused by ZFNs, can be fixed using HDR. With genome editing, knock-out (KO) and knock-in (KI) alterations were created in this way. The development of transcription activator-like effector nucleases (TALENs) in 2010 was another big breakthrough in the field of designer nucleases. The DNA binding domain of ZFN may be replaced with more versatile and easy-to-generate DNA binding modules, according to researchers. TALE proteins were found in plant pathogenic bacteria of the genus Xanthomonas, which gave rise to these modules. The sequence-specific binding ability of TALEs is used by these bacteria to control the gene expression of the infected plant cell in their favor.

TALEs are also easier to make than zinc finger proteins since the nucleotide specificity is determined by only two amino acids. TALENs are created by fusing a sequence-tailored TALE DNA binding domain with the FokI endonuclease domain for genome editing (analogous to that used for ZFN). TALENs quickly replaced ZFN for genome editing because their design and production needed less work and were more adaptable. [11, 14, 15, 19-21]. However, due to inefficient transfection, design complexity, and constraints on multiplexed mutations, these technologies' uses are restricted [12, 16, 22-24].

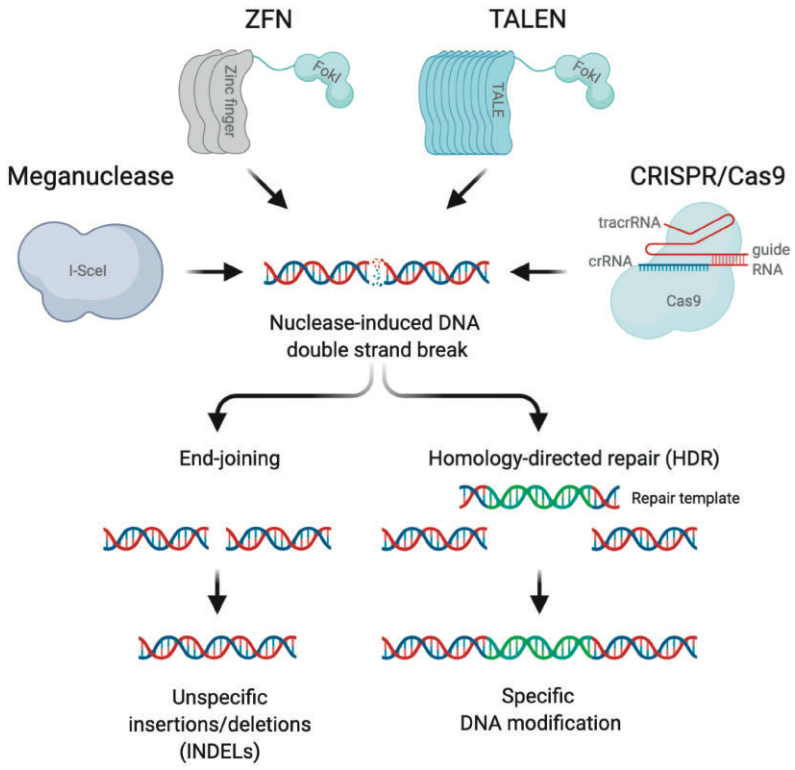

Figure 1. Mechanism of major genome editing platforms. All four major genome editing systems lead to double-strand breaks (DSBs) at the desired locus. These DSBs breaks are either repaired by error-prone end-joining pathways resulting in random insertions and deletions (INDELs) or if a DNA repair template is provided, by homology-directed repair (HDR). The latter mechanism can be exploited to introduce site-specific mutations into the locus of interest. While meganucleases, zinc finger nucleases (ZFN), and transcription activator-like effector nucleases (TALENs) rely on protein-DNA interaction for target recognition CRISPR/Cas9 utilizes a dual guide RNA composed of a generic tracrRNA and a specific crRNA [15].

4.1.3. Rise of CRISPR/Cas9 genome editing; The history of CRISPR genome editing

Scientists were looking for a protein that, like TALE and zinc finger proteins, has programmable DNA binding capabilities without requiring the time-consuming creation of a new protein domain. This protein should, in theory, already exist in nature as a nuclease. Unbeknownst to them, a mechanism similar to the CRISPR/Cas system in bacteria had previously been found. In the late 1980s, non-repeating spacer sequences were discovered in the genome of *Escherichia coli*, which, unlike conventional tandem repeats, were separated by repetitions. For more than a decade, the nature of these repetitions remained a mystery until new sequencing methods permitted the decoding of many more genomes.

In the year 2000, Mojica and coworkers discovered that similar repetitions are found in many different bacteria and practically all archaea, indicating that they play a significant role. Furthermore, these repetitions were shown to be linked to conserved genes known as CRISPR-associated or Cas genes, which are endonucleases that exist naturally. CRISPR stands for clustered regularly interspaced short palindromic repeats, and this was the first time the acronym was used [15].

Three research groups separately established in 2005 that the spacer sequences are identical to phage and other alien genetic elements' genomes. When Horvath and coworkers revealed that infected bacteria include spacers originating from phage, the Cas protein is directed to the invaders' genome, where it precisely chops the phage DNA, the nature of CRISPR was ultimately confirmed in 2007.

As a result, analogous to the adaptive immune response seen in higher species, the CRISPR/Cas system provides a defense mechanism against pathogens. It was quickly discovered that CRISPR RNA (crRNA) is produced from the spacer to direct the Cas protein to its target, which is DNA. The protospacer adjacent motif (PAM), a short sequence motif next to the crRNA targeted region on the target DNA, has been found to be crucial for cleavage as well as self vs. non-self-discrimination of the CRISPR system [15, 18]. Furthermore, it was shown that in a CRISPR system, only

one Cas protein, Cas9, imparts DNA cleavage and that trans-activating crRNAs (tracrRNAs), which play a role in crRNA maturation, are required for crRNA activity. Without a doubt, the most essential experiments were carried out independently by Emmanuelle Charpentier's group in partnership with Jennifer Doudna, and almost simultaneously by Virginijus Siksnys' group. They showed that the CRISPR/Cas9 system can be rebuilt in vitro and programmed to target desired regions in different species, demonstrating its use as a genome editing programmable nuclease (**Fig. 2**).

Charpentier and Doudna, along with Martin Jinek, further simplified the system by combining the crRNA and tracrRNA into a single guide RNA (sgRNA) with complete activity. This resulted in a simple two-component system in which modifications to the sgRNA's guide sequence (20 nucleotides in native RNA) may be used to program CRISPR-Cas9 to target any DNA sequence that is next to a PAM.

Despite ZFNs and TALENs, which need continual target-specific re-engineering of protein modules to modify each DNA target site, the CRISPR-Cas9 method just necessitates a modification in the guide RNA sequence for genome editing at a given location. As a result, the scientific community has quickly and widely accepted the CRISPR-Cas9 technology based on the S. pyogenes system to target, edit, or change the genomes of a wide range of cells and creatures. In addition to its simple low-cost design, this approach can also swiftly be multiplexed to target multiple genes at the same time. As this system was easy to adapt by any biomedical laboratory, CRISPR genome editing revolutionized the field, and subsequently, Emmanuelle Charpentier and Jennifer Doudna were given the Nobel Prize in Chemistry in 2020 for their discovery [15, 22].

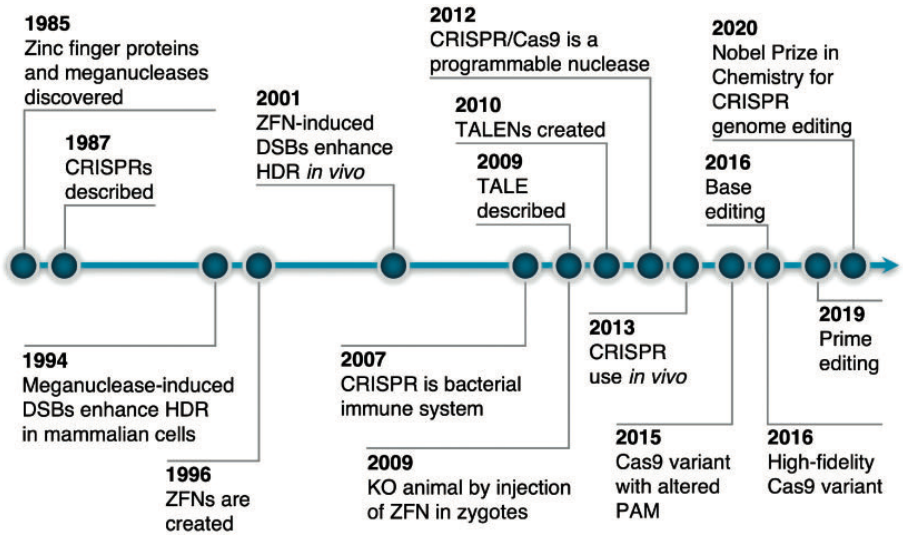

Figure 2. Key developments in the history of genome editing. DSBs, double-strand breaks; HDR, homology-directed repair; KO, knock-out; PAM, protospacer adjacent motif; TALENs, transcription activator-like effector nucleases; ZFN, zinc finger nucleases [15].

4.1.4. CRISPR-Cas system

CRISPR-Cas system is typically comprised of two parts: (i) Cas proteins—involved in the protection and the acquisition of invading nucleotides, and (ii) the CRISPR array—which consists of conserved recurrent domains known as direct repeats and embedded variable sequences with the same length known as spacers. The CRISPR-Cas system recognizes and cleaves foreign nucleotides in a sequence-dependent manner.

CRISPR-Cas contains three phases as a defense system: (i) adaptation: The host organism captures invading nucleotide fragments, and successive insertions are constructed inside the CRISPR array, (ii) expression/crRNA biogenesis: The CRISPR array is translated into a big precursor crRNA (pre-crRNA), which is then cleaved by RNases into mature guide crRNA, (iii) interference: effector complexes that capture and degrade invading nucleotides are guided by the mature crRNA (**Fig. 3**).

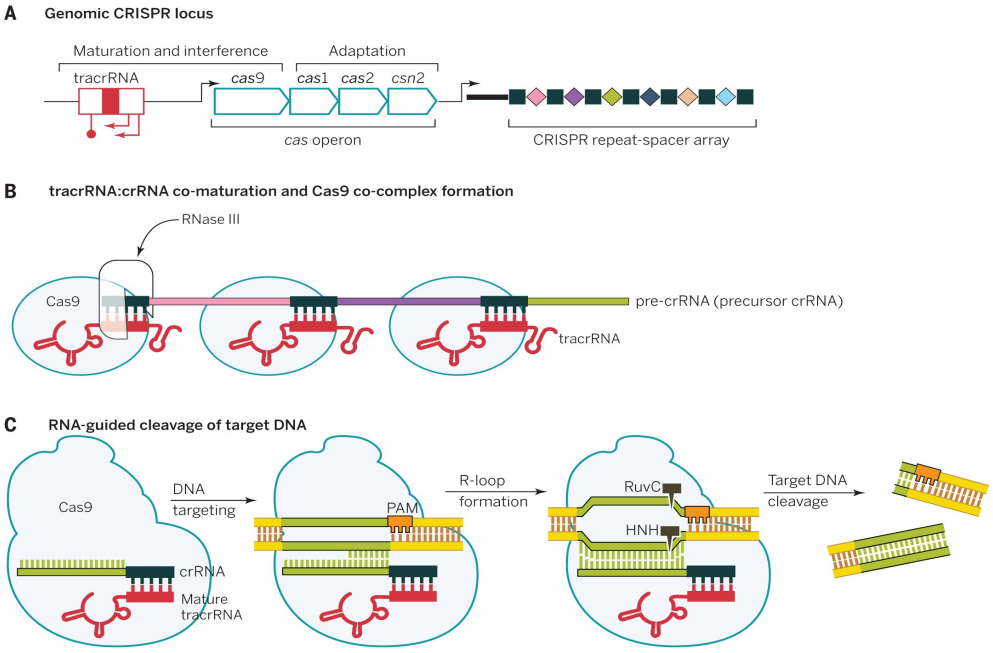

Figure 3. Biology of the type II-A CRISPR-Cas system. The type II-A system from S. pyogenes is shown as an example. (A) The Cas gene operon with tracrRNA and the CRISPR array. (B) The natural pathway of antiviral defense involves the association of Cas9 with the antirepeat-repeat RNA (tracrRNA: crRNA) duplexes, RNA co-processing by ribonuclease III, further trimming, R-loop formation, and target DNA cleavage. (C) Details of the natural DNA cleavage with the duplex tracrRNA:crRNA [22, 25].

Based on their effector proteins, the CRISPR-Cas system has been divided into two categories. Class 1 systems have four to seven Cas proteins in effector complexes, whereas class 2 systems have just one Cas protein with several sub-domains. In the class 1 system, there are three subtypes: type I, III, and IV; in the class 2 system, there are three subtypes: type II, V, and VI. Type I, II, and V target DNA, and type VI targets RNA, Type III recognizes and cleaves both DNA and RNA. Class 2 CRISPR-Cas systems have been a popular alternative for developing a new generation of genome

editing technology due to the straightforward structural design of effector complexes [16].

The best-studied and most frequent multiple-domain protein is Cas9, a crRNA-dependent endonuclease—containing two distinct nuclease domains, that is HNH and RuvC. The target and non-target DNA strands are cleaved by these nuclease domains, respectively. CRISPR-Cas9 may create double-stranded breaks (DSB) in genomic DNA at specific locations. Cas9 is guided to a specific DNA sequence by guide RNA (gRNA), where it breaks both strands. Once the right target has been found, the Cas9 will bind at the targeted genomic locus adjacent to a short DNA sequence known as PAM (for SpCas9 is the triplet NGG) and generate a DSB with the effect of introducing insertion/deletion (INDEL) mutations in the specific gene sequence and if the target region is homologous to gRNA [16, 17, 26].

Because of its simplicity of use and efficacy, the CRISPR/Cas9 system is the most powerful gene-editing technology currently accessible.

The CRISPR/Cas9 system is a versatile and strong biotechnological tool for identifying and targeting specific DNA and RNA sequences in the genome. It can be used to target a sequence for gene knockin, knockout, and replacement as well as monitoring and regulating gene expression at the genome and epigenome levels by binding a specific sequence [11]. (**Fig. 4**).

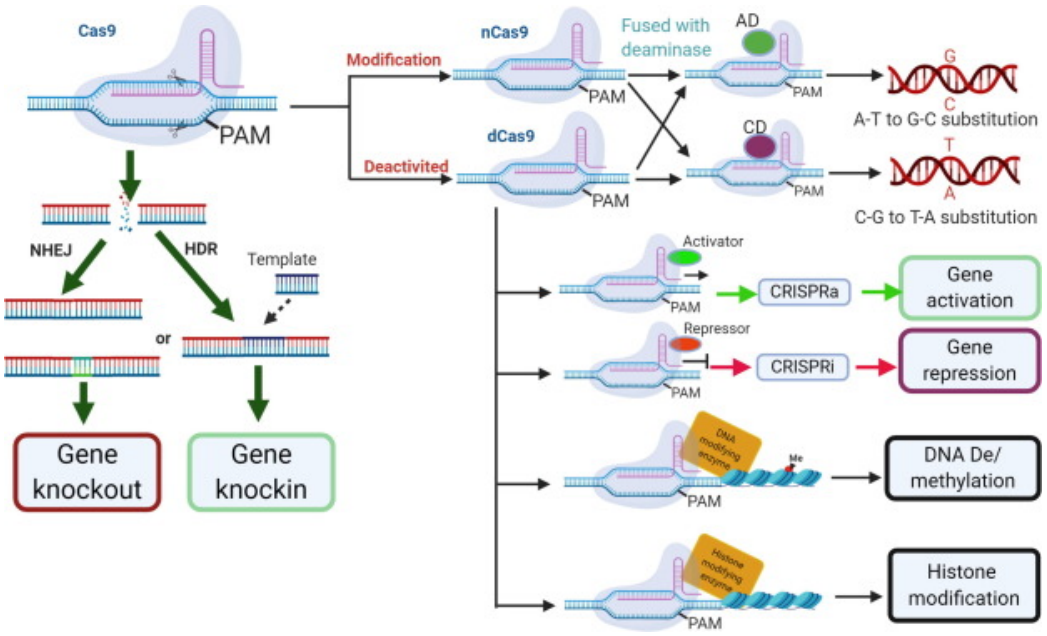

Figure 4. Application of CRISPR/Cas genome editing in gene functional study. CRISPR/Cas system has a diversity of applications in gene functional study. Based on the DNA double-strand break (DSB) repair mechanism, CRISPR can directly cause gene knockout (silencing) by insertion or deletion of a couple of nucleotides and repaired by non-homologous end join (NHEJ); however, if the homologue-directed repair (HDR) happened, with a DNA donor, CRISPR/Cas genome editing can be used to replace an undesirable gene or overexpress (knock-in) and an individual gene. If deactivating the Cas9 enzyme, and with transcription effector or other enzymes fused with the dCas9, CRISPR/Cas system also can be used to base editing, epigenome editing, and imaging [11].

4.1.5. CRISPR/Cas9 technology in grapevine

The use of these new technologies in grapevine breeding could be particularly beneficial because they produce minimal and precise modifications in selected genotypes of interest, such as elite cultivars sought after by the wine market, without altering the genetic background as traditional breeding does. CRISPR/Cas9 technology has been successfully applied to generate edited grapevine plants (Osakabe et al., 2018) [54].

Two distinct delivery techniques were used: one based on A. tumefaciens gene transfer to ensure stable integration of genetic components in the genome, and the other based on direct delivery of pure Cas9 protein and gRNAs. Ren et al. (2016) inserted point mutations in the L-idonate dehydrogenase gene into 'Chardonnay' embryogenic cell suspension. They were able to resurrect plants with altered tartaric acid and vitamin C production [27]. Plants with albino leaves were created when ‘Neo Muscat’ somatic embryos were transformed with a CRISPR/Cas9 editing construct, targeting the phytoene desaturase gene, in 2017 [28]. Transgenic 'Sultana' plants have recently been generated using mutant variants of the WRKY52 transcription factor gene under both mono- and bi-allelic circumstances by Wang et al., 2018 [29]. To develop non-transgenic altered grapevines Malnoy and colleagues (2016) directly transported pure Cas9 and gRNAs into 'Chardonnay' protoplasts, resulting in edited protoplasts but not entire plants. However, the proportion of protoplasts with a mutation was modest, about 0.5 percent [17, 30].

4.1.6. Understanding CRISPR

The words "genome engineering," "genome editing," and "gene editing" were defined and compared by Robb et al., 2019. Genome engineering is the science of designing and modifying the sequence of genomic DNA. Genome editing and gene editing are genome engineering approaches that use DNA repair mechanisms to integrate site-specific alterations into genomic DNA. Gene editing is distinct from

genome editing in that it only affects one gene [18]. The CRISPR (Clustered, Regularly Interspaced, Short Palindromic Repeats) system provides the foundation for a suite of gene-editing technologies that are advancing research in fields ranging from health to diagnostics to agriculture and energy.

Gene editing includes the insertion, removal, or modification of DNA sequences with a precise and targeted change. The CRISPR system uses two essential components to carry out gene editing: a bacterially derived nuclease (*Streptococcus pyogenes*), a guide RNA (gRNA).

The gRNA, which is made up of two parts: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), is a particular RNA sequence that recognizes and directs the nuclease to the target DNA region. crRNA is a 17-20 nucleotide sequence that is complementary to the target DNA and varies according to the target gene. The tracrRNA, on the other hand, is a constant sequence that acts as a scaffold for the Cas nuclease to connect to the crRNA. The earlier CRISPR editing techniques used a two-part gRNA complex with distinct crRNA and tracrRNA, but currently, it's common to employ a single gRNA (sgRNA) strategy, which combines the crRNA and tracrRNA into a single RNA molecule (**Fig. 5)**.

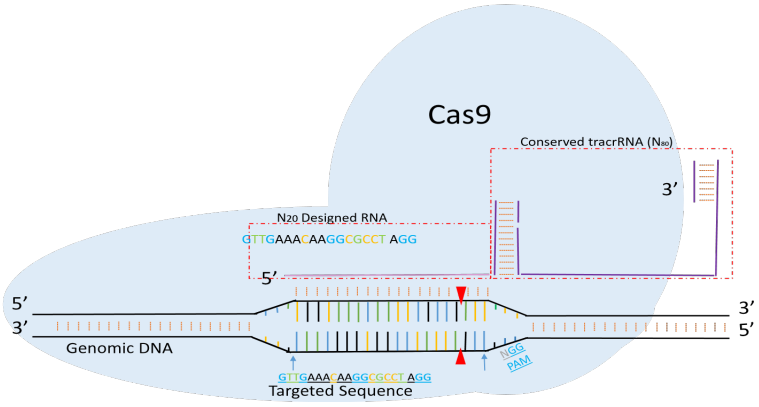

Figure 5. The schematic representation of precise cleavage of the targeted site using the CRISPR–Cas9 system. The CRISPR–Cas9 system consists of two components, sgRNA, and Cas9. sgRNA further consists of two-component, one that is designed and shows complementary to opposite stand of targeted DNA

sequence, while the other is conserved and labeled as tracrRNA. The Cas9 nuclease contains two activity sites and induces cleavage at 3′ nucleotide before the PAM site on target [19].

The CRISPR complex works in three stages to edit genes: **targeting**, **cleavage**, and **repair** [19, 21, 25, 31].

CRISPR Complex Targeting; The crRNA is complementary to the target DNA by design, allowing the gRNA to guide the CRISPR complex to the right genomic site for gene editing. A protospacer adjacent motif (PAM) must be present downstream of the target site for the CRISPR complex to successfully connect to the DNA. PAMs are short sequences of 3 to 8 nucleotides in length that vary in nucleotide sequence based on the nuclease utilized, with nucleases isolated from different species needing various PAMs. Cas9, the most commonly used nuclease, derived from *Streptococcus pyogenes* (spCas9), recognizes a PAM sequence of 5'-NGG-3' (where 'N' is any nucleotide).

Cleavage of the DNA; The nuclease can cut the DNA after the CRISPR complex connects to the target site. The CRISPR complex has two nuclease domains, each of which cuts a different strand of DNA. The HNH nuclease domain cuts the complementary strand of the gRNA, whereas the RuvC nuclease domain cuts the non-complementary strand. The Cas9/sgRNA complex generates blunt-end DSBs at the 3-bp upstream of the PAM [32].

Repair of DSB; The native cellular DNA repair machinery tries to repair the DSB in one of two ways after the nuclease cleaves the DNA: non-homologous end-joining (NHEJ) and homology-directed repair (HDR) [33]. NHEJ tends to be the primary cellular DSB repair mechanism. Since the DNA ends are re-ligated in the absence of a homologous DNA template, it might introduce insertion or deletion mistakes (indel) into DNA. A loss-of-function (LOF) mutation is caused by indels that result in frameshift mutations and premature stop codons, and it is the most common way for CRISPR to disrupt (knockout) a gene. HDR may be used by CRISPR to conduct knock-in replacement and expression of a certain genomic sequence. HDR-mediated CRISPR editing requires a DNA donor template with the new desired sequence flanked by

homology regions, in addition to the primary CRISPR components. The cells may repair the DSB by homologous recombination after introducing this donor template together with the CRISPR components. As a result, the novel sequence gets incorporated into the target gene.

4.1.7. Cas9 protein

Researchers are continuously looking for ways to improve Cas9's specificity. In an initial effort, the Cas9 protein was employed in tandem, similar to how ZFNs and TALENs were used previously. To do this, one of Cas9's two nuclease domains was inactivated, resulting in a nickase that only cuts one strand of DNA. Feng Zhang's group created the first high-fidelity Cas9 protein variation three years later, in 2016. They used structure-guided engineering in order to change residues in the protein to prevent non-specific DNA binding. Many additional variations have since been developed that can distinguish even single base pair mismatches. However, none of these variants appear to preserve the wild-type SpCas9's on-target activity [15].

Bioinformatic analyses first identified Cas9 as a large multifunctional protein with two putative nuclease domains, HNH and RuvC-like. Cas9's HNH domain cleaves the complementary DNA strand to the crRNA's 20-nucleotide sequence, while its RuvC-like domain cleaves the DNA strand opposite it. Mutating aspartate to alanine substitution either the HNH or the RuvC-like domain in Cas9 generates a variant protein with single-stranded DNA cleavage (nickase; Cas9n) activity [34], whereas mutating both domains (dCas9; Asp10 → Ala, His840 → Ala) [35, 36] results in an RNA- guided DNA binding protein which can activate gene expression instead of cutting the DNA and allow researchers to study the gene's function [22] (**Fig. 6**).

Cas9's nuclease activity can be triggered even if the RNA guide sequence and an off-target genomic region have inadequate complementarity, especially if mismatches are distal to the protospacer adjacent motif (PAM), a short stretch of nucleotides

essential for target selection. These off-target consequences make genome-editing applications difficult [37].

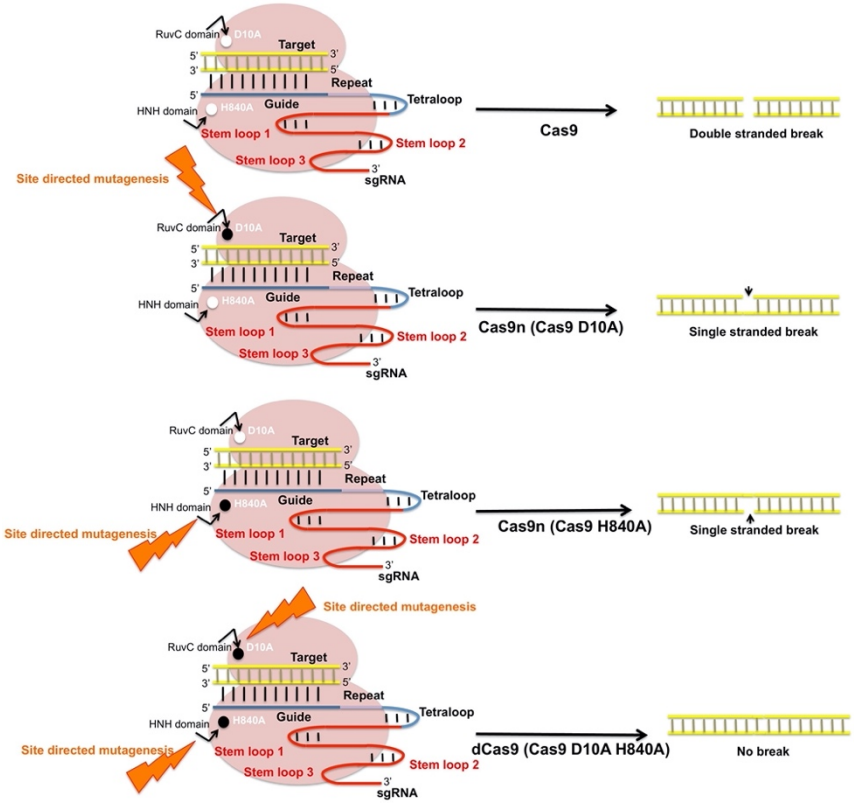

Figure 6. Schematic representation of Cas9 nuclease activity and its modifications. SpCas9 endonucleases create DSBs in target DNA through the activity of RuvC and HNH nuclease domains. SpCas9 nucleases can be converted into DNA nickase by substitution of its key amino acids D10A and H840A that produce single-stranded breaks. Site-directed mutagenesis in D10A produces Cas9n D10A and mutation in the HNH domain produces Cas9n (H840A). Mutations in both catalytic residues modify Cas9 to an inactive dead Cas9 (dCas9) [20].

Several methods for improving Cas9 specificity have been reported, for example, decreasing the amounts of active Cas9 in the cell, using Cas9 nickase mutants,

truncating the guide sequence at the 5′ end, and using a pair of catalytically inactive Cas9 nucleases, each fused to a FokI nuclease domain. Although each of these methods lowers off-target mutagenesis, they all have drawbacks: Reduced Cas9 levels can diminish on-target cleavage efficiency, double nicking necessitates the delivery of two single-guide RNAs (sgRNAs) at the same time, and shortened guides can boost indel formation at some off-target loci while reducing the number of target sites in the genome. DNA strand separation is required for Cas9-mediated DNA cleavage [20, 37].

Nuclease activity can be inhibited by mismatches between the sgRNA and its DNA target in the first 8 to 12 PAM-proximal nucleotides. This nuclease activity can be restored by introducing a DNA: DNA mismatch at that location.

The crystal structure of SpCas9 in association with guide RNA and target DNA provides a foundation for rational engineering to increase selectivity. The structure reveals a positively charged groove in SpCas9 that is likely important in stabilizing the nontarget strand of the target DNA. It is located between the HNH, RuvC, and PAM-interacting domains. Therefore, positively charged residues within the nontarget strand groove (nt-groove) might reduce nontarget strand binding and stimulate rehybridization between the target and non-target DNA strands, necessitating more rigorous Watson-Crick base pairing between the RNA guide and the target DNA strand. Slaymaker et al., 2015 have demonstrated “enhanced specificity” SpCas9 (eSpCas9) through structure-guided design that neutralization of positive charges in the nt-groove can dramatically decrease off-target indel formation while preserving on-target activity. To engineer this protein, alanine point mutations were made in the chromosome-binding motif of SpCas9, (mutants consisting of individual alanine substitutions at 31 positively charged residues within the nt-groove). These findings suggest that eSpCas9 can improve the specificity of genome-editing applications. [37, 38] (**Fig. 7**).

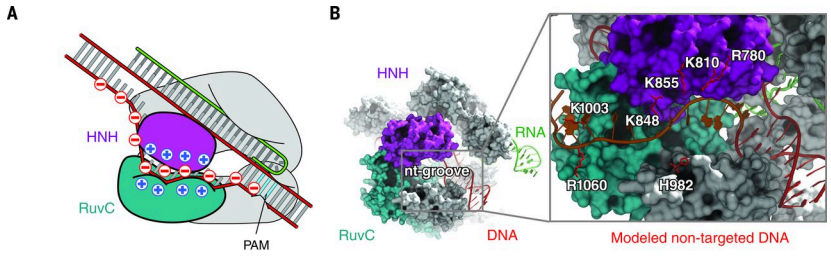

Figure 7. Structure-guided mutagenesis improves the specificity of SpCas9. (**A**) A model of Cas9 unwinding highlighting locations of charge on DNA and the nt-groove. The nt-groove between the RuvC (teal) and HNH (magenta) domains stabilizes DNA unwinding through nonspecific DNA interactions with the noncomplementary strand. RNA:cDNA and Cas9:ncDNA interactions drive DNA unwinding in competition against cDNA:ncDNA rehybridization. (**B**) A crystal structure of SpCas9 (Protein Data Bank ID 4UN3) shows the nt-groove situated between the HNH (magenta) and RuvC (teal) domains. The non-target DNA strand (red) was manually modeled into the nt-groove (inset) [37].

4.1.8. gRNA

Cas9 can reliably produce insertion or deletion mutations (indels) or precise modifications by using nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) to mend Cas9-induced double-stranded breaks. Unwanted indel mutations can, however, be produced at off-target locations with sequence similarities to the on-target region. Several methods for improving the specificity of RNA-guided Cas9 have recently been described, including truncation of the 3′ end of gRNA (which is derived from the trans-activating CRISPR RNA or 'tracrRNA' domain that is believed to mediate interaction with Cas9), or addition of two guanine nucleotides to the 5′ end of the gRNA (just before the 20-nt complementarity region); however, (RNA-guided nucleases using these altered gRNAs can have decreased on-target activities [39]. A 'paired nicking' approach is another option originally implemented with pairs of closely spaced zinc finger nickases, in which two gRNAs targeted to neighboring regions on opposing DNA strands each recruit a Cas9 variation (Cas9-

D10A) that nicks DNA rather than cutting both strands, might lower mutation rates at known off-target sites of single gRNA-guided Cas9 nuclease. However, indels can still be found at certain off-target locations and adding a second gRNA might bring new off-target alterations because a single gRNA-directed Cas9 nickase can successfully induce indels at some loci. Finally, the paired nickase method will not increase the specificity of catalytically inactive Cas9 (dCas9) coupled to heterologous effectors such as transcriptional activation domains. Off-target effects of RNA-guided nucleases might be minimized by decreasing the length of the gRNA-DNA interface, although it may seem paradoxical, studies have shown that lengthening the complementary region's 5′ end might lower on-target editing efficiency. Certain gRNAs, on the other hand, have been demonstrated to preserve substantial Cas9-mediated, on-target cleavage capabilities despite having truncations or increasing numbers of mismatches at the 5′ end of their targeted complementarity regions [11, 40, 41]

Fu et al., 2014 have shown that these 5′-end nucleotides may not be required for complete gRNA activity and that they generally compensate for mismatches at other points along with the gRNA-target DNA interface; Shorter gRNAs are consequently more sensitive to mismatches and hence more specific. The activities of gRNAs with 17 or 19 nucleotides of target complementarity were comparable to those of a full-length gRNA with 20 nucleotides of complementarity, but a gRNA with 15 nucleotides of complementarity failed to exhibit activity [42]. Truncated gRNAs with 17-nt complementarity have been shown to reduce unwanted mutagenesis at some off-target locations without losing genome-editing effectiveness on-target [40].

4.1.9. Design the gRNA

When choosing a CRISPR/Cas system for genome editing, it's critical to consider which Cas will be used, as well as the guide RNA design, to ensure that they target the proper place, and that the gRNA is effective. Due to the fact that sgRNAs are exclusively responsible for recruiting Cas9 and guiding it to a specific genomic region,

optimizing sgRNA design is critical for effective gnome editing operations. In order to design an effective sgRNA, the DNA sequences must contain an appropriate PAM and be immediately adjacent to the gRNAs designed.

Other important factors for designing high-activity gRNAs include:

gRNAs have a length of approximately 20 base pairs, gRNAs with an intermediate GC concentration are preferable to those with a high or low GC content, that putting a purine in the PAM-proximal location can improve Cas9 cutting efficiency, Target Location; The gRNA must target an exon that is required for protein function in order to successfully generate a LOF mutation, Chromatin Accessibility; Successful sgRNA binding in vivo is influenced by chromatin accessibility, with successful binding happening more frequently in areas of DNA with accessible chromatin, and off-target complementarity; The best gRNA sequences are those that are specific to the target DNA. Even if complementarity isn't perfect, gRNAs can nevertheless bind to other areas. Select gRNAs having at least three base pairs of mismatches from any other gRNA sequences in the genome when possible [11, 31, 43, 44].

The good news is that now we can design gRNAs using a variety of web-based computational tools that will automatically search for the proper DNA sequences that meet the PAM requirements [11].

4.1.10. Determine of T-DNA copy number in transgenic plants

The use of stable plant transformations to test a variety of hypotheses is quickly rising, and it has been suggested as a critical element in addressing future food supply security as well as responding to global change [45]. One of the most important responsibilities of a transgenic plant research project is validation of transformations and identification of particular lines deserving of detailed analysis. This normally involves determining the copy number, for each independent event for transgenes

inserted in the genome. Developing a method for recognizing single copy transgene insertion events in a population of independent transgenic lines would be beneficial [46]. This can usually be tested by self- pollination or cross-pollination to a nontransgenic line. However, this way is time-consuming and laborious procedure [47]. Southern blot analysis, quantitative polymerase chain reaction (qPCR) thermal asymmetric interlaced-PCR (TAIL-PCR) and most recently digital droplet PCR (ddPCR) have all been used to provide information about the integration status of a transgenic allele(s) in genomes [45].

While Southern blot analysis is a strong tool, it is not without its drawbacks. It necessitates considerable expertise, particularly in species with big genomes, such as many agricultural plants also process is more labor intensive, and less well suited for automation [46]. The use of quantitative polymerase chain reaction (qPCR) allows for a faster investigation of target sequences in genomic DNA, despite this, the error rate between technical replicates is frequently significant.

Recently, Droplet digital PCR (ddPCR) method has been developed [48] which is a novel technology that provides much more data from a single reaction mixture than qPCR [46]. Digital PCR (dPCR) divides reactions into partitions, transforming PCR's exponential, analogue nature into a linear, digital signal that enables precise estimation of the frequency of recurrence of certain sequences. The number of partitions enhances confidence; thus, the availability of emulsion technologies that enable reactions to be divided into tens of thousands of nanodroplets allows accurate determination of copy number in what has become known as digital droplet PCR (ddPCR) [49].

4.1.11. Determination of Successful Gene Editing

The identification of insertions or deletions (indels) introduced by the CRISPR experiment is required to confirm effective gene targeting. Sanger sequencing, mismatch detection tests, next-generation sequencing (NGS), phenotypic evaluation, and measuring mRNA and protein levels for the targeted gene are all possibilities for

determining the effectiveness of a gene-editing project. The sensitivity, scalability, resolution, and cost of these approaches vary. For example, while NGS has extremely high sensitivity and resolution, it is expensive and needs a great deal of technical competence to perform. Mismatch detection, on the other hand, is simple to use but lacks the sensitivity of Sanger sequencing and next-generation sequencing (NGS). Detecting the existence of an indel is frequently seen to be best practice. A variety of approaches, including restriction enzyme/PCR-based methods, can be used to screen for the presence of mutations [31, 50].

Direct sequencing of PCR amplicons that cover the target locations, on the other hand, has shown to be a reasonably rapid and straightforward method of obtaining detailed information on the events occurring at the target locus [51].

4.1.12. Research aims

In this chapter, the main aim is to perform the knockout of a green fluorescent protein (GFP) reporter gene, that is already integrated into the grape genome, in the Sultana cultivar by direct delivery of RNPs into protoplast.

The subsequent aim was to investigate the ability of regeneration of grape plants engineered by DNA-free gene editing, using two independent sgRNAs.

Eventually, to provide an optimized protocol to target important native genes in the grape plant in the future.

4.2. Materials and methods

4.2.1. Design gRNA

To identify guide RNA (gRNA) sequences within the GFP gene tow web tools published by Concordet and Haeussler 2018 [52] (http://crispor.org/), and Bae et al. 2014 (http://rgenome.net/) [53] were used. Two different target sites were selected from these lists for the current study. gRNA target2 (gRNA target2: 102–119 bp), and gRNA target4 (gRNA target4: 48–65 bp).

http://crispor.org/; CRISPOR.org is a web tool for genome editing experiments with the CRISPR–Cas9 system. It finds guide RNAs in an input sequence and ranks them according to different scores that evaluate potential off-targets in the genome of interest and predict on-target activity. On the first page of CRISPOR.org, you have to enter only three pieces of information: step 1, paste the target sequence into the input sequence box, in our case GFP, step 2, Select your genome of interest from the list, so *Vitis vinifera* – wine grape NCBI GCF_000003745.3 (12x) was chosen from the list, and corresponding PAMs 20bp-NGG - SpCas9-HF1, eSpCas91.1. as the Protospacer Adjacent Motif (PAM) from the dropdown box has been chosen for step 3 and finally submitted (**Fig. 6, 7, 10).**

And for http://rgenome.net/ choose 'Cas-Designer' from the menu and select 'Endonuclease type' (SpCas9 from Streptococcus pyogenes: 5′-NGG-3′ in this study) and 'Target Genome' (Plant - *Vitis vinifera* (IGGP_12×/Ensplant26) - European grapevine. Enter the target sequence and press the 'Submit' button below [54].

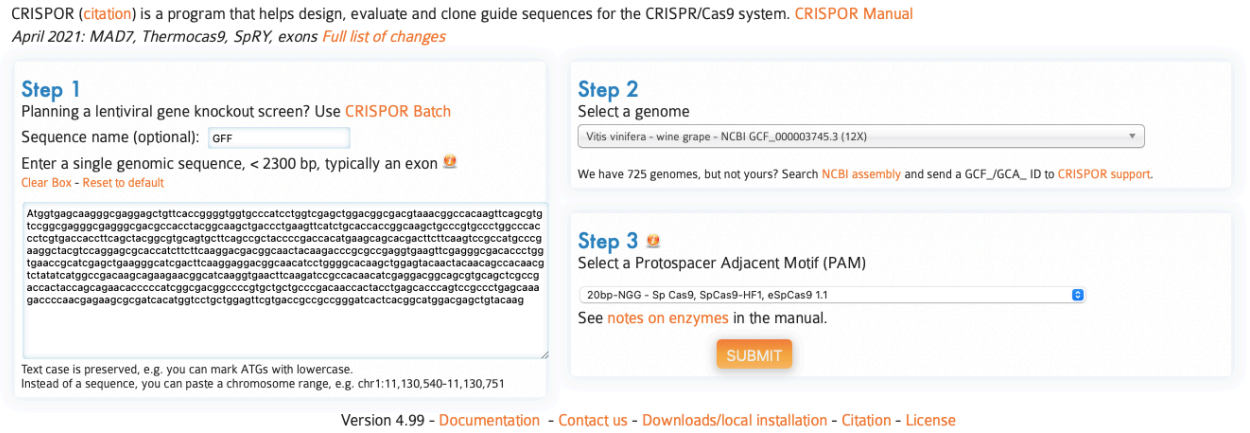

Figure 6. The sequence input page of crispor.org

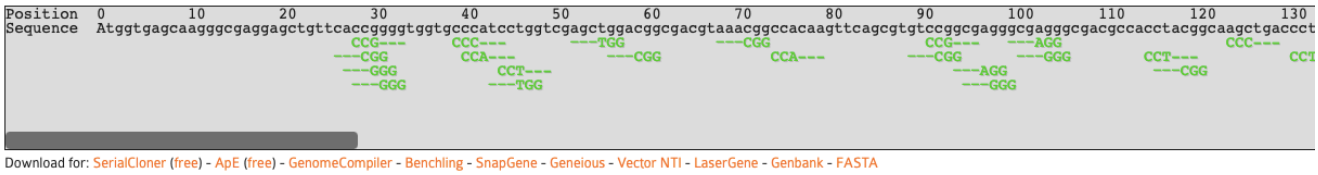

Figure 7. Output 1: Annotated input sequence

4.2.2. gRNA synthesis

In this study for *de novo* CRISPR gRNA synthesis GeneArtTM, Precision gRNA Synthesis Kit was used. The first step in de novo CRISPR gRNA synthesis is the analysis of the sequence of interest to identify potential CRISPR targets. Once the CRISPR target sequence has been selected, specific forward and reverse primers have been designed (**Table 1**) for the PCR assembly of the gRNA DNA template. At this point can be confirmed the template assembly by running 5 µL of the PCR product

against a size marker on a 2% E-GelTM EX Agarose Gel. gRNA DNA template will then be used to generate the gRNA by in vitro transcription (IVT) [42]. A gRNA synthesized is including 5' Gs + 18 nucleotide sequence complementary to the target DNA + 80-nt constant region of the crRNA/tracrRNA.

Table 1. Tow gRNA target sequences have been designed.

gRNA target	PAM- number	Primers for de novo synthesis
TCGCCGTCCAGCTCGACC (is in the rev-3'-5')	AGG-45	Forward: TAATACGACTCACTATAGTCGCCGTCCAGCTCGACC Reverse: TTCTAGCTCTAAAACGGTCGAGCTGGACGGCGA
CGAGGGCGACGCCACCTA (is in the fw-5'-3')	CGG-120	Forward: TAATACGACTCACTATAGCGAGGGCGACGCCACCTA Reverse: TTCTAGCTCTAAAACTAGGTGGCGTCGCCCTCG

4.2.3. eSpCas9

Ready-to-use recombinant eSpCas9 protein from *Streptococcus pyogenes* (ESPCAS9PRO: Enhanced Specificity SpCas9 compared to wild type Cas9) purchased from Sigma-Aldrich, which has been engineered to further increase the specificity of Cas9 and contain one nuclear localization sequence positioned for optimal activity (1X NLS). eSpCas9 has ~160 KD.

4.2.4. Protoplast isolation

Protoplast isolation of Sultana GFP-embryogenic calli has done the same to the section **2.2.5.** GFP-embryogenic calli subcultured to $C1^{P}k70$ media 7-10 days before isolation and GFP positive - calli have selected at the stereomicroscope before isolation. EC (1 g FW) was incubated in 10 ml of filter-sterilized enzyme solution on a gyratory shaker (30 cycles/min) at RT and darkness for 5 h. After digestion, protoplasts were filtered through a nylon mesh (60 μm) and harvested by centrifugation at 100g for 5 min. protoplast pellets were resuspended in washing solution.

In our study in parallel of the protoplast transfection by RNPs and then cultivation experiment, the other two experiments have been done, as a negative control. the first protoplast was transfected with only PEG in the same condition of RNP transfection (*Control*), and the second protoplast was cultivated after cell wall digestion, without any transfection treatment (*N.T*).

4.2.5. RNP direct delivery

2×10^5 of protoplast used for transfection by RNPs. recombinant eSpCas9 mixed with gRNA in the molar mass ratio of espCas9/gRNA; 1:5 = 60µg:60 µg.

eSpCas9 and gRNA were pre-mixed before transfection and incubated at RT for 10 min in darkness. Transfection has been done as described in section **3.2.2.** Shortly, protoplasts resuspend in 200 μl MMg solution and transfected by RNP, 200 µL of PEG 4000 added and the tube gently mixed, immediately before aggregation occurs and incubated for 20 min at RT and darkness.

The PEG solution rapidly settles on the bottom of the well and thus complete mixing of protoplasts, RNPs, and PEG requires additional mixing. The PEG solution at the concentration required for transformation, 40%, is toxic to protoplasts after prolonged exposure, therefore it is necessary to dilute the PEG after the transformation procedure has been completed. thus 400 µL of W5 washing solution was added, mixed, and incubate at RT for a further 10 min. Added again 800 µL of W5 washing solution, mix, and incubate at RT for a further 10 min. In all steps mix gently the mixture with a pipette tip. Protoplast's mixture was centrifuge at 100g for 5 min at RT, the supernatant discarded, and then washed two times with WI.

4.2.6. Protoplast cultivation and plant regeneration

Pelletized protoplasts were resuspended in 2 mL of solid medium to reach a final concentration of 1×10^5/mL and transferred to Petri dishes, after the solidification, liquid medium was added followed by incubation in the dark at 27 °C.

Transfected PPTs derived SEs observed under a stereomicroscope using a GFP filter for pre-selection of potential edited plants with either lower GFP signal or without detectable GFP signal. Whole plant regeneration was done as described in section **2.2.6.**

4.2.7. Measurement of GFP gene copy number Using Droplet Digital PCR

The transgene copy number was determined using QX200TM Droplet Generator (Bio-Rad), by running two PCR reactions for each sample. The first amplifies a transgenic area (target gene, in this study GFP gene), whereas the second amplifies a region of an endogenous gene (reference gene) of known copy number. Restriction endonuclease (RE) has been used to separate potential tandem gene copies ensuring proper random portioning into droplets and reduce sample viscosity improving template accessibility. The RE detection site should not be between the target or reference gene primers. Ubiquitin as a reference gene and EcoRV as a RE have been used. UBQ and GFP primers sequences are displayed in **Table 2**.

Table 1. UBQ and GFP primers sequences.

Primer	Sequence 5'-3'
UBQ forward	TCTGAGGCTTCGTGGTGGTA
UBQ reverse	AGGCGTGCATAACATTTGCG
GFP forward	GAAGTTCGAGGGCGACAC
GFP reverse	CCGTCCTCCTTGAAGTCG

Assemble the PCR reactions and condition are shown in **Table 3** and **4**. In addition to the sample reactions, control reactions with genomic DNA from a wild-type (non-transgenic plant-(sample C1 CTR negative GFP in **Table 4**.)) and no DNA (sample NTC-GFP in **Table 4**.) have been set up.

Table 3. PCR reaction.

Components	**Vol for 1 reaction**	**Final concentration for 22 μl**
Supermix (2X)	11 μl	1
Forward GFP primer (10μM)	0.22 μl	100 nM
Reverse GFP primer (10μM)	0.22 μl	100 nM
Restriction Enzyme (5U/ μl)	1 μl	5U/reaction (0.23 U/ul)
Genomic DNA (0.8ng/ μl)	5 μl	4 ng/reaction
Nuclease-free Water	4.56	
Total volume	22 μl	

Table 4. PCR cycling conditions.

Step	**Temperature ℃**	**Time**	**Number of cycles**
1	95	5 min	
2	95	30 sec	40 times
3	53 (for GFP), 55 (for UBQ)	1 min	
4	4	5 min	
5	90	5 min	
6	4	Hold	

4.2.8. Mutation detection

Young leaves of GFP signal absent plantlets were used for DNA extraction. For analysis of mutation in the target region, primer pairs that amplify the GFP gene have been designed and PCR performed using a high-fidelity polymerase enzyme (**Table 5**).

Table 5. Detection of the genes of interest by PCR.

Reaction preparation, Final volume: 50 µL		
DNA template	1 – 2 µL	22 – 150 ng/µL
Hi-fi buffer	10 µL	5x
Forward	1 µL	20 pmol
Reverse	1 µL	20 pmol
Hi-fi polymerase enzyme	0.5 µL	
H_2O	35.5 - 36.5 µL	
Cycle		
1	95 °C	1 minute (01:00)
2	95 °C	15 second (00:15)
3	65 °C	15 second (00:15)
4	72 °C	30 second (00:30)
5	Go to step 2	29 times
6	72 °C	5 minutes (05:00)

PCR production has been purified by the Wizard® SV Gel and PCR Clean-Up System from Promega and send to Custom DNA Sequencing service of Eurofins for sequencing using Mix2Seq Kit.

4.3. Results

Protoplasts were isolated from GFP EC of Sultana cultivar (**Fig. 8**) and used for transfection of RNPs. The GFP fluorescence expression of EC was controlled by a stereomicroscope. The yield of protoplasts isolated was 6.5×10^7 protoplast/gr.

Cell wall digestion assay by calcofluor staining showed that the majority of the cell wall has been digested, although a complete cell wall digestion in woody plants is difficult; on the other hand, a long-time treatment with the digestion enzymes could decrease the totipotency or viability of protoplasts. Our results showed that an incubation of 5 h in digestion solution is appropriately optimized for both cell wall digestion and keeping protoplasts viable and totipotent. Protoplasts were cultivated in solid medium, surrounded by liquid medium, and monitored every week for the first cell division and embryogenesis development.

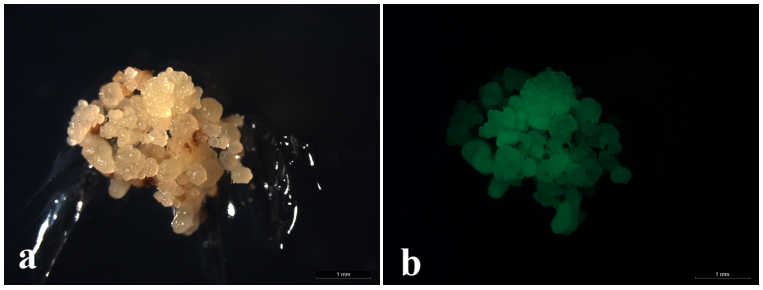

Figure 8. Embryogenic calli that used for protoplasts isolation, **a**. White light, **b**. GFP filter.

4.3.1. RNPs preparation

To induce site-directed mutations in the GFP gene two web tools published by Concordet and Haeussler 2018 (http://crispor.org/), and Bae et al. 2014 (http://rgenome.net/) were used. Two different target sites were selected from these lists for the current study. gRNA target2 (gRNA target2: 102–119 bp) and target4 (gRNA target4: 48–65 bp) (Figure 9). These sgRNAs were designed to pair with their corresponding 18 nucleotides at target sites in the GFP gene locus and to help the Cas9 system to create site-specific DSBs at 3 bp upstream of the PAM motifs. To disturb exogenous GFP genes in grape protoplasts, we used an RNP complex consisting of recombinant Cas9 protein and in vitro synthesized target site-specific sgRNA (Figure 10). These targets were chosen based on their predicted ranking of different scores that evaluate potential off-targets in the genome of interest and on-target activity; high probability of frameshift, low off-target rate, and high specificity score to minimize the off-target effects in the genome. The higher efficiency score indicates the more likely cleavage at this position (Figure 11).

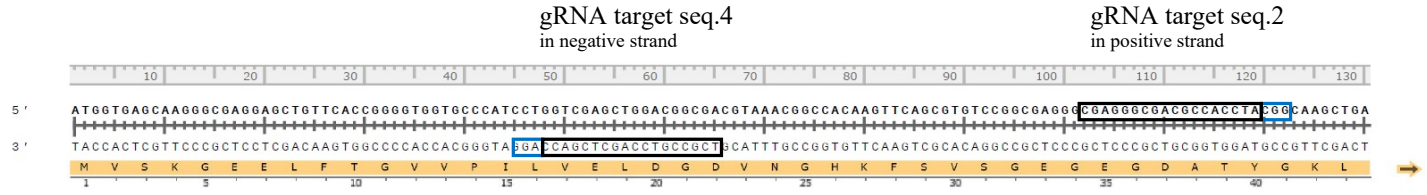

Figure 9. gRNA target sequences position in GFP sequence. gRNA.2 has selected in positive strand (PAM position 120) and gRNA.4 in negative strand (PAM position 45).

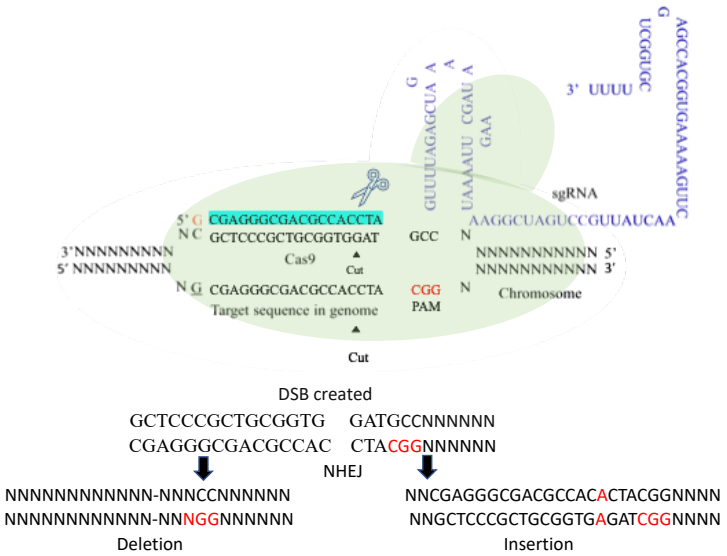

Figure 10. CRISPR-/Cas9-targeted mutagenesis system. Schematic of Cas9/sgRNA (number 2) complex targeting a sequence in chromosomal DNA. The 5' 18 nucleotides of the sgRNA (protospacer) are complementary to the top strand of the chromosome and are directly followed by a PAM which allows Cas9 to make a DSB. The non-variable section of sgRNA remains identical regardless of the intended target. DSBs are repaired by error prone NHEJ resulting in small insertions and deletions (indels).

Predicted guide sequences for PAMs

Ranked by default from highest to lowest specificity score (Hsu et al., Nat Biot 2013). Click on a column title to rank by a score.
If you use this website, please cite our paper in NAR 2018. Too much information? Look at the CRISPOR manual.

Download as Excel tables: Guides / Guides, all scores / Off-targets / Saturating mutagenesis assistant

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes (Only G- / Only GG- / Only A-)	MIT Specificity Score	CFD Spec. score	Predicted Efficiency (Show all scores) Doench '16	Mor.-Mateos	Outcome Out-of-Frame	Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score (exons only; No match, no chrom filter)
32 / fw	GGGCGAGGAGCTGTTCACCG *GGG* Enzymes: *SmiMI, MspI, Hpy166II, BseDI, NciI, LpnPI, OliI, StyD4I* Cloning / PCR primers	100	100	--	--	--	--	0-0-0-0-1 0-0-0-0-1 1 off-targets	4:exon:XM_019225329.1
40 / rev	CCGTCCAGCTCGACCAGGAT *GGG* Enzymes: *BaeGI, NlaIV, BtsCI, BanI, BstNI, Bsp1286I, StyD4I* Cloning / PCR primers	100	100	--	--	--	--	0-0-0-0-3 0-0-0-0-0 3 off-targets	4:intergenic:XM_019226285.1/XM_019226284.1-XM_010664134.2/XM_010664136.2/XM_010664137.2/XM_010664135.2 4:intergenic:XM_019222886.1-XM_010658204.2/XM_002264645.4 4:intergenic:XM_010656272.2/XM_010656271.2/XM_002281515.4/XM_010656273.2-XM_019221998.1/XM_002276677.4
45 / rev	CGTCGCCGTCCAGCTCGACC *AGG* Enzymes: *StyD4I, LpnPI, BstNI, TaqI, BtsCI* Cloning / PCR primers	100	100	--	--	--	--	0-0-0-0-1 0-0-0-0-0 1 off-targets	4:exon:XM_002271124.4
47 / fw	CACCGGGGTGGTGCCCATCC *TGG* Enzymes: *StyD4I, LpnPI, BstNI, TaqI, BccI* Cloning / PCR primers	100	100	--	--	--	--	0-0-0-0-1 0-0-0-0-0 1 off-targets	4:exon:XM_019220386.1/XM_002271697.3
72 / fw	GAGCTGGACGGCGACGTAAA *CGG* Enzymes: *BshFI, BceAI, Hpy166II, AcoI* Cloning / PCR primers	100	100	38	80	29	66	0-0-0-0-5 0-0-0-0-0 5 off-targets	4:exon:XM_019216449.1 4:intergenic:XM_010662995.2/XM_010662994.2/XM_010662993.1/XM_010662992.2/XM_00XM_010662996.2 4:intron:XM_002282702.4 show all...
98 / fw	CAAGTTCAGCGTGTCCGGCG *AGG* Enzymes: *MspI, LpnPI, MwoI* Cloning / PCR primers	100	100	55	66	28	70	0-0-0-0-1 0-0-0-0-0 1 off-targets	4:intron:XR_786832.2/XR_002031157.1/XM_002279163.3
99 / fw	AAGTTCAGCGTGTCCGGCGA *GGG* Enzymes: *MwoI* Cloning / PCR primers	100	100	58	62	26	63	0-0-0-0-1 0-0-0-0-0 1 off-targets	4:exon:XM_019219582.1
120 / fw	GGCGAGGGCGACGCCACCTA *CGG* Enzymes: *BceAI, BstC8I* Cloning / PCR primers	100	100	47	85	46	65	0-0-0-0-3 0-0-0-0-0 3 off-targets	4:intergenic:XM_010660697.1/XR_002031570.1-NR_127874.1 4:exon:XM_019221187.1 4:intergenic:NM_001281128.1-XM_019217053.1/XM_010655627.2/NM_001281185.1
175 / rev	CTGAAGGTGGTCACGAGGGT *GGG* Enzymes: *BshFI, SmiMI, PspPI, LpnPI, BstNI, OliI, StyD4I* Cloning / PCR primers	100	100	56	65	53	66	0-0-0-0-2 0-0-0-0-0 2 off-targets	4:exon:XM_010657890.2 4:intergenic:XM_010656735.2/XM_010656734.2/XM_010656733.2-XR_002030942.1/XR_002030941.1/XR_002030940.1/XR_002030939.1/XR_002030938.1/XR_(
188 / rev	CTGCACGCCGTAGCTGAAGG *TGG* Enzymes: *Tsp45I, AcuI, MaeIII* Cloning / PCR primers	100	100	65	61	49	63	0-0-0-0-1 0-0-0-0-0 1 off-targets	4:exon:XM_002274807.4
191 / rev	GCACTGCACGCCGTAGCTGA *AGG* Enzymes: *AluBI, AcuI* Cloning / PCR primers	100	100	58	51	52	76	0-0-0-0-2 0-0-0-0-0 2 off-targets	4:intergenic:XM_019224058.1-XM_010660492.2 4:exon:XM_002265911.3
201 / fw	CTCGTGACCACCTTCAGCTA *CGG* Enzymes: *AluBI, BceAI, BstC8I* Cloning / PCR primers	100	100	57	56	49	69	0-0-0-0-2 0-0-0-0-0 2 off-targets	4:exon:XM_002280083.4 4:intergenic:XR_002029924.1-XM_002262983.4/XM_002262947.4
226 / rev	TCGTGCTGCTTCATGTGGTC *GGG* Cloning / PCR primers	100	100	37	55	49	60	0-0-0-0-4 0-0-0-0-0 4 off-targets	4:exon:XM_010653124.2 4:exon:XM_002264161.2 4:exon:XM_019222763.1/XM_019222762.1/XM_002269532.3 show all...
260 / rev	GACGTAGCCTTCGGGCATGG *CGG* Enzymes: *NlaIII, EciI* Cloning / PCR primers	100	100	66	59	37	82	0-0-0-0-2 0-0-0-0-0 2 off-targets	4:exon:XM_002277085.3 4:intron:XM_010655009.2/XM_002264069.4

Figure 11. The guide list, the table rows are sorted by specificity score.

Two gRNA target sequences are highlighted by yellow quadrate.

gRNA synthesized by in vitro transcribed (IVT) as described in the Kit, in vitro transcribed gRNA quality determined by running in the gel, the expected gRNA transcript size is 100 bases. A discreet band at 100 bases indicates intact RNA (**Fig. 12)**. The concentration of the gRNA transcript was determined using the NanoDrop^{TM} spectrophotometer and the concentration for each gRNA was different, we have seen up to a 2x variation in concentration estimation. For example, the concentration of gRNA number 2 was ∼1950 ng/µL while the concentration of gRNA number 4 was 3700 ng/µL.

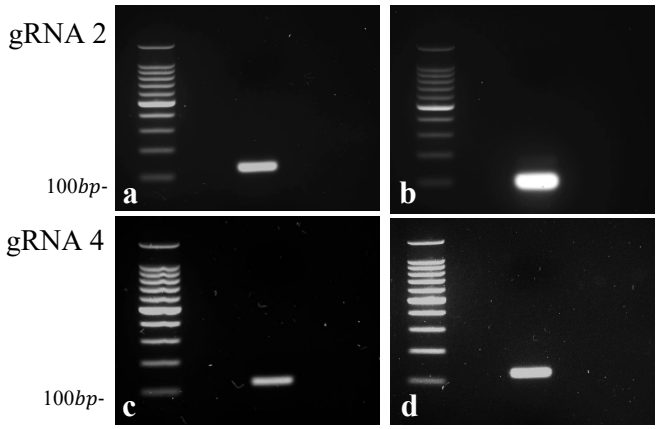

Figure 12. **a**. DNA gRNA template 2 ≈100bp, **b**. DNA gRNA template 4 ≈100bp **c**. gRNA2 synthesized by in vitro transcribed (IVT) ≈ 100bp, **d**. gRNA4 synthesized by in vitro transcribed (IVT) ≈100bp.

Enhanced specificity" SpCas9 (eSpCas9) was used, consisting of an individual alanine substitution at 31 positively charged residues within the nt-groove, which can dramatically decrease off-target indel formation while preserving on-target activity.

Premix of Cas9 and gRNA is essential 10 min before transfection to make the RNP complex. Cas9 undergoes a conformational change upon gRNA binding that shifts

the molecule from an inactive, non-DNA binding conformation into an active DNA-binding conformation. Importantly, the spacer region of the gRNA remains free to interact with target DNA [55].

4.3.2. direct delivery of RNP into protoplasts

The transfection process of RNPs was performed according to [54]. The protoplasts were then washed by WI and cultured immediately after transfection by RNPs. After two months of culture, the grown somatic embryo was transferred to a regenerative medium for 4 weeks for further growth.

By following the loss of GFP fluorescence, we were able to observe the somatic embryos that undergone targeted mutations caused by CRISPR/ Cas9 activity (**Fig. 9**). After two months of cultivation, well-developed SEs were transferred to regeneration medium for further growth for 4 weeks. 23 regenerated well-shaped mature SEs have derived from each RNP2 and RNP4 SEs transfection and transferred to shoot regeneration media for 4 weeks under the photoperiod. To obtain whole plants regenerated shoots transferred to root regeneration medium. In every step, the GFP expression signal has been controlled by the stereomicroscope and in case of absent expression of the GFP signal, SE has been a candidate as an edited plant. Finally, in RNP2 transfected group 8 and RNP4 transfected group 10 plantlets have obtained respectively (**Fig. 14).** On the other hand, in the negative controls including *Control* (transfected only by PEG) and *N.T* (no transfected) any loos in GFP signal have been observed (**Fig. 13**).

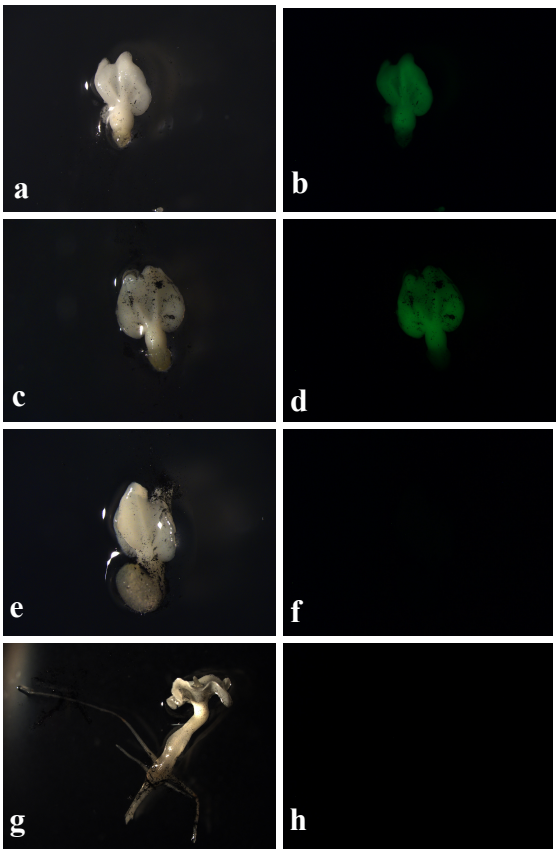

Figure 13. Somatic derived-protoplast in regeneration medium; white light and GFP filter. **a**-**b**. somatic derived protoplasts of N.T. **c**-**d**. somatic derived protoplasts of Control. **e**-**d**. somatic derived protoplasts of gRNA 2. **g**-**h**. somatic derived protoplasts of gRNA 4.

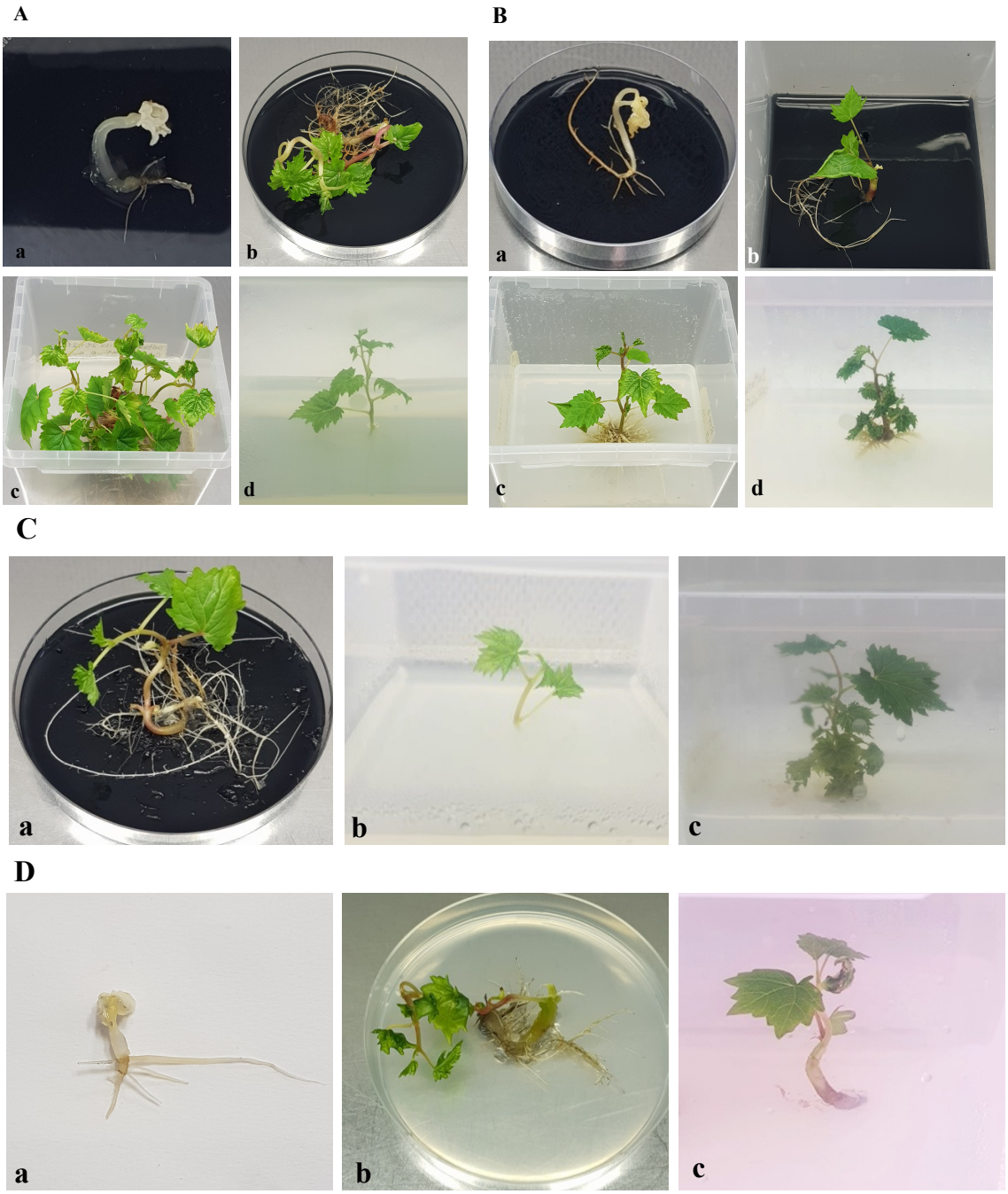

Figure 14. Plant regenerated derived protoplast. **A**. N.T, a. well-developed somatic, b. shoot regenerated in MG1BAP, c. in C2D, d. whole plant in MSN. **B.** gRNA2, a. well-developed somatic, b. shoot regenerated in MG1BAP, c and d. whole plant in RIM. **C.** Control, a and b. shoot regenerated in MG1, c. whole plant in RIM. **D**. gRNA4. a. well-developed somatic, b. shoot regenerated in C2DBAP, c. whole plant in MSN.

4.3.3. Measurement of GFP gene copy number Using Droplet Digital PCR

During ddPCR process, each sample is divided into a large number of partitions and PCR reactions are carried out on each partition individually. Fluorescence is incorporated into the target amplicon, as it is in qPCR procedures. Instead of measuring fluorescence intensity at a specified cycle during the exponential phase of the reaction, each partition is assessed for the presence or absence of fluorescence (and thus the target) at the reaction endpoint. The target gene's copy number is obtained by calculating the proportion of partitions in which the target gene was amplified relative to partitions in which the reference gene, of known copy number, was amplified. A single reaction should produce 12,000-20,000 droplets. The calculation of copy number relies on the ratio of positive to negative droplets (**Fig. 15**).

The copy number of the target gene (GFP) is calculated by $(C^{target}/C^{ref}) * N^{ref}$, where C^{target} is the concentration of the transgene, C^{ref} is the concentration of the endogenous gene, and N^{ref} is the copy number of the reference gene [49].

Concentration data for each sample appear in the wells in the plate map and are tabulated in the results table. These data have been exported to Microsoft Excel for analysis of gene copy number. The reference gene (Ubiquitin) is a single copy gene in the *V. vinifera* genome, therefore, based on the concentration of reference gene and target gene provided by ddPCR (**Table 6**), there is a single copy of GFP gene in the cell line of transformed Sultana cultivar.

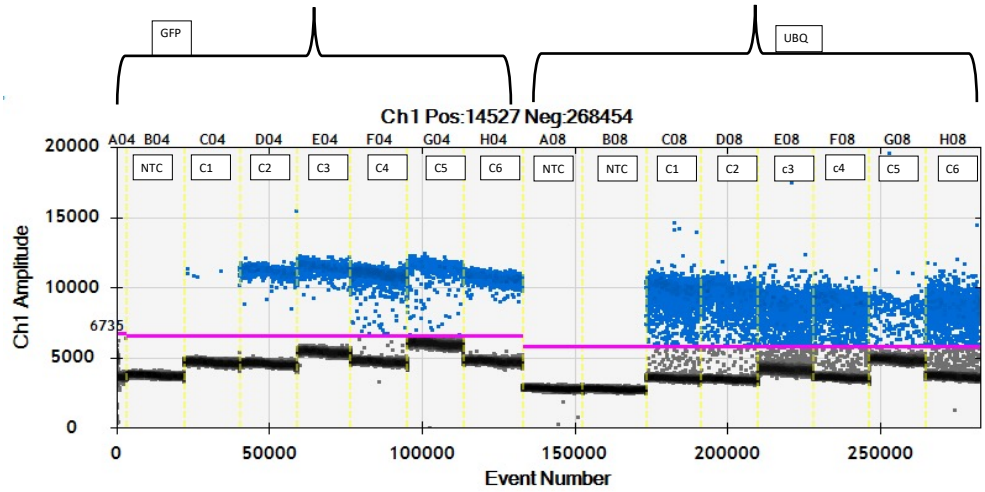

Figure 15. Plot showing positive (blue, those above the red threshold line) and negative (grey, below the red threshold line) droplets for eight samples; A04-A08 droplets of GFP gene and B08-H08 ddPCR droplets of ubiquitin gene.

Table 6. ddPCR data from transgenic plants.

Well	Samples	Concentration of GFP gene	Concentration of reference gene	Copy number of GFP gene
A04	NTC-GFP	0	0	-
B04	NTC-GFP	0	0	-
C04	C1 CTR negative GFP*	7,04	1520,2	0,005
D04	C2 CTR positive GFP	1267,2	997,7	1,270
E04	C3 GFP	1696,2	1123,1	1,510
G04	C5 GFP	840,4	547,8	1,534
H04	C6 GFP	1478,4	1169,3	1,264

*C1 CTR negative GFP is wild-type (non-transgenic) plant.

4.3.4. Mutation evaluation

No expression of GFP in the preliminary step of somatic embryogenesis can show that the GFP gene is knocked out but it's necessary to confirm the edition by molecular methods. Screening for the presence of mutations can be carried out using several methodologies including restriction enzyme/ PCR-based methods. However, we have found that direct sequencing of PCR amplicons that cover the target sites (**Fig. 16**) is relatively quick and simple and gives detailed information on the events occurring at the target locus [51]. We employed a common mutation detection service of Eurofins for sequencing (Custom DNA Sequencing).

The samples putatively carrying a mutation were used for DNA extraction and sequencing. Confirmation of successful gene targeting by RNP requires the detection of insertions or deletions (indels) that commonly introduced three nucleotides upstream of PAM. The sequencing results demonstrated that one candidate in each group has mutated successfully. In both cases, an insertion of one base has been occurred three nucleotides upstream of PAM. An Adenine and a Thymidine have been inserted in the RNP2 and in the RNP4 transfected sample, respecitvely. Both insertions, resulting in frameshift mutations that produce a loss-of-function (LOF) mutation and disrupt (knockout) the GFP gene (**Fig. 17**).

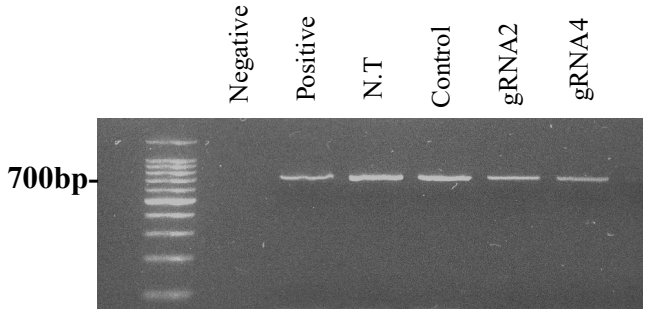

Figure 16. PCR amplification of GFP gene using total DNA extracted from different sample as a template.

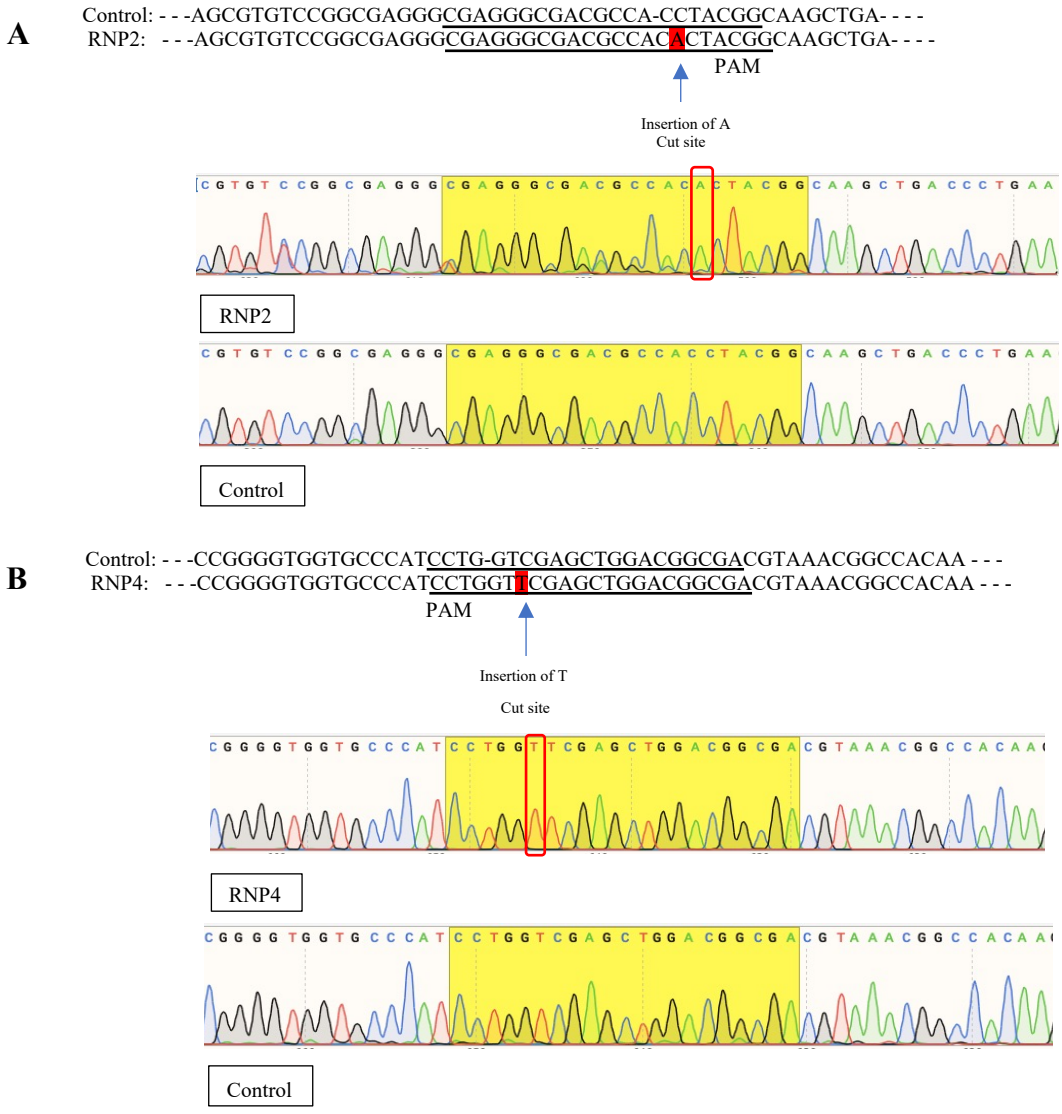

Figure 17. Cleavage the DNA strands 3 bases upstream of the PAM caused by nuclease domains in Cas9 generating a blunt-end DNA double stranded break (DSB). Sequencing results of both mutants demonstrated **A.** Adenine insertion in RNP2 and **B.** Thymidine insertion in RNP4 transfection which are incorporated into DSBs via NHEJ pathway.

Regenerated plantlets derived from gene-edited protoplasts exhibited a normal phenotype concerning leaf shape and color and growth habits compared to wild-type plants (**Fig. 18**).

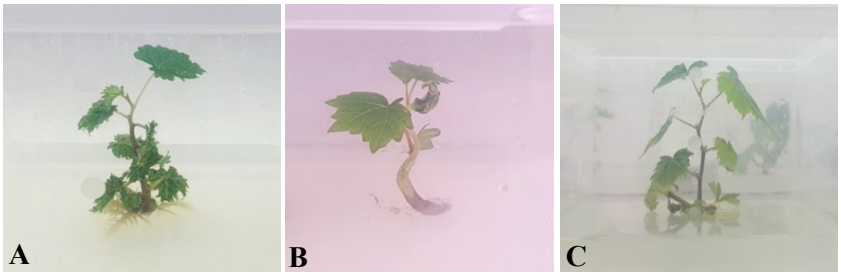

Figure 18. Phenotype of regenerated plantlet derived from gene edited protoplasts using **A**. RNP2, **B**. RNP4 and **C**. wild type.

4.4. Discussion

In this chapter, we described a successful knockout of a green fluorescent protein (GFP) reporter gene, that is already integrated into the grape genome, in the Sultana variety by direct delivery of RNPs into protoplast. We demonstrated the use of this powerful new tool in targeted knockout of a gene settled in the grape genome. By following the loss of the GFP fluorescence signal, we were able to observe the cells that had endured targeted mutations as a result of CRISPR/ Cas9 activity. In addition, we presented examples of the various types of indels obtained by Cas9-mediated cleavage of the GFP gene, guided by two independent sgRNAs. The application of the CRISPR/Cas9 RNP system enables the generation of grape plants engineered by DNA-free gene editing. Eventually, we provided an optimized protocol to target important native genes in the grape plant in the future.

The growing demand for agriculture to stay sustainable and productive in the face of changing climatic circumstances and the pressures of a growing global population could be helped by using gene-editing technologies. The development of target-specific genome engineering tools, including zinc finger nucleases (ZFNs), mega-nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein 9 (Cas9), have ushered forth a new era of gene editing. CRISPR is a well-known bacterial immune defense system that serves as the foundation for CRISPR-Cas genome editing biotechnologies. In 2012, a key publication demonstrated how the CRISPR-Cas9 system may be programmed for site-specific DNA cleavage, signaling a breakthrough in genome editing. The high efficiency, simplicity, precision, user-friendly, and versatility of the CRISPR-Cas9 system have surpassed earlier genome editing biotechnologies such as ZFNs and TALENs [12, 13, 56]. The revolutionary CRISPR-Cas system offers enormous potential for modifying gene expression for crop improvement and food production. CRISPR-Cas-based technology and applications have being rapidly developed. Base editing, for example, introduces desired point

mutations in a target region using cytosine base editors (CBEs) and adenine base editors (ABEs) [56]. However, when plasmid vectors are used, all endonucleases have been reported to cause off-target effects and unwanted genome integration due to the persistence of plasmids. To tackle this issue, recent studies have been demonstrated that direct delivery of purified recombinant nuclease proteins such as Cas9 and TALENs combined with guide RNA can be used in plant and animal systems to cleave target DNA sequences. One of the most significant advantages of employing direct Cas9 protein delivery is the ability to induce mutations quickly and precisely. Furthermore, since the Cas9 protein-guide RNA complexes rapidly degrade in regenerating cell cultures, the new kinds obtained with this method may be exempt from current GMO regulations in plants [8, 10, 12, 13, 56].

In this study, to optimize a protocol for direct delivery of RNPs into protoplast, several experiments have been done. The common method used for plant protoplast transfection is PEG-mediated transfection. The PEG-mediated method is widely used due to its easy operation, low cost, lack of requirements for specific equipment, and generation of stable results [8, 17, 57].

The first transfection of protoplasts by RNPs has been done as described by [58] with some modification. The same protocol that was used previously for transfection of protoplast by a vector carrying out YFP gene marker (see chapter 2), were used for direct delivery of RNPs into the protoplast. Protoplast remained in the WI medium for 72h post-transfection and was then cultivated. In this condition, protoplast did not grow at all, although the integrity of some of the protoplasts was preserved, some protoplasts showed bulging of the cytoplasm. This could happen due to a lack of nutrients in WI and partly weak cell wall reconstruction.

Then, fresher (younger) GFP EC were used and the transfection process was performed according to [54] with a small modification, they improved their protocol by increasing the time of PEG treatment to 40 min. To avoid a toxic effect of PEG for the protoplasts, after 20 min of PEG treatment, the PEG solution was diluted using W5

in two steps. The post-transfection incubation in WI was reduced from 72 hours to overnight, with adding glucose to the WI. Results showed that protoplast growth very well and plant regenerated with high efficiency.

In the next step, to increase the efficiency of protoplast-derived regenerated plants, protoplasts were cultivated immediately after transfection without incubation in WI. Since the GFP protein is highly stable in the grape cells and the complete disappearing of fluorescence signal takes 1–2 weeks [59], the incubation in WI medium does not make sense in this approach and it can affect both viability and totipotency.

Recent studies have reported that off-target mutations induced by RNA-guided endonuclease RNPs are rarely found or limited when Cas9 is used at 2–10-fold molar excess of gRNA [12]. In the present study, we used only a low ratio of Cas9 protein to sgRNA (1:5) which was within the ratios used in previous studies, suggesting that its off-target effects might be very low. Nevertheless, there is mounting evidence that off-target events in plants may not be a major issue because the chance of them occurring via tissue culture-based transformation or other mutagenesis approaches is minimal [60].

Droplet digital PCR (ddPCR) method has been used to determined copy number of GFP gene in transformed plants. Digital PCR shares the qualities of rapidity and sensitivity with qPCR. At the same time, it exhibits increased tolerance to inhibitory substances, making it an attractive alternative to qPCR [47]. ddPCR has low costs and scalability in common with other PCR procedures, but it has greater precision and reliability [49].

This is the first study that presented an efficient protocol for foreign DNA-free CRISPR/Cas9 mediated gene editing in *Vitis vinifera* cv. Sultana, including detailed protoplast-to-plant steps. Recently, many groups in the world published some applications of CRISPR technology to grape varieties genome editing using the vector-mediated transformation [17]. Ren et al. (2016) stably transformed embryogenic cell suspension in Chardonnay to introduce point mutation in the L-idonate dehydrogenase

gene. They succussed to regenerate mutants with altered production of tartaric acid and vitamin C [27]. In another study in 2017, the phytoene desaturase gene was targeted with a CRISPR/Cas9 editing construct in Neo Muscat somatic embryos and albino leaves were observed in regenerated plants [28]. Recently, Wang et al. reported that transgenic Sultana plants have obtained carrying a mutation in WRKY52 transcription factor gene [29]. In an attempt to produce non-transgenic edited grape varieties, Malnoy and colleagues (2016) have used a direct delivery method to transfect Chardonnay protoplasts with RNP complex. they gained edited protoplasts, but not regenerated whole plants [30].

In this study for the first time, we managed to achieve whole plants regenerated from DNA-free genome edited protoplasts. Monitoring the protoplast to whole plant developmental stages demonstrated that regenerated plantlets derived from gene-edited protoplasts exhibited a normal phenotype concerning leaf shape and color and growth habits compared to wild-type plants.

For the consumer, it is critical to guarantee that regulations are clear and that products are safe. It is easy for activist groups to spread data-free ideas and anecdotal reasoning to promote dread of any new technology, particularly when it includes food production, as was the scenario with GM crops. Additionally, market access for gene editing goods must be considered, which poses a risk to business investment in the technology. Many jurisdictions have yet to rule on gene-edited crops and foods, which continues to be a challenge for plant breeding firms and researchers. The European Union continues to be a significant hurdle to the development of new markets for sustainable food production technologies [13].

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Future plan

Optimization of plant recovery from somatic embryos in *Vitis vinifera* using artificial electric fields

Grapevine is one of the most economically important fruit crops worldwide and is widely used for producing wine, juice, and dried and fresh fruit [1]. However, its yield and quality are adversely affected by abiotic and biotic factors including climate change and various diseases [2]. One of the most cost-effective and environmentally friendly strategies for limiting losses due to biotic and abiotic stresses is the development of disease/stress-resistant cultivars through new biotechnological approaches such as genome editing technology [3].

However, the capacity to obtain plants from modified somatic embryos is critical for the effective application of the techniques, which requires an efficient and reliable plant regeneration system. Plant regeneration from grapevine (*Vitis* spp.) via somatic embryogenesis typically is poor and problematic due to extremely low efficiency, including extended culture durations required for embryo–plant conversion. Poor plant recovery especially in recalcitrant varieties is a bottleneck to the selection of improved genetically modified lines [4]. Although we have improved the efficacy of plant regeneration by modifying the ratios of the specific phytohormones auxin and cytokinin in the medium [5] , some studies indicate other molecular and physical perturbations can induce plant regeneration in planta.

Long exposure to a weak electric current, both continuous [6] and alternating [7], has been found to improve in vitro regeneration efficacy in tobacco tissue cultured in a shoot-inducing media. Other results have also suggested that a brief pulse of an electric field parallel to the root can enhance the probability of its regeneration by up to double and perturb the local distribution of the hormone auxin, as well as cell division regulation [8].

Finally, electrostimulation showed the potential to improve the development of the root systems and shoot growth in two kale cultivars [9]. The mechanism

of the electrical stimulation of growth is poorly understood, but one interesting hypothesis is that the electric field stimulates the biosynthesis or transport of growth regulators such as IAA [10]. and as a consequence, the expression of genes related to stress responses and secondary metabolism.

Currently, we have indicated VvAGO10 as one of the main genes which regulate shoot apical meristem development during grapevine somatic embryogenesis [11]. To clarify the detail of this mechanism in this experiment we propose to investigate the efficacy of regeneration and the expression level of the following gene in response to external electric fields in two grapevine varieties.

The lengthy history of research in this field at the Imperial College London, as well as The Laboratory of Plant Morphogenesis's multidisciplinary skills and expertise, along with state-of-the-art equipment available in the Department of Life Sciences at Imperial, will complement our skills and know-how. This collaborative project will advance plant regeneration protocols and produce preliminary data that will be used for future joint grant proposal, for example within the Horizon Europe scheme.

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