11 Aug 2021 12:08

SATI tarryn@satgi.co.za Tel: 021 863-0366	CFPA inmaak@mweb.co.za Tel: 021 872-1501	HORTGRO anita@hortgro.co.za Tel: 021 870 2958	RAISIN SA ferdieb@raisinsa.co.za Tel: 054 495 0283	WINETECH andraga@winetech.co.za Tel: 021 276 0499
				X

FINAL REPORT 2021

Winetech Number : GenUS 17/02

1. PROGRAMME & PROJECT LEADER INFORMATION

	Research Organisation leader	Project leader
Title, initials, surname	Profs J Burger / M Vivier	Drs M Campa / P Young
Present position	Professors (Molecular Genetics & Grapevine Molecular Biology and Biotechnology)	Senior Researcher / Senior Researcher
Address	Dept. of Genetics / South African Grape and Wine Research Institute Stellenbosch University 7600 Stellenbosch	Dept. of Genetics / South African Grape and Wine Research Institute Stellenbosch University 7600 Stellenbosch
Tel. / Cell no.	021-8085858 / 8083773	021-8085885/021-8089204
E-mail	jtb@sun.ac.za; mav@sun.ac.za	jtb@sun.ac.za

2. PROJECT INFORMATION

Project title	Grapevine genome editing: development of tools and implementation of the technology
Short title	Grapevine CRISPR

Fruit kind(s)	Wine grape		
Start date (mm/yyyy)	2017-07	**End date** (mm/yyyy)	2020-12

Key words	Genome editing, CRISPR/Cas-9; targeted gene silencing, tissue culture; grapevine

	CFPA	RAISIN SA	HORTGRO	SATI	WINETECH	ARC	OTHER
TOTALS All years	R 0	R 0	R 0	R 0	R 4 001 516	R 0	R 0

Total cost of entire project	R 4 001 516

3. EXECUTIVE SUMMARY

Objectives and Rationale

The immense potential of CRISPR/Cas9 technology as a genome editing tool suggests that it can be used in grapevine improvement strategies. This project aims to establish CRISPR technology in grapevine. We will establish conventional CRISPR/Cas-based protocols for editing the grapevine genome by targeting a single-copy carotenoid gene at both the genome (DNA) and transcript (RNA) levels. The project comprises four major objectives:

1. Construction of appropriate Cas9- and Cas13a-based editing vectors.
2. Preparation of plant materials for genetic transformation of *Nicotiana benthamiana* and grapevine.
3. Transformation of *N. benthamiana* and grapevine and regeneration of plantlets.
4. Evaluation of transformants, both phenotypically, and at a molecular level.

The reason for using *N. benthamiana* in parallel with grapevine is that it is a fast-growing herbaceous plant that is genetically well-characterised, and amenable to genetic transformation. Moreover, the gene selected for optimising the genome editing system in grapevine, is identical between the two species, suggesting that the editing constructs can be shared, thus saving time and money. Successful achievement of this goal would be an excellent justification to take this project to the next level in which we propose to target economically important traits like drought tolerance and virus resistance in grapevine.

Methods

Binary vector backbones for genome editing were acquired from Addgene. The targets for the selected LBCY gene were designed using the CRISPR-P and RGEN software. Constructs were assembled using a Gibson assembly strategy, and validated by Sanger sequencing before being transformed into Agrobacterium for plant transformation. Genetic transformation of *N. benthamiana* leaf disks and grapevine embryogenic callus was done according to routine protocols established at SAGWRI. *N. benthamiana* and grapevine plantlets were phenotypically (visually), genetically (PCR, RT-qPCR and sequencing) and biochemically (UPLC) analysed, according to established protocols.

Key Results

All molecular tools required for the editing of the proposed genes in *N. benthamiana* and grapevine were designed and constructed. These were used to generate a population of edited *N. benthamiana* plants were comprehensively characterised and demonstrated successful editing of the target gene. Both mono- and bi-allelic editing were observed.

In spite of diminished regeneration ability, transformation with the editing constructs yielded 43 Chardonnay lines. However, molecular analysis performed on these showed that these were not transformed nor were they edited. The same construct was also transformed to grapevine in Italy, also leading to no edited plants, indicating that editing the specific gene could be lethal in grapevine. Another construct, targeting a different gene (VvPDS) was

however successful, leading to the characteristic bleached phenotype, confirming that successful editing was achieved in this case.

Transformation experiments with both Cas13a-LBCY vectors yielded working populations of *N. benthamiana* plants for both target sites. Preliminary RT-qPCR results on these lines showed reduction in the expression of LBCY in some samples but not consistently among all lines.

Key Conclusion of Discussion

Transformation experiments with a Cas9-based vector in *N. benthamiana* and comprehensive phenotypic, genetic and biochemical analysis confirmed the successful editing of these plants. Similar experiments with grapevine yielded regenerated Chardonnay plants, but genetic analysis on DNA from these plants showed failure of the transformation and thus also no mutations were introduced. Successful editing was obtained in a repeat experiment targeting a different gene that was conducted in Italy. Cas13a-based editing in *N. benthamiana* showed a reduction in expression of the target gene, but results were inconsistent and should be repeated with larger sample numbers.

Take Home message for Industry

In this project, we have demonstrated an ability to design and assemble constructs for the editing of *N. benthamiana* and grapevine, and in a very short time re-established a grapevine transformation and regeneration capability. This opens up possibilities for editing of grapevines to address biotic and abiotic stress issues of economic importance, such as virus resistance and drought.

4. PROBLEM IDENTIFICATION AND MOTIVATION

Problem Identification

The negative economic impact of biotic and abiotic stresses in vines are recognised by the international viticulture industry. Natural tolerance, resistance or cures for these afflictions are rare, and best practises still rely on prevention of these stresses and diseases. Rapid developments in genome editing technologies, and especially the versatility demonstrated in many applications of CRISPR/Cas9-based technology, may impact radically in the ongoing battle with most of these conditions in vineyards all over the world.

TThis project will aim to establish CRISPR technology in grapevine. During this proof-of-concept phase, we will establish a conventional CRISPR/Cas9-based protocol for editing the grapevine genome by targeting a single copy carotenoid gene that is central to grapevine secondary plant metabolism and will lead to a readily monitored phenotype. Considering the negative impact of viruses on the local and international grapevine industries, and the fact that they have RNA genomes, we will also attempt to silence an RNA transcript of an easily-monitored grapevine

gene - the latter acting as proof-of-concept proxy to a virus like Grapevine leafroll-associated virus 3. The project will comprise four major objectives, each comprising of a number of tasks with related milestones. The first objective will be the construction of all molecular vectors to introduce the different genetic elements required for CRISPR-based genome editing. The second objective will comprise the preparation of plant materials for routine genetic transformation protocols for Vitis cultivar(s), while the third will attempt to introduce the different genetic elements into appropriate grapevine tissues, and to regenerate grapevine plantlets for subsequent analysis. Finally, primary transformants will be evaluated, both phenotypically, and at a molecular level for efficacy and accuracy of the editing.

Motivation

CRISPR is based on an innate (yet adaptive) archaeal and bacterial immune system, but has been developed to its current commercial status by manipulating the *Streptococcus pyogenes* system. The technology allows for the editing of genomes with unprecedented precision, efficiency, and flexibility, and has found applications in disciplines ranging from medicine to agriculture. Crops that have been bioengineered by CRISPR and field-tested include maize, wheat, rice, sorghum, soybeans, cucumber, potatoes, tomatoes and oranges. Commercial CRISPR crops include mushrooms and oilseed rape, both of which have been deregulated (*i.e.* are not considered GM) in the USA. In grapevine, only three applications of CRISPR technology have been reported. These include targeting a gene involved in the biosynthesis of tartaric acid in ‘Chardonnay’; knocking out the Vitis MLO-7 gene in ‘Chardonnay’ protoplasts, in order to increase resistance to powdery mildew; and targeting the *Vitis vinifera* phytoene desaturase gene, in which plants with albino leaves were regenerated.

The proposed study aims to create grapevine plants that will constitutively express SpCas9, and guide-RNAs (gRNAs) that will target *VvLBCY,* a single-copy gene that functions at a very specific branch-point in the carotenoid pathway in the Vitis genome. The success of the approach could be assessed visually by a distinct phenotype, as well as by molecular analyses using techniques well established in the SU laboratories. An exciting potential application of this technology would be to target virus diseases of grapevine. For this reason, an RNA-targeting nuclease (Cas13a), will be expressed in grapevine. For proof-of-principle, we will target a grapevine RNA transcript that will be easy to observe phenotypically (*e.g*. lycopene β-cyclase, VvLBCY), which will serve as a proxy for the genome of an RNA virus.

5. ACCUMULATED PROGRESS TABLE

Objectives	**Milestones (Significant event or stage in a project)**	**Date Achieved**
O1. Construction of CRISPR vectors.	M1.1 Construct an Agrobacterium-based binary plant expression vector containing the Cas9 gene from Streptococcus pyogenes (SpCas9) – for silencing an RNA	2018-09-30

	transcript of the Vitis vinifera lycopene beta-cyclase gene (VviLBCY) - 03/2018. M1.2 Construct an Agrobacterium-based binary plant expression vector containing the Cas13a (C2C2) gene from Leptotrichia shahii – for silencing the RNA transcript of an easily-monitored grapevine gene (e.g. LBCY) - 03/2018. M1.3 Design, synthesise and clone a number of sgRNAs and crRNAs, targeting the abovementioned Vitis metabolic gene and the RNA transcript, into the respective CRISPR vectors - 07/2018. M1.4 Do in vivo screens of the designed gRNAs against their selected targets in a model plant (N. benthamina) using multiplexed targeting (four targets) approach, using plantlets generated in M3.1 - 09/2018.	
O2. Establish appropriate plant materials and routine genetic transformation protocols for Vitis cultivar(s).	M2.1 Sample immature flowers and excise anther filaments from Sultana and Chardonnay and establish callus cultures - 12/2018. M2.2 Select, synchronise and proliferate pro-embryogenic masses - 12/2018. M2.3 Induce embryo formation and test regeneration potential of the material - 04/2019.	2019-04-30
O3.Transformation of N. benthamiana and grapevine and regeneration of plantlets.	M3.1 Transform N. benthamiana leaf disks and regenerate putative transgenic plantlets for in vivo evaluation as described in M1.4 - 10/2018. M3.2 Transform grapevine embryogenic callus and regenerate putative transgenic plantlets for evaluation - 12/2019. M3.3 Transform Cas13a::crRNA constructs into both N. benthamiana leaf disks and grapevine embryogenic callus, select for transformed material and regenerate plants - 09/2019.	2019-12-31
O4. Evaluation of transformants, both phenotypically, and at a molecular level.	M4.1 Evaluation of N. benthamiana population (VviLBCY) - 12/2019. M4.2 Evaluation of grapevine population (VviLBCY) - 12/2020. M4.3 Evaluation of N. benthamiana and grapevine populations transformed with Cas13a::crRNA constructs - 09/2019.	2020-12-31

6. WORKPLAN (MATERIALS AND METHODS)

W1 to match M1.1 - M1.4 Construction and testing of CRISPR vectors.

1.1 A binary vector backbone was acquired from a commercial source (Addgene). The binary vector has

an NPTII gene (Kanamycin resistance gene) and is designed for a multiplexed targeting approach, meaning that multiple gRNAs are expressed from a single transcript - these polycistronic mRNAs are processed posttranscriptionally into individual gRNAs by RNA-cleaving enzymes. These enzymes include the CRISPR-associated RNA endoribonuclease Csy4. This approach allows for a single gene to be targeted at different positions, or multiple genes to be targeted at the same time. The plasmid used was pDIRECT_22C, which includes a Cas9, codon-optimised for *Arabidopsis thaliana* (AtCas9).
1.2 Previously published SpCas9 targets in the *Vitis* genome (Wang et al. 2016; BMC Plant Biology), and publicly available software (http://biodb.sdau.edu.cn/gc/ and http://cbi.hzau.edu.cn/CRISPR2/) were used to identify potential target sites common between *N. benthamiana* (used as a model system) and grapevine.
1.3 The gRNAs were inserted via Golden Gate cloning in pDIRECT_22C. The vector was verified by Sanger sequencing.
1.4 Fifty leaf explants of *N. benthamiana* were transformed with pDIRECT_22C+LCBY gRNAs for testing the mutation efficacy of the four designed targets. Evaluation of these plants are described under W4 (M4.1).
1.5 (Milestone modified) Two target sites in the lycopene β-cyclase (LBCY) transcript were selected using bioinformatics and the CRISPR-RT online software. Primers were designed to generate each crRNA (target) and after oligo annealing each crRNA was ligated into a pjjb308 plasmid (Addgene). The plasmid was transformed into *E. coli* strain DH5α and colonies were selected for Sanger sequencing. Once the presence of the target in pjjb308 was confirmed, the plasmid was included in a Golden Gate cloning reaction that also comprised of three additional plasmids: pjjb296 (Addgene), pMOD_C00 and pTRANS_220d (Voytas Lab). The resultant reaction was transformed into *E. coli* strain DH5α and colonies were selected for PCR screening and Sanger sequencing. Those that returned positive were used for transformation into *Agrobacterium* strain EHA105. Colonies obtained from the transformation were screened by PCR and stored in glycerol stocks.

W2 to match M2.1 - M2.3: Establish appropriate plant materials and routine genetic transformation protocols for *Vitis* cultivar(s).
Standard methods to sample immature flowers and excise anther filaments from Sultana and Chardonnay were used to establish callus cultures. The cultures were subjected to regular transfers to fresh media and selection for callus with embryogenic potential during the sub-culturing. In addition to the explants from the flowers, embryogenic cultures were also initiated from *in vitro* buds and leaf explants. The cultures were maintained, bulked up and subjected to embryo induction according to published methods.

W3 to match M3.1 - M3.3: Transformation of grapevine and *N. benthamiana* and regeneration of plantlets.
3.1 Fifty leaf explants of *N. benthamiana* were incubated with *Agrobacterium* EHA105 containing pDIRECT_22C+LCBY gRNAs. The explants were then subcultured on regeneration medium every two weeks. Regenerating shoots were then transferred to the rooting medium. Fully regenerated *N. benthamiana* plantlets were maintained on MS medium and stock and working populations of the plantlets were established and maintained through *in vitro* propagation. The working population was used for further analyses.
3.2 Transform grapevine embryogenic callus and regenerate putative transgenic plantlets for evaluation. A standard grapevine transformation protocol was used for grapevine somatic embryos, using *Agrobacterium* EHA105 containing DIRECT_22C+LCBY gRNAs. Selection started approximately three weeks after the exposure to *Agrobacterium* and will proceed throughout the embryo regeneration processes until plants are recovered (approximately 2-4 months). Fully regenerated plantlets will be maintained and subcultured on MS medium to generate a stock and working population of putative transgenics for further analyses.
3.3 Cas13a::crRNA constructs were used to transform *N. benthamiana* leaf disks as described above. Equivalent transformation of grapevine embryogenic callus will commence shortly.

W4 to match M4.1 -M4.2: Evaluation of transformants, both phenotypically, and at a molecular level.
4.1 Evaluation of *N. benthamiana* population (VviLBCY). Regenerated *N. benthamiana* plants were analysed for the presence of the Cas9 gene using PCR. In positive plants and in control plants, the target gene (LBCY) was amplified using PCR and then Sanger sequenced. Some of the edited (knock-out

LBCY) plants were also analysed for gene expression using RT-qPCR. Possible off-targets were identified using RGEN software and subsequently mutation-specific primers were designed. PCR products were Sanger sequenced and analysed for mutations. The plants were also phenotyped for pigment content and profiles, particularly in terms of chlorophyll and carotenoid contents and profiles to indicate the presence or absence of pigments.
4.2 Evaluation of grapevine population (VviLBCY). Similar methods will be used to analyse the putative grapevine transformants, as described for the tobacco population.
4.3 Evaluation of LCBY mRNA levels in both *N. benthamiana* and grapevine populations will be done using RT-PCR and/or RT-qPCR.

7. RESULTS AND DISCUSSIONS

Progress towards Objective 1. Construction of CRISPR vectors.

LBCY was selected as a suitable target gene since the grapevine LBCY (*VvLBCY*) has been isolated and analysed at the SAGWRI. The gene has been functionally characterised and is highly conserved. In addition, *VvLBCY* contains no introns, the gene can therefore be isolated directly from genomic DNA of additional grapevine genotypes (instead of isolation from cDNA, with the inherent associated difficulties). This provided us with a suitable gene to test the vectors and target predictions that needed to be evaluated in order to successfully implement this technology in grapevine.

The initial plan to do *in vitro* screens to test the efficacy of our targets was aborted when it was decided to use a multiplex targeting approach in the grapevine *LBCY* gene. The highly conserved nature of *LBCY* genes in plants allowed for single constructs to be designed that in theory would be functional in grapevine and in *N. benthamiana*. To increase our chances of total knock-out of the target gene, we decided to use a multiplexed targeting (four targets) approach, which largely negates the *in vitro* screen initially planned. This vector was successfully constructed and evaluated in transgenic *N. benthamiana* plants as described in Objective 4.

M1.1 Construct an Agrobacterium-based binary plant expression vector containing the Cas9 gene from *Streptococcus pyogenes* (SpCas9) – for silencing an RNA transcript of the *Vitis vinifera* lycopene beta cyclase gene (*VvLBCY*). (COMPLETED)

A multiplexed gene editing approach, using the pDIRECT_22C backbone vector, was chosen to knock-out *LBCY* in grapevine, based on the high mutagenesis efficiency reported by Cemark et al. (2017). Since grapevine transformation is a time-intensive process, *N. benthamiana* was considered as a model system to assess the functionality of the chosen editing approach and the targets selected. To this end, four targets that are common between *N*. *benthamiana* and grapevine *LBCY* cDNAs were identified using CRISPR-P and RGEN software. The corresponding guide RNAs (gRNAs) were cloned into pDIRECT_22C and transformed into *Agrobacterium tumefaciens* (EHA105) (Figure 1).

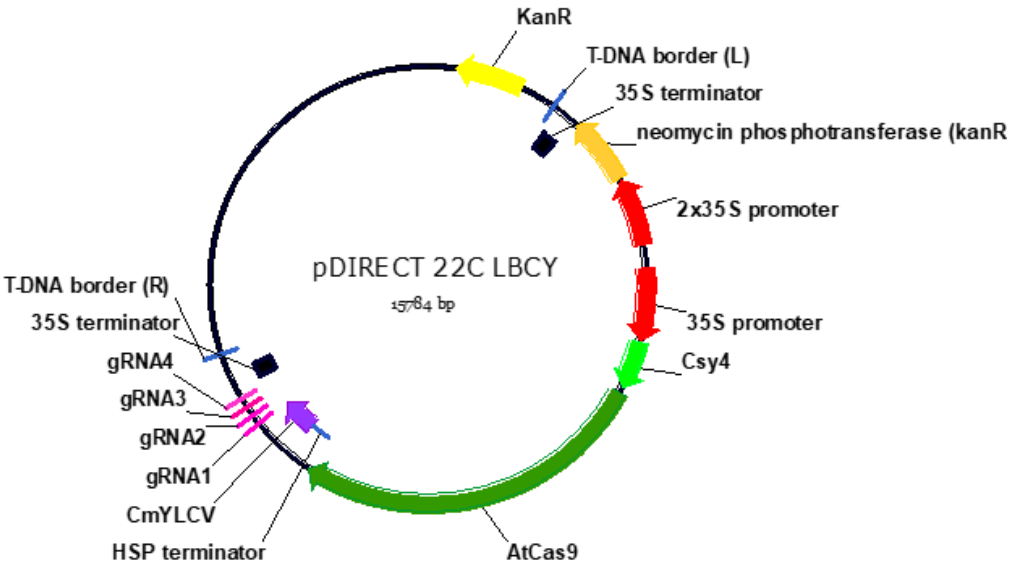

Figure 1. Schematic representation of the construct pDIRECT_22C-LBCY obtained for *Agrobacterium*-mediated transformation.

M1.2 Construct an Agrobacterium-based binary plant expression vector containing the Cas13a (C2C2) gene from *Leptotrichia wadei* (LwaCas13a) – for silencing the RNA transcript of an easily-monitored grapevine gene (*e.g. LBCY*). (COMPLETED)

AND

M1.3 Design, synthesise and clone a number of sgRNAs and crRNAs, targeting the abovementioned Vitis metabolic gene and the RNA transcript, into the respective CRISPR vectors. (COMPLETED)

Towards evaluating the ability to target mRNAs in host plants, Cas13a-based vectors were designed to two different target regions in the grapevine *LCBY* transcript. Previous designs of nuclear DNA targets (M1.1) showed that levels of sequence similarity between grapevine and *N. benthamiana* were adequate so that single constructs in theory should work in both species. This approach was therefore followed in the design and construction of the Cas13a vectors. Cas13a-LBCY Target 1 and Cas13a-LBCY Target2 plasmids have been successfully assembled and validated by Sanger sequencing (Figure 2). These plasmids were then transferred into *Agrobacterium* strain EHA105, for subsequent transformation into *N. benthamiana* and grapevine.

Recently, Mahas et al. showed that the different signals, *i.e.* NLS and NES, could localise Cas13a variants to either nuclear or cytoplasmic subcellular areas, therefore improving activity against the target transcripts (Mahas et al., 2019). In light of this report, the existing construct Cas13a-LBCY Target1 was modified to investigate if subcellular localisation of the CRISPR modules could improve RNA-targeting efficiency. Henceforth, the Gibson assembly method was used to modify the existing Cas13a-LBCY constructs by replacing the Cas13a open reading

frame with a Cas13a fragment flanked by an NLS/NES sequence on both the N- and C- termini (Figure 2).

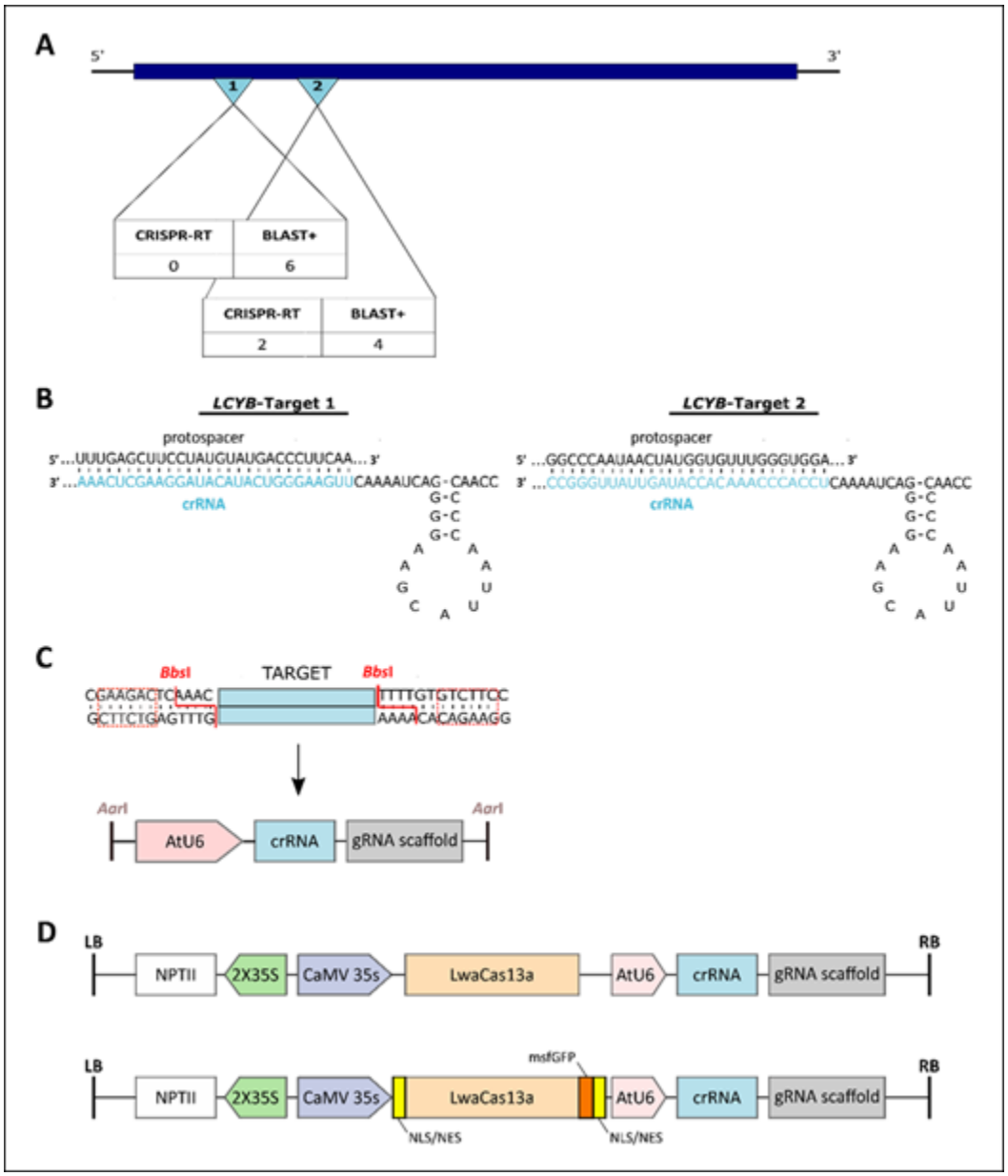

Figure 2. Target design and the CRISPR/Cas13 machinery assembled for plant transformations. (A) The *LBCY* transcript of *N. benthamiana* and *V. vinifera* with the estimated positions of the selected crRNA targets and their CRISPR-RT and BLAST+ scores across both species. (B) The selected RNA targets (28 nucleotides in length) paired with their respective crRNAs. The stem-loop sequence is obtained from Abudayyeh et al., 2016. (C) A schematic representation of the *Bbs*I cut sites adjacent to the target sequence that was followed by ligation into the intermediate vector pjjb308. (D) Schematic representation of the T-DNA region of the two assembled Cas13a/crRNA constructs with their respective components. The CaMV_35S promoter and the Arabidopsis U6-26 gene promoter (AtU6) are responsible for the expression of LwaCas13a and the crRNA, respectively. For selection purposes, it contains a neomycin phosphotransferase type II gene (NPTII) driven by a 35S promoter. NLS, nuclear localisation sequence; NES, nuclear export signal; msfGFP, monomeric superfolder GFP; LB, left border; RB, right border.

Progress towards Objective 2: Establish appropriate plant materials and routine genetic transformation protocols for Vitis cultivar(s)

M2.1 Sample immature flowers and excise anther filaments from Sultana and Chardonnay and establish callus cultures. (COMPLETED)

AND

M2.2 Select, synchronise and proliferate pro-embryogenic masses. (COMPLETED)

AND

M2.3 Induce embryo formation and test regeneration potential of the material. (COMPLETED)

Somatic embryogenesis was successfully carried out in *V. vinifera* cultivars Chardonnay, Pinotage and Hanepoot (Muscat). Figure 3 shows a compilation of representative pictures obtained from the cultivar Chardonnay, showing some of the typical steps involved in initiating SEC cultures, inducing embryogenesis and the appearance of somatic embryos in culture. Representative pictures of the embryogenic process in Pinotage and Hanepoot are shown in Figure 4.

The efficiency of embryogenesis was different between cultivars, between explants within the same cultivar, as well as the specific season in which the somatic embryogenesis initiation was conducted (2018 versus 2019) (Figure 5 and Table 1).

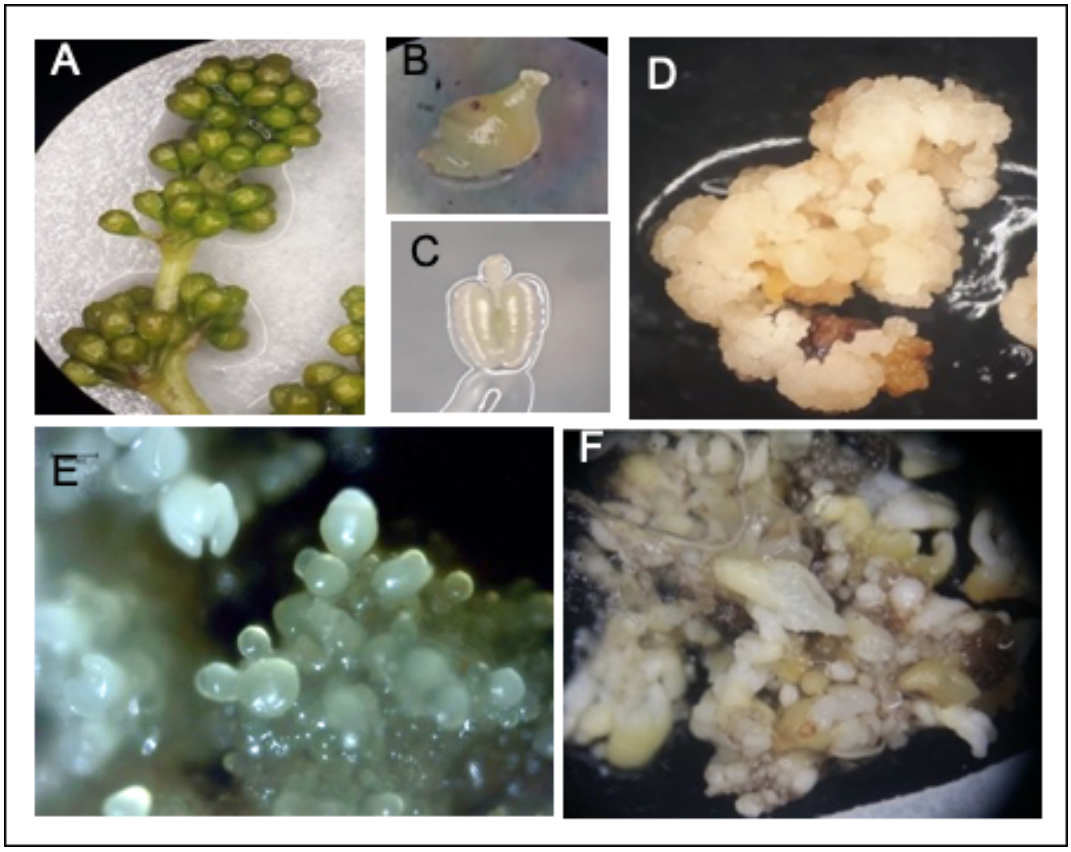

Figure 3. Somatic embryogenesis of Chardonnay initiated from immature flowers. (A) Chardonnay immature inflorescence after sterilisation. (B) A Chardonnay ovary directly after being excised from the inflorescence. (C) A Chardonnay anther directly after being excised from the inflorescence. (D) Chardonnay somatic embryogenic calli produced after roughly 5 months on callus induction medium. (E) Chardonnay somatic embryo formation after being placed on embryo induction medium for roughly 3 weeks. (F) Chardonnay somatic embryos after being placed on embryo induction medium for roughly another 2 months.

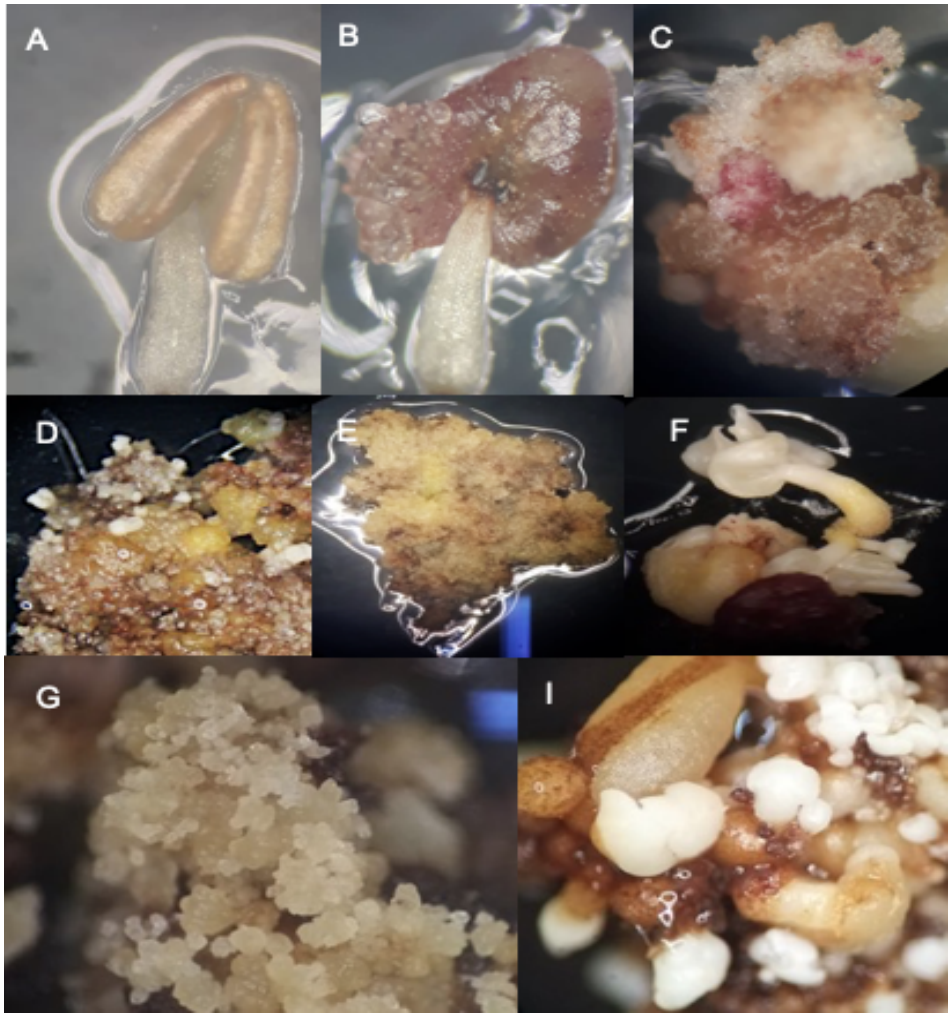

Figure 4. (A) Pinotage anthers after a week of culture on callus induction medium. (B) Callus formation initiated after three weeks after isolating the anthers. (C) Non-embryogenic calli produced on a Pinotage ovary. (D) Initiation of embryo production in Pinotage on callus induction medium. (E) Pinotage SEC calli on embryo induction medium after being pooled from explants (anther, ovary and whole flowers). (F) Full embryo production seen in Pinotage calli on callus induction medium. (G) Hanepoot Somatic embryogenic calli produced on callus induction medium (H) Embryo initiation of Hanepoot on embryo induction medium.

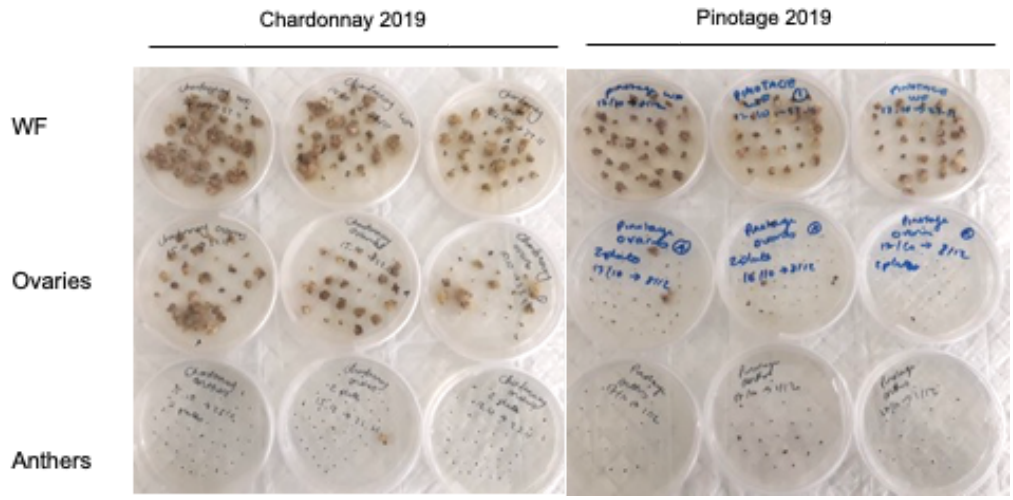

Figure 5. Callus formation from different immature inflorescence-derived explants (anthers, ovaries and whole flowers) after ± one month of culture during the 2019 season. WF=Whole flowers.

All explants from all three cultivars in both seasons showed the ability to produce callus (non-embryogenic and embryogenic) (Table 1). During the first season, whole flowers from all three cultivars showed a callus-producing success rate of over 74%, whereas in the second season, the highest callus producing Chardonnay explant was again whole flowers at a rate of 82.9%, and in Pinotage, the ovaries at a maximum of 58.4%. Despite the high success rates of the production of callus across all three cultivars, the embryogenic potential of this callus varied across cultivars. In general, Chardonnay showed the highest conversion of callus into embryogenic callus, with success rates as high as 74.1% and 66.7 % for anther-derived callus in 2018 and 2019 respectively. Considering the rate of success from explant to embryogenic calli, Chardonnay proved to be the most responsive cultivar to the induction of embryogenesis irrespective of the season.

Pinotage on the other hand, showed maximum success rates in the conversion of callus into embryogenic callus of 13.3% from whole-flower derived callus in 2018, and 0.5 % from anther-derived callus in 2019. This data suggests that although Pinotage is capable of readily producing callus, the embryogenic potential of this callus is low.

When combining the data in a genotype-specific manner (pooling the three explants per cultivar), Chardonnay showed the highest production of embryogenic calli in both seasons, with a total of 18.7 % success in 2018 and 4 % in 2019. Furthermore, Pinotage showed an overall total of 3.6 % in 2018 and 0 % in 2019, with the collective total of Hanepoot being 8.4% success in 2018. The between-cultivar discrepancies are well described for grapevine and were expected (Martinelli & Gribaudo, 2009; Nakajima *et al*., 2000; Bouamama *et al*., 2007; Vidal *et al*., 2009), with Chardonnay known to be a cultivar that readily undergoes embryogenesis (Martinelli *et al*., 2001).

Table 1 The success rate of the induction of embryogenesis in three different cultivars (Chardonnay and Pinotage and Muscat) and three different explants (anthers, ovaries and whole flower) in 2018 and 2019. Ch=Chardonnay, Pi=Pinotage, Ha=Hanepoot, A=anther, O=ovary, WF=Whole flower.

Year	Explant type		Explants plated	Callus formation (embryogenic and non-embryogenic)	Success rate of explant into callus (%)	Explants forming embryogenic callus	Success rate of callus into SEC (%)	Success rate (explant to SEC) (%)	Success rate of explant into SEC per cultivar (%)
2018	Ch	A	1998	424	21.2	314	74.1	15,7	18.7
		O	1150	375	32.6	280	74.7	24,3	
		WF	1100	821	74.6	201	24.5	18,3	
	Pi	A	3100	1333	43.0	25	1.9	0,8	3.6
		O	1675	1255	74.9	64	5.1	3,8	
		WF	1100	913	83.0	121	13.3	11,0	
	Ha	A	2600	620	23.8	87	14.0	3,3	8.4
		O	750	388	51.7	221	57.0	29,5	
		WF	1225	1081	88.2	75	6.9	6,1	
2019	Ch	A	5500	309	5.6	206	66.7	3,7	4.0
		O	1531	1072	70.0	64	6.0	4,2	
		WF	350	290	82.9	25	8.6	7,1	
	Pi	A	5375	645	12.0	3	0.5	0,1	0.0
		O	1438	840	58.4	0	0.0	0,0	
		WF	375	178	47.5	0	0.0	0,0	

Progress towards Objective 3. Transformation of *N. benthamiana* and grapevine and regeneration of plantlets.

M3.1 Transform *N. benthamiana* leaf disks and regenerate putative transgenic plantlets for *in vivo* evaluation as described in M1.4. (COMPLETED)

Leaf disks of *in vitro N. benthamiana* plants were transformed with the empty vector (negative control), as well as the pDIREC_22C-LBCY construct and putative transgenic lines were regenerated under selection. The transformations with the empty vector yielded plantlets that had a normal (comparable to untransformed) appearance (referred to as the “control” plants), whereas two types of phenotypes were observed from the plantlets that were regenerated from leaf disks exposed to the pDIREC_22C-LBCY construct. These plantlets were either visibly bleached and displaying a range of growth and morphological abnormalities (referred to as the

“bleached” population), or a mostly normal and green phenotype was observed (referred to as the “green” population). The putative plantlets were micropropagated to establish plant stocks for further analyses (reported on in Objective 4).

M3.2 Transform grapevine embryogenic callus and regenerate putative transgenic plantlets for evaluation. (COMPLETED)

The Chardonnay cultures generated and tested in South Africa were used as source material for a grapevine transformation with the empty vector and the pDIREC_22C-LBCY construct during July 2019 and the cultures were subjected to regeneration under selection.

The regenerative ability of the cultures was confirmed with the regeneration of many plantlets from the untransformed control (Figure 6). It was clear that the transformations with both the empty vector control (expressing the CAS9, but no guide RNAs), as well as pDIREC_22C-LBCY plasmid, had some difficulty in regenerating plantlets from the embryos, even though the embryos progressed through their developmental stages under selection (Figure 7).

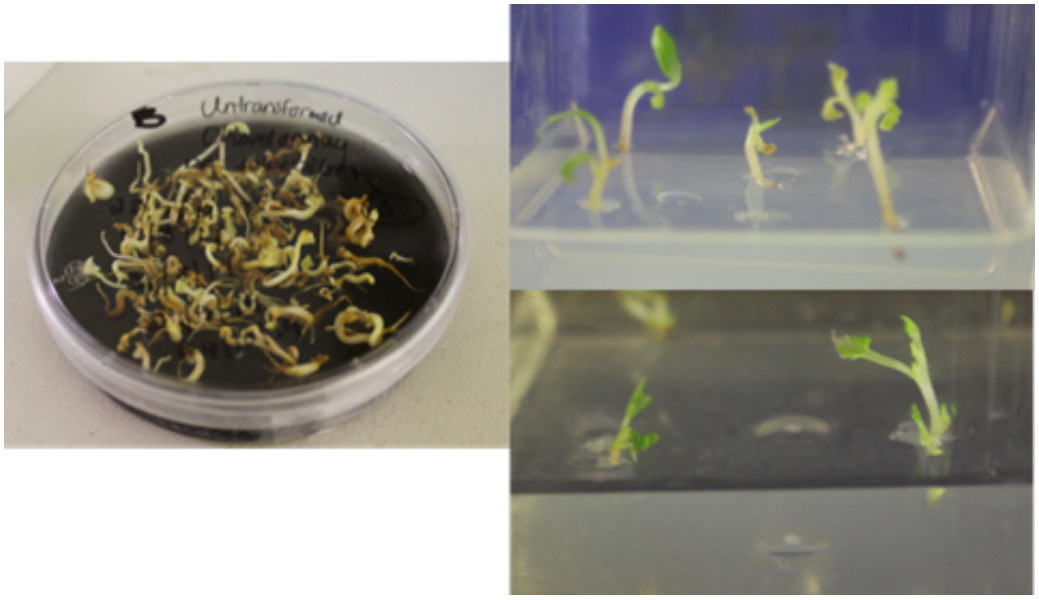

Figure 6. Regenerating embryos of the untransformed control (no selection).

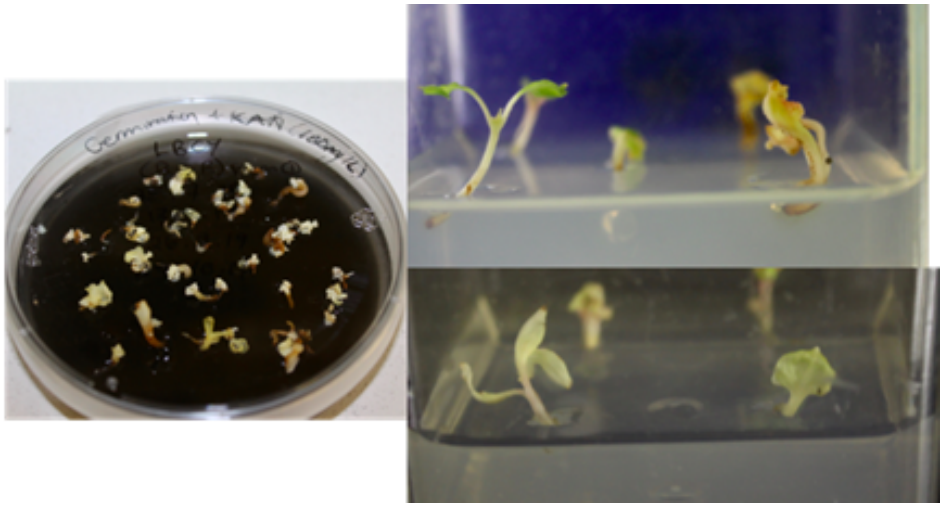

Figure 7. Regenerating embryos under selection after transformation with the pDIREC_22C-LBCY construct.

The working population for the grapevine transformations were as follows:

- 43 putatively transformed lines (maintained in triplicate) growing on Woody Plant Medium, supplemented with Kanamycin (100 mg/L) (Figure 8 as an example).

- Many (>30) untransformed Chardonnay plants as controls growing on Woody Plant Medium.

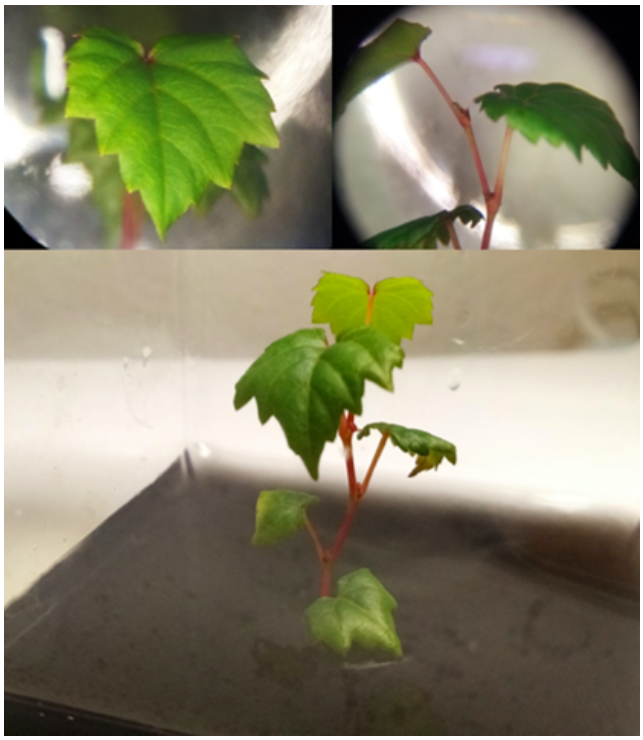

Figure 8: Leaves, Growth tip and rooted whole plant of LBCY GV 15.

M3.3 Transform Cas13a::crRNA constructs into both *N. benthamiana* leaf disks and grapevine embryogenic callus, select for transformed material and regenerate plants. (COMPLETED)

Two transformation experiments, one with Cas13a-LBCY Target 1 and one with Cas13a-LBCY Target 2, were conducted. Several regenerated *N. benthamiana* plants were obtained (Table 2), and following molecular screening, a working population of micropropagated plants transformed with Cas13a::crRNA constructs was established: five lines with empty vector (controls), four lines with Cas13a::target1 and four lines with Cas13a::target2. For each line, at least three clonal replicates were propagated.

Subsequently, because of new data in literature, another Agrobacterium-mediated transformation experiment was conducted with the LwaCas13a_T1 constructs modified for cellular localisation: Cas13/NLS-T1 and Cas13a/NES-T1. The Cas13/NES-EMPTY plasmid was also included as a negative control. A total of 12 LwaCas13/NLS-T1, nine LwaCas13a/NES-T1 and eight LwaCas13a/NES-EMPTY rooting *N. benthamiana* plants were obtained from the Agrobacterium-mediated transformation events (Table 2). Among these T0 plants, screening by PCR amplification of the Cas13a transgene revealed 11 LwaCas13a/NLS-T1, seven LwaCas13a/NES-T1 and five LwaCas13a/NES-EMPTY plants to be transgenic. Each transgenic plant line was propagated in triplicate and maintained on rooting media in a growth chamber.

Table 2 Plant count of the explants, regenerated plantlets and transgenic T0 lines from the Agrobacterium-mediated stable transformation experiments of *N. benthamiana.*

Constructs used for *A. tumefaciens* transformation	Total number of leaf discs	Total number of regenerated plantlets	Total number of confirmed transgenic plants
Cas13a-LBCY Target1	80	20	10
Cas13a-LBCY Target2	80	16	8
Cas13a_EMPTY	50	8	8
Cas13a/NLS-T1	80	12	11
Cas13a/NES-T1	60	9	7
Cas13a/NES-EMPTY	50	8	5

Across both experiments, no phenotypic differences were observed between the plants transformed with Cas13a constructs and the control plants transformed with the Cas13a harbouring no crRNA (Figure 9). Notably, a proportion of plants from the transgenic lines displayed phenotypic abnormalities such as vitrification and a lack of apical dominance during the regeneration process and post-micropropagation, when compared to wild-type plants.

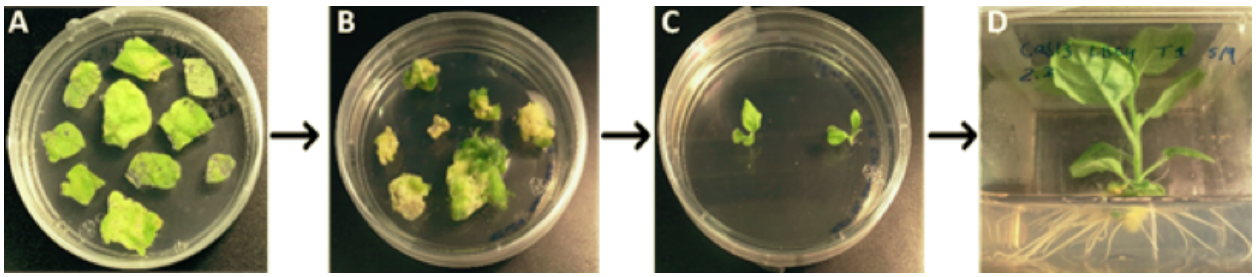

Figure 9. Regeneration process of the *N. benthamiana* leaf discs transformed with a binary vector. (A) The growth of leaf discs on co-cultivation medium after incubation with Agrobacterium (B) Calli formation stage with shoots. (C) Isolation of shoots onto root induction medium. (D) Fully regenerated putative transgenic plant.

Grapevine transformations have not been conducted with these constructs, since basic analysis of the tobacco population will provide important information regarding the efficacy of the constructs. These analyses were concluded too late to proceed with the grapevine transformation (see M4.3).

Progress towards Objective 4. Evaluation of transformants, both phenotypically and at a molecular level.

M4.1 Evaluation of *N. benthamiana* population (VvLBCY). (COMPLETED)

Several regenerated *N. benthamiana* plantlets showed a very distinct bleached phenotype at the first phases of the regeneration process. The plantlets were screened for the presence of the construct, using *Cas9*-specific primers, and a total of 84 *N. benthamiana* transformed plants were obtained. Of these, 52 plants displayed a complete bleached phenotype of leaves, stem and roots (hereafter referred to as a *white*, Figure 10 a, b, c). Interestingly the remaining 32 plants did not show any bleached phenotype (hereafter referred to as *green*, Figure 10 d, e, f). Negative control transformations (plants transformed with the empty pDIRECT22C plasmid) led to regenerated plants displaying a phenotype identical to wild-type plants grown *in vitro* (Figure 10 g, h, i).

Figure 10: Phenotype of the regenerated *N. benthamiana* plants. (a, b, c) - complete bleached phenotype of regenerated plants transformed with the pDIRECT22C-LBCY vector (W44, W45, W63 edited lines respectively). (d,e,f) - wild-type green phenotype of regenerated plants transformed with pDIRECT22C-LBCY vector (G17, G18, G21 edited lines respectively). (g, h, i) - wild-type green phenotype of the negative control plants transformed with empty vector pDIRECT22C (E3, E15, E24 control lines respectively).

Molecular characterisation of the *N. benthamiana* plant lines was performed on a subset of the transformed lines (7 *white* lines, 5 *green* lines and 4 control lines). Target sequences 1 and 2 overlap and are situated approximately 350 bases downstream of the start codon, while the overlapping target sequences 3 and 4 are located approximately 1340 bases downstream from the start codon. To analyse the mutations that potentially occurred from the editing; a region (~1 kb) encompassing all four of the targets was amplified. Sanger sequencing of clones derived from the PCR products was used to identify specific mutations in the target sequences of all the edited lines. No mutations were observed in any of the control lines (Figure 11). Interestingly, two of the five *green* lines had mutations, but importantly, not in all the clones that were sequenced. For the *green* lines with mutations, approximately half of the clones had mutations. Moreover, only a single type of mutation was observed in each mutated *green* line (a deletion of 4 nts in both lines G18 and G21), and these mutations occurred only in target area 1-2. In contrast, in the *white* lines there were several types of mutations within the same line, ranging from an insertion of a single nucleotide (+1) to short deletions (-2, -4, -5, -6, -7 and -8 nts) for both target areas. All the mutations listed above caused a frameshift and/or stop codon.

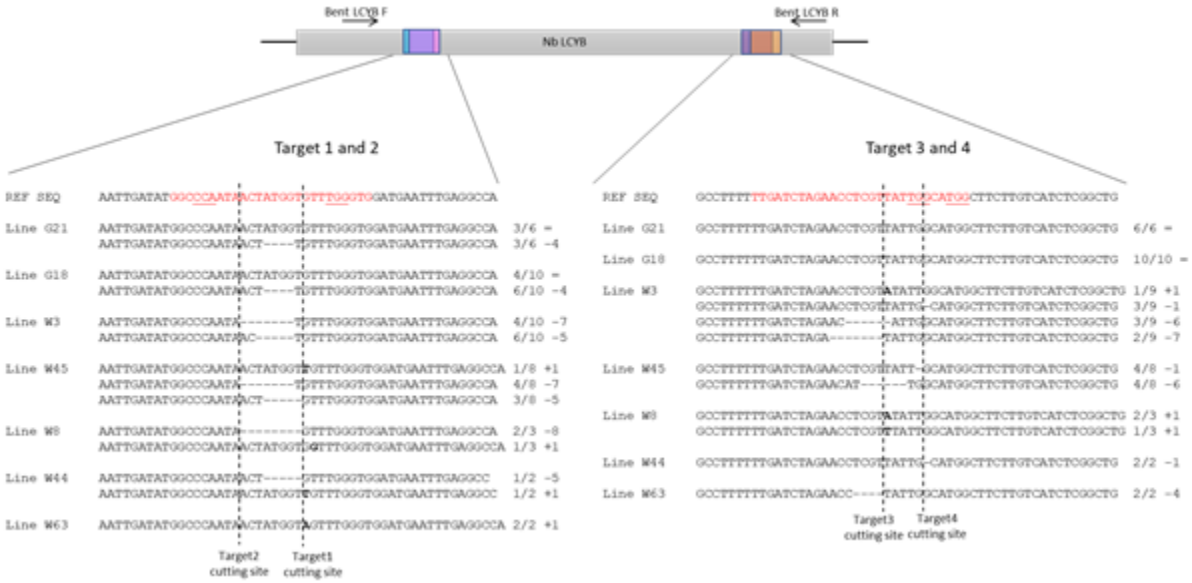

Figure 11: Mutation analysis by Sanger sequencing of LBCY-edited plants. The area containing target 1-2 and target 3-4 amplified with Bent LBCY F and Bent LBCY R aligned to wild type *LBCY* sequence. Type of mutations and the frequency are listed on the right of the sequence of the transgenic edited lines analysed.

Plant lines that displayed the leaf bleaching phenotype and that were genetically verified to be edited (as described above), were selected for further *in vitro* propagation and verification by chemical analysis. Photosynthetic pigments were extracted from the leaves of bleached and

control plants and analysed by reverse-phase UPLC. Leaf pigment analysis indicated that the LBCY-CRISPR plants had significantly reduced carotenoid and chlorophyll levels compared to the control plants. Interestingly, the edited plants are not devoid of carotenoids and chlorophylls, and have accumulated a unique carotenoid, delta-carotene. This can be attributed to the editing of the lycopene beta-cyclase encoding gene in the edited plants (LBCY-CRISPR) that consequently prevents the major leaf carotenoids, beta-carotene and lutein, being produced and accumulating the precursor, delta-carotene. The individual pigments were quantified and verified the observed phenotypes in the plant population analysed. The edited plants had significantly reduced major photosynthetic pigments (chlorophyll a, chlorophyll b, beta-carotene and lutein).

RNA expression analysis for *Cas9*, *LBCY* and the gRNAs was conducted on three edited *white* lines (W44, W45, W63), four edited *green* lines (G17, G18, G21, G37) and three control lines (E3, E15, E24). *Cas9* was expressed in all the lines except for G17 and the different levels of expression were not linked with either the bleached or green phenotype. As expected, the gRNAs (targets) were not expressed in the control lines, while the levels of expression in the *white* lines were very high, although highly variable between the lines. Lines G17 and G18 showed no gRNA expression, while interestingly, line G37 showed a high level of gRNA expression. All the lines analysed, expressed *LBCY*, but in the *white* lines the level of expression was significantly decreased when compared to both control and *green* lines (Figure 12).

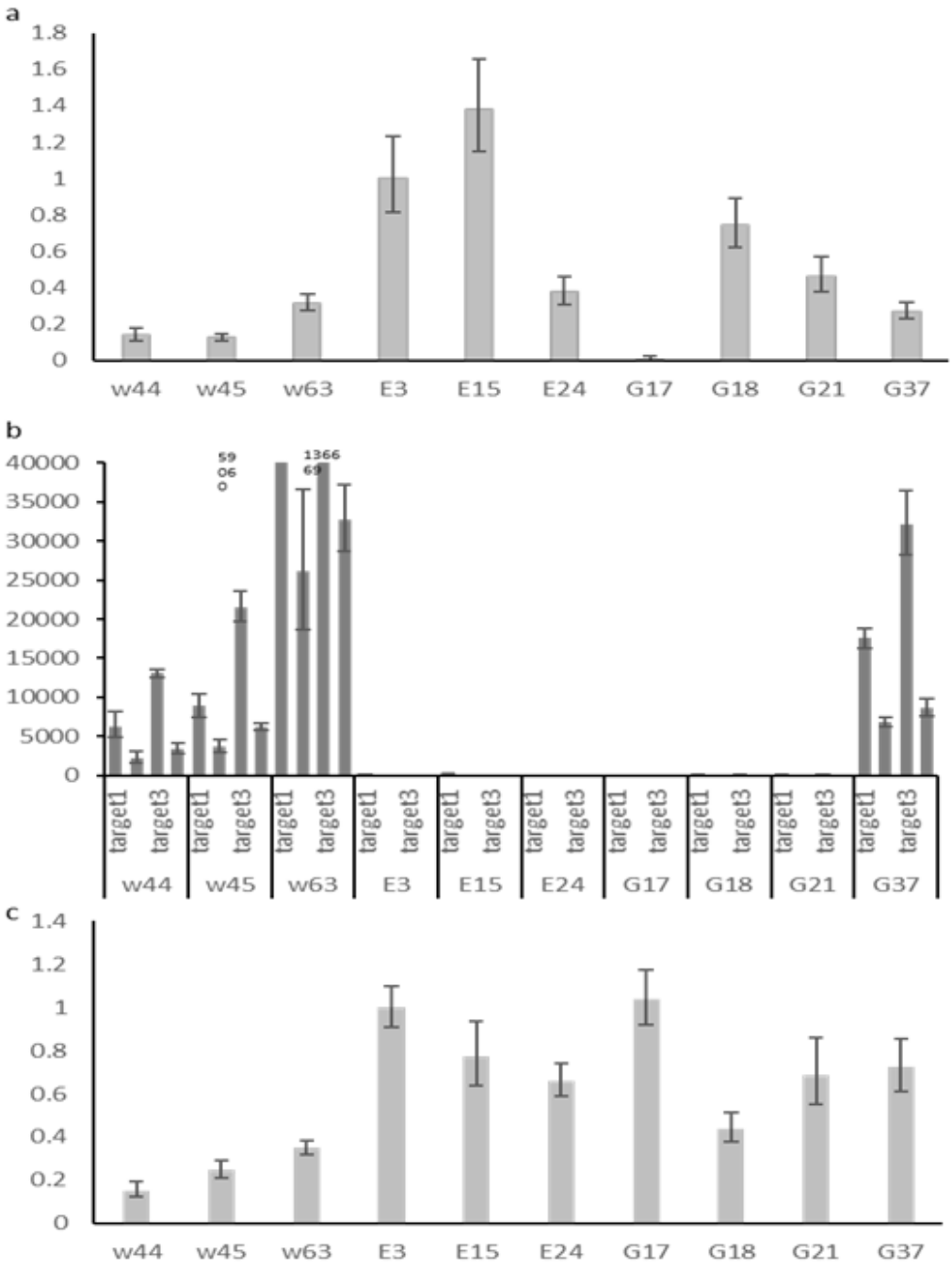

Figure 12: RNA expression analysis. Relative quantification of (a) Cas9, (b) gRNAs (targets) and (c) LBCY determined by RT-qPCR. Expression normalised to sample E3. n=3 Error bar represents 95% confidence interval.

Gene expression analysis of the plant populations revealed three distinct groupings for the transformed tobacco plants: (1) white (bleached) edited plants, (2) green transformed plants, and (3) green control plants (*i.e.* transformed with an empty vector - no sgRNAs). A similar trend was confirmed by additional carotenoid and chlorophyll analysis of plants from these three groupings, revealing distinct differences in the composition of the photosynthetic pigments in these plants (Figure 13).

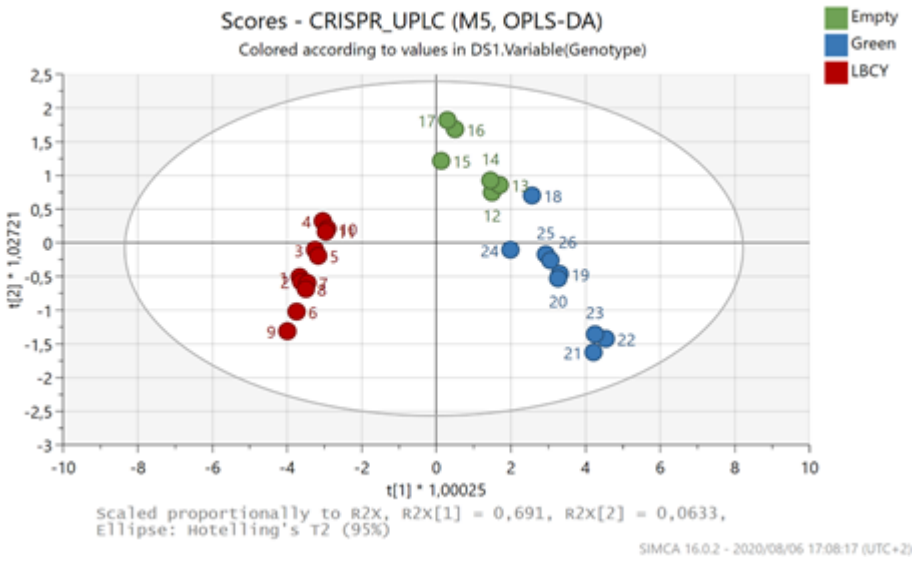

Figure 13. Leaf carotenoid and chlorophyll analysis of the *N. benthamiana* populations. Multivariate data analysis (OPLS) of the Control plants ("Empty"; empty vector control), transformed plants that are phenotypically green ("Green"), and the LBCY-edited bleached (white) phenotype ("LBCY") showing unique differences between the populations.

As expected, the bleached LBCY-edited plants accumulated delta–carotene, a carotenoid that does not occur in the phenotypically green plants (Figure 14). Interestingly, although the absolute concentrations of the photosynthetic pigments were markedly affected, the ratios of the major photosynthetic pigments (Chla:Chlb, b-carotene: Chlb and lutein:Chla)) were maintained irrespective of the genotype (Empty/Green/LBCY) (Figure 15).

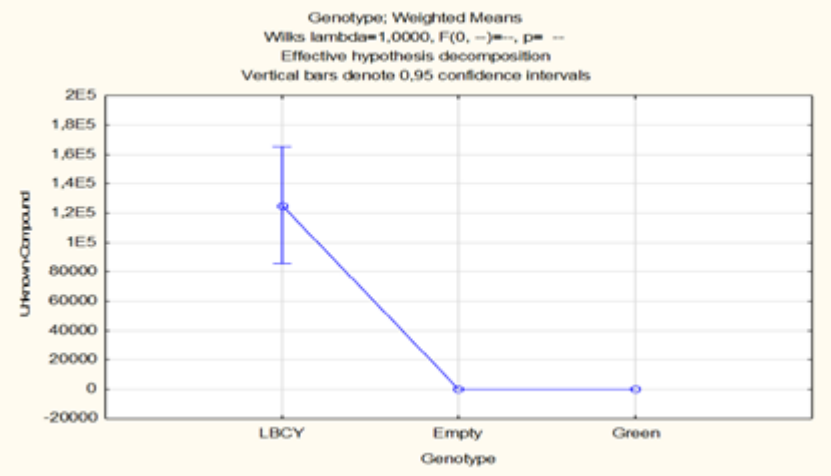

Figure 14. Accumulation of the carotenoid delta–carotene in the leaves of the white LBCY-edited *N. benthamiana* plants.

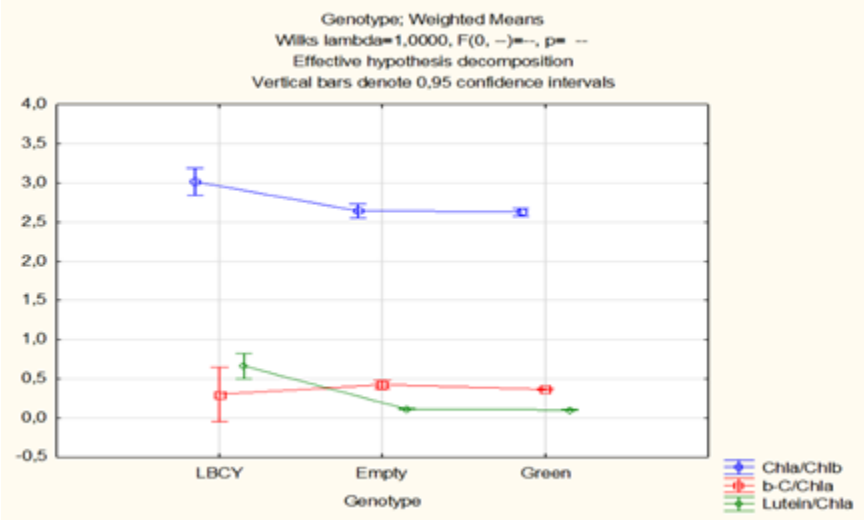

Figure 15. The ratios of the major photosynthetic pigments required for normal photosynthetic functioning (*i.e.* Chla:Chlb, beta-carotene: Chlb and lutein:Chla) were relatively constant, irrespective of the genotype in *N. benthamiana* leaves.

This is an interesting finding and it is most likely due to the frequency of the editing event(s) in the *N. benthamiana* genome. Bleached, white plants are most likely due to both genomic alleles of LBCY being successfully edited (and thereby completely disrupting LBCY functioning). Transformed plants with a green phenotype most likely have one edited (non-functional) LBCY allele, as well as an unedited functional LBCY allele. The functional LBCY allele is sufficient to maintain a relatively normal visual leaf phenotype, but the mutation in the other allele affects the individual carotenoid and chlorophyll composition.

To demonstrate our hypothesis and that the mutations are stable and heritable, *N. benthamiana* transformed plants were hardened-off to self-pollinate and produce seeds. As expected, *white* lines were not able to survive in soil and it was not possible to obtain T1 plants. Interestingly, seeds from G18 and G21 *green* lines germinated *in vitro* on selection medium, resulted in a segregation pattern, with some of the T1 seedlings displaying a bleached phenotype (Figure 16), while G37 and E15 T1 plants have the same green phenotype as the T0 plants. G17 seedlings were not able to grow on selection medium.

In order to characterise the editing of these T1 lines, high-throughput sequencing (HTS) sequencing was performed as follows: one plant from the control line (E12), three plants each from lines G37 and G17 (grown without selection, and both displaying only the green phenotype), three green and three white plants from line G18, and three green and two white plants from line G21. The data were analysed with the CRISPResso pipeline and, as expected, E12, G37 and G17 don't have any mutations in the target areas while G18 and G21 in both green and white phenotypes have mutations but only for targets 1 and 2. Noticeably the green plants in both lines had a partially edited genotype as a wild-type background was maintained except for one plant G18_3G that had only wild-type alleles. White plants have multiple mutation (heterozygous) profiles, and in total two types of mutations were identified: a deletion of four nucleotides, and a one-nucleotide insertion (Figure 17). The data are partially consistent with

the Sanger sequence of T0, indeed the four-nucleotide deletion was observed before (Figure 11) while the insertion was not detected in the Sanger sequencing. As previously shown for T0, also T1 plants have no mutations in the area of target 3 and 4. The segregation results together with the sequencing confirm that the presence of wild-type alleles results in the green phenotype, while when all the alleles are mutated, the plants are albino and not viable.

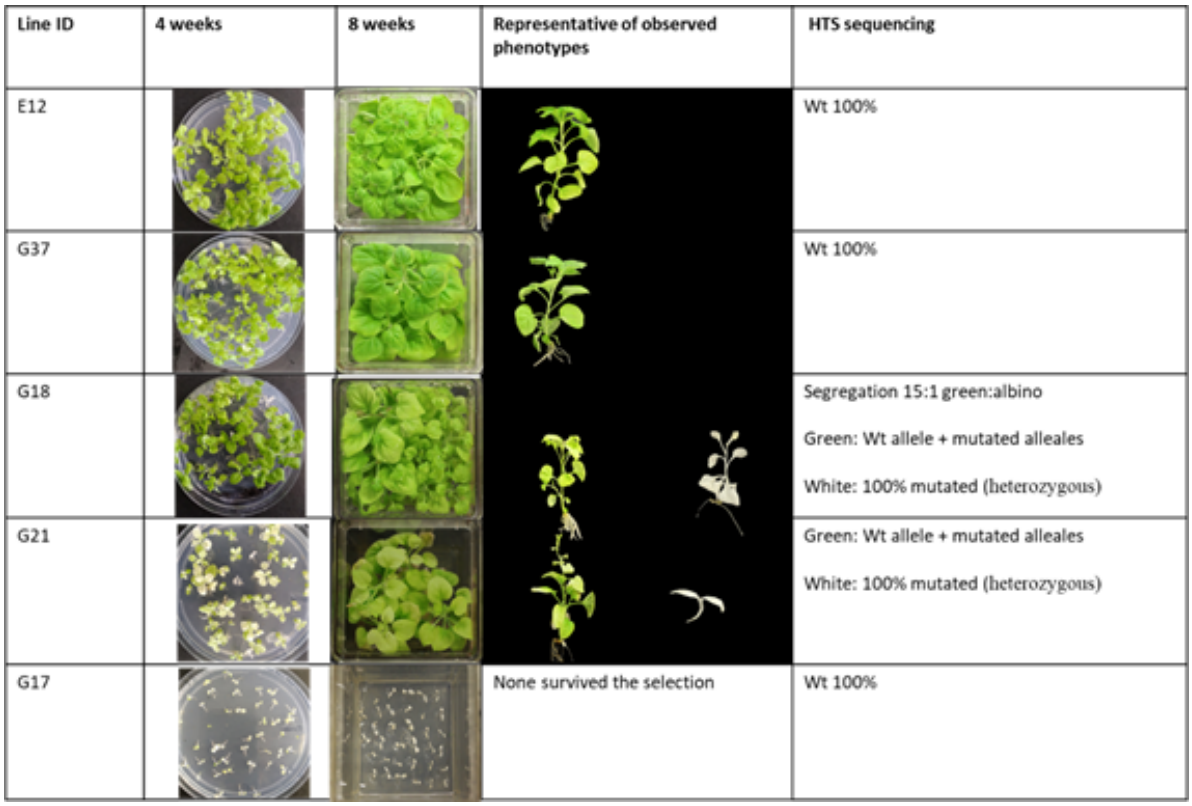

Figure 16. T1 *green* lines germinated on selective media. G18 and G21 segregate in bleached and green phenotypes. G17 seedlings do not grow. G37 and E15 are all green.

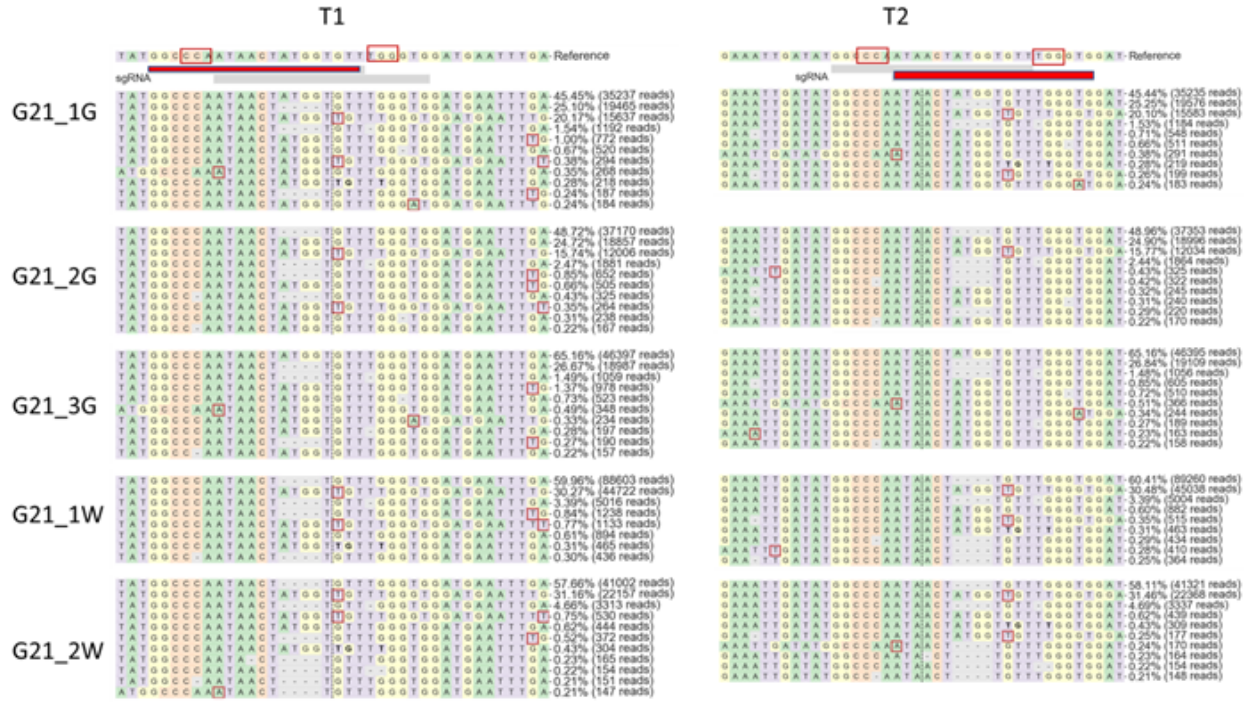

Figure 17. Example of CRISPRESSO output. The % on the right of the sequence represents the frequency of that particular sequence/allele. White plants (G21_W) have 100% of mutated alleles (either a deletion of four nucleotides or a substitution, G-to-T), while green plants (G21_G) have both wild type and mutated alleles (different % in different lines).

M4.2 Evaluation of grapevine population (VvLBCY). (COMPLETED)

Some embryos demonstrated abnormalities and/or showed recalcitrance with regards to plantlet regeneration. In order to test the possibility that the editing of the gene was affecting the regenerative capability of the embryos, DNA extraction and PCR analysis were performed on grapevine embryos. Results showed that these embryos were not transformed, nor were they edited. Leaves from five regenerated plants were collected and DNA was extracted for a pre-screen, but due to the lock-down regulations, this and further analysis of the regenerated plantlets have been delayed. This work has been re-started recently and a three-month delay with regards to the original target date (from 31/12/2020, until 31/03/2021) occurred.

DNA was extracted from 10 regenerated grapevine plants and analysed by PCR for the presence of Cas9 and NPTII genes. The PCRs were negative for all the plants for both genes. Because it is possible that editing occurs even without integration of DNA, the plants were also analysed for the editing in the *VvLBCY* gene. To this end, VvLBCY was amplified from three lines (GV9, GV11, GV13) and cloned into pGEM-T Easy. Ten colonies for each plant line were sequenced. From the sequencing results, no mutations were found (Figure 18).

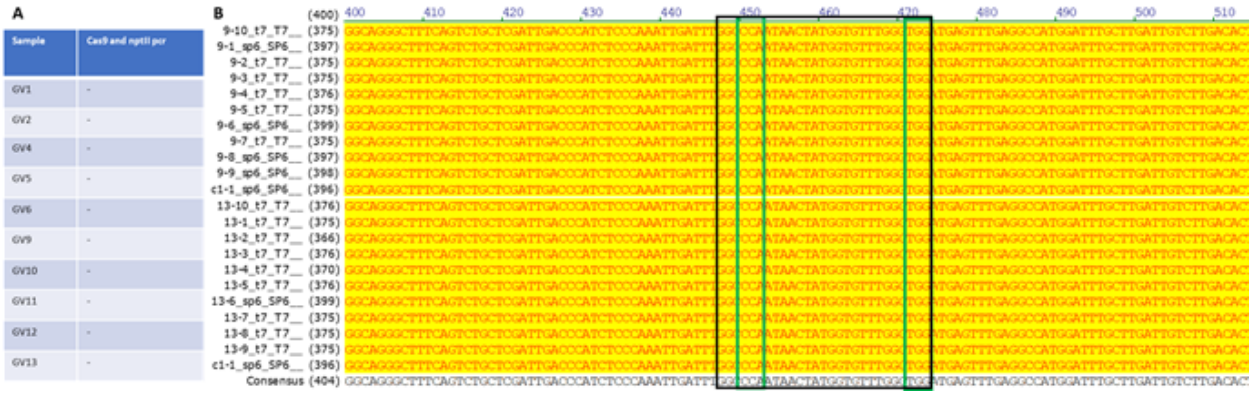

Figure 18: Molecular analysis of grapevine regenerated plants transformed with pDIRECT_22C+LCBY. (A) PCR results for Cas9 and NPTII genes of ten plants (GV1, GV2, GV4, GV5, GV6, GV9, GV10, GV11, GV12, GV13). (B) Example of editing analysis of the VvLBCY sequences of ten colonies per plant (GV9 and GV13), in the black box is the area were the sgRNA binds and in the green boxes are the PAM sequences.

Because no edited grapevine plants for LBCY were obtained, we speculated that the knock-out of this gene could be lethal in grapevines. Since COVID lockdowns and building renovations didn't allow us to perform additional grapevine transformation experiments, the same construct was tested in Italy (FEM) to edit both VvLBCY and VvPDS. No plants could be regenerated for the VvLBCY construct, but four lines were regenerated for VvPDS. The four VvPDS plants displayed a clear bleached phenotype demonstrating editing of the PDS gene, while the molecular analysis confirmed that they were transformed (Figure 19). So in both transformation experiments the editing of VvLBCY resulted in no viable plants being obtained, but it is very encouraging that our vector succeeded in the editing of VvPDS.

B

Plant line	NPTII copy number
GT125-1	1.27
GT125-2	0.49
GT125-3	0.66
GT125-4	1.31

Figure 19: VvPDS grapevine edited plants. (A) Three lines (GT125-1, GT125-2, GT125-3) displaying a clear bleached phenotype caused by the editing of the VvPDS gene (B) molecular analysis via RT-qPCR to determine the copy number of NPTII gene in the edited lines.

M4.3 Evaluation of *N. benthamiana* and grapevine populations transformed with Cas13a::crRNA constructs. (*N. benthamiana* COMPLETED, grapevine DELAYED)

Molecular analysis was conducted on the regenerated *N. benthamiana* plants to confirm the presence of Cas13 constructs. Five lines transformed with empty vector, four lines with Cas13a::target1 and four lines with Cas13a::target2 were identified. Three of the lines (9T1, 8 T2 and 14T2) displayed a stunted phenotype and some discoloration of the leaves compared to the controls (empty vector). The following subsets of transgenic plant lines were selected for gene expression analyses by RT-qPCR: lines 9, 16 and 20 transformed with the Cas13a-LBCY Target 1 construct; lines 8, 13 and 14 transformed with the Cas13a-LBCY Target 2 construct and lines E1, E2 and E3 transformed with the control plasmid Cas13a-EMPTY. Three plants per transgenic line were selected and as another negative control, three wild-type *N. benthamiana* samples were included.

Thereafter, LBCY gene expression was quantified by RT-qPCR and normalised with the housekeeping gene APR. In Figure 20A, the expression of LBCY is observed in all the transgenic lines and control plants. The control samples E1, E2 and E3 present highly variable levels of expression, with E1 being the only sample expressing a similar level of LBCY expression to the wild-type sample. This variability is also true for the samples within each transgenic line and across both the LwaCas13a_T1 and LwaCas13a_T2 transgenic lines. Notably, independent plants from line 9 and line 8 appear to indicate a reduced level of LBCY expression (approximately 2-fold lower) compared to the reference sample E1 and the wild-type sample. However, once all plants for each line were biologically grouped, no significant difference in LBCY expression was observed for any of the transgenic lines when compared to the control group (Figure 20B).

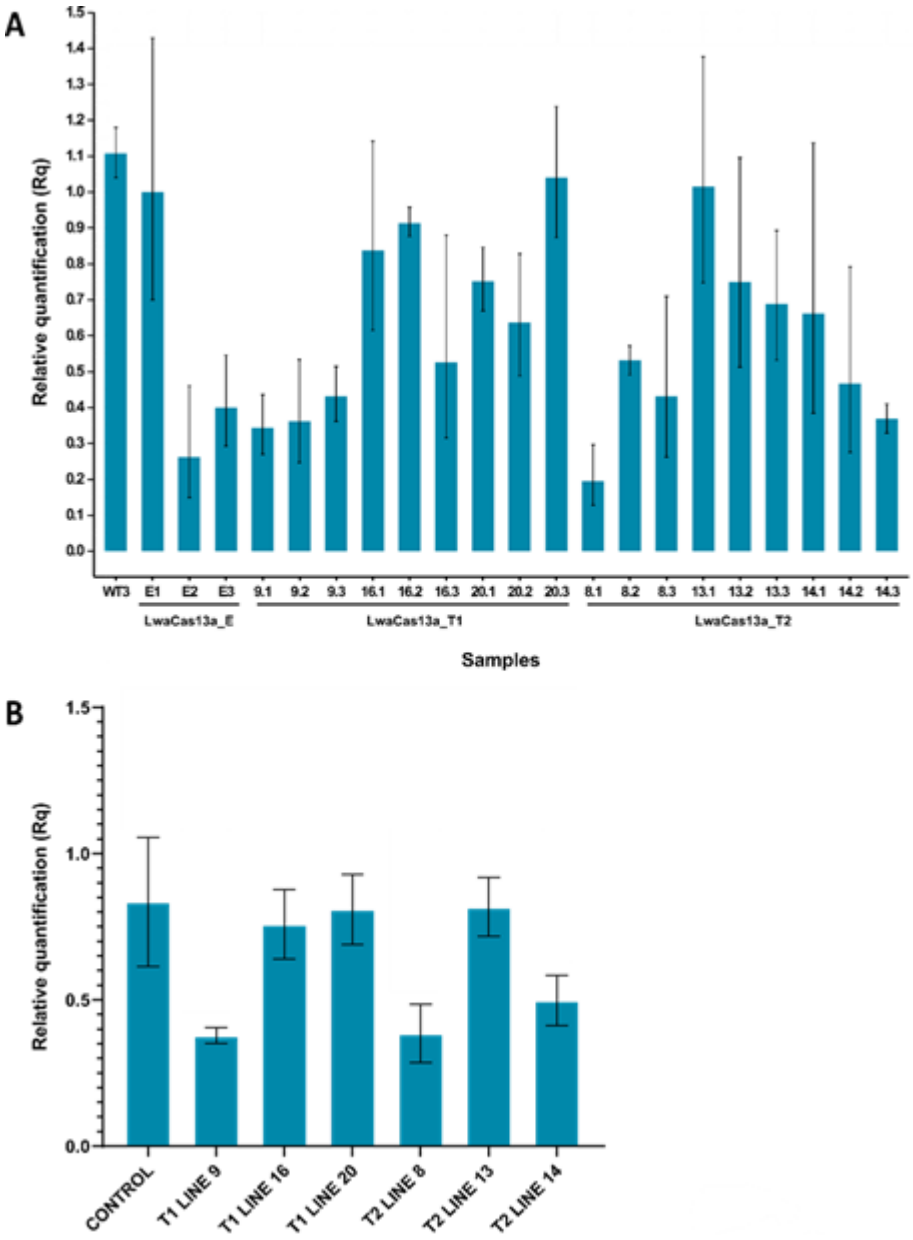

Figure 20. RT-qPCR analysis of LBCY gene expression in the selected transgenic lines. (A) Relative fold-expression is normalised to reference sample E1 and the error bars represent 95% confidence intervals of n=3 technical replicates. The samples are grouped according to the constructs they were transformed with: control plasmid Cas13a_EMPTY (LwaCas13a_E), Cas13a-LBCY Target 1 (Cas13a_T1) and Cas13a-LBCY Target 2 (LwaCas13a_T2). (B) Data is shown as mean fold-expression ± SEM of the transgenic lines (n=3 biological repeats per line). Statistical analysis was performed using the Student's unpaired t-test and significant difference was determined at $p \leq 0.05$ with respect to the control group.

Ten transgenic lines that were transformed with the Cas13a/NLS-T1 construct (NLS1-NLS10), seven transgenic lines that were transformed with the Cas13a/NES-T1 construct (NES1-NES7) and five transgenic control lines transformed with Cas13a/NES-EMPTY (E1-E5) were selected for quantitative analyses. As a preliminary assessment, one plant per transgenic line was selected. To determine the transcriptional regulation of the gene encoding LBCY, a RT-qPCR

analysis was performed on the selected transgenic lines and normalised with the endogenous control gene APR. All of the selected samples expressed LBCY, with variation in the transcript's abundance seen across both the NLS and NES lines. In particular, the results show a potential reduction in LBCY expression, equal to or more than a ~1.7-fold reduction, for independent plants NLS1, NLS4 and NES1, when compared to their respective reference control samples.

Based on these preliminary results, two more plants from each of these three transgenic plant lines were selected for the same RT-qPCR analysis to statistically elucidate if this reduction in expression is consistent across each transgenic line. Three transgenic control plants, which were transformed with the Cas13a_NES EMPTY construct, were included to form a control group. The results suggest that the transcript encoding LBCY is significantly downregulated in line NES1 as the relative expression is ~>2 fold lower when compared to the control group (Figure 21A). No significant changes in LBCY expression were observed for the two NLS lines 1 and 4.

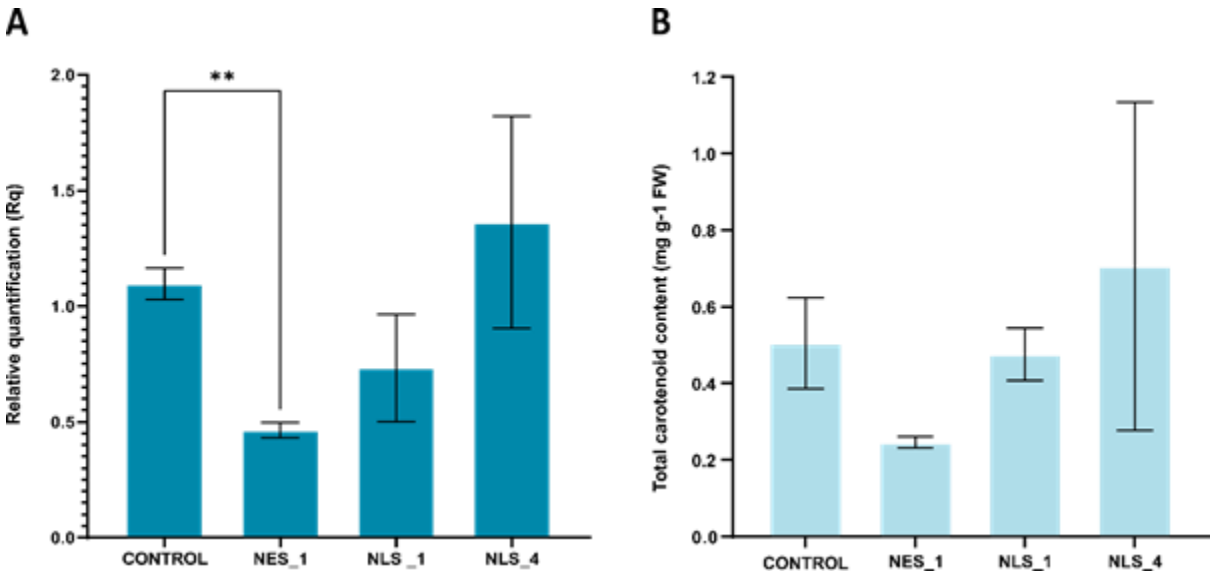

Figure 21. Assessment of CRISPR/Cas13a-mediated targeting of the LBCY transcript in selected transgenic lines. (A) RT-qPCR analysis of LBCY gene expression in the transgenic lines. The mean fold expression of the Cas13a_NLS and Cas13_NES transgenics lines (n=3 biological repeats per line) is compared to that of the control group. Statistical analysis was performed using the Student's unpaired t-test ($p < 0.05$) and the error bars represent ±SEM. ** = $p \leq 0.01$ (B) Total carotenoid content in selected transgenic lines. Data is representative of mean Cx+c values (n=2) and the error bars represent ±SEM.

In order to physiologically characterise these transgenic lines, the main photosynthetic pigments were evaluated using measurements from UV/vis absorption spectra as a preliminary quantification. Shown in Figure 21B, the mean carotenoid content levels are representative of two transgenic plants per line. The levels of carotenoid content in the transgenic lines appear to correlate with the same trend in results obtained from the RT-qPCR analysis of NES line 1, as it

is evident that a lower level of total carotenoid content is recorded for NES line 1, while lines NLS 1 and 6 do not show a decrease in total carotenoid content when compared to the control group.

8. CONCLUSIONS AND RECOMMENDATIONS

Transformation experiments with a Cas9-based vector in *N. benthamiana* and comprehensive phenotypic, genetic and biochemical analysis confirmed the successful editing of these plants. Similar experiments with grapevine yielded regenerated Chardonnay plants, but genetic analysis on DNA from these plants showed failure of the transformation and thus also no mutations were introduced. Successful editing was obtained in a repeat experiment targeting a different gene that was conducted in Italy. Cas13a-based editing in *N. benthamiana* showed a reduction in expression of the target gene, but results were inconsistent and should be repeated with larger sample numbers.

In this project, we have demonstrated an ability to design and assemble constructs for the editing of *N. benthamiana* and grapevine, and in a very short time re-established a grapevine transformation and regeneration capability. This opens up possibilities for editing of grapevine to address biotic and abiotic stress issues of economic importance, such as virus resistance and drought. It is recommended that this technology that has been established now, be further explored to address economically important issues in grapevine production in South Africa.

9. PLANNED OUTPUTS

a) TECHNOLOGY DEVELOPMENT, PRODUCTS AND PATENTS

The project established a capability to use genome editing technology to improve grapevine, using CRISPR/Cas technology. It also re-established the ability to genetically modify grapevines.

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

c) HUMAN RESOURCES DEVELOPMENT / TRAINING (STUDENTS)

Student Name and Surname	Student Nationality	Degree (eg Hons, MSc)	Level of studies in final year of project	Total Bursary Cost for Industry for entire project
Honours				
Ms Shannon Derman	SA	Hons (Wine Biotechnology)	BSc (Hons)	R 50000
Ms Gaelle Robertson	SA	BSc(Hons) Genetics	BSc (Hons)	R 50000
Masters				
Ms Gaelle Robertson	SA	MSc (Genetics)	MSc	R 160000
Ms Katarina Spencer	SA	MSc (Genetics)	MSc	R 160000
Ms Shannon Derman	SA	MSc (Wine Biotech)	MSc	R 0
PhD				
Postdocs				
Dr Manuela Campa	Italy		final	R 520000

d) LIKELY PUBLICATIONS (POPULAR, PRESS RELEASES, SCIENTIFIC)

Publication in a relevant high-ranking scientific journal, e.g. PNAS, is underway.

e) PRESENTATIONS/PAPERS THAT COULD BE DELIVERED

Oral/Poster presentations planned for the ISGPB2021 conference in Oct 21 in South Africa.

10. PROJECT OUTCOME AND IMPACT

New Knowledge	Benefits Supply Chain	Direct Grower Application	Direct Packhouse/Winery/ Cellar Application	Other
X		X		

Other is:

The Value of the project to industry

In this project, we have demonstrated an ability to design and assemble constructs for the editing of *N. benthamiana* and grapevine, and in a very short time re-established a grapevine transformation and regeneration capability. This opens up possibilities for editing of grapevines to address biotic and abiotic stress issues of economic importance, such as virus resistance and drought.

11. PERSONS PARTICIPATING IN THE PROJECT:

INITIALS AND SURNAME	HIGHEST QUALIFICATION	RACE (M,W)	GENDER (M,F)	INSTITUTE DEPARTM	POSITION	TOTAL COST TO PROJECT
RESEARCH PERSONNEL						R 450000
PR Young	PhD	W	M	SAGWRI	Researcher	R 450000
SUPPORT PERSONNEL						R 150000
Michelle Korkie	Matric	B	F	SAGWRI	Tech Assistant	R 150000

POSITION: Co = Co-worker (other researcher at your institution)
Coll = Collaborator (participating researcher that does not receive funding for this project from industry)
PF = Post-doctoral fellow
PL = Project leader
RA = Research assistant
TA = Technical assistant/ technician

12. TOTAL COST OF PROJECT

TOTAL ANNUAL COSTS (ALL YEARS)	CFPA	RAISINSA	HORTGRO	SATI	WINETECH	ARC	OTHER	TOTAL
2016	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0
2017	R 0	R 0	R 0	R 0	R 850000	R 0	R 0	R 850000
2018	R 0	R 0	R 0	R 0	R 457500	R 0	R 0	R 457500
2019	R 0	R 0	R 0	R 0	R 1298250	R 0	R 0	R 1298250
2020	R 0	R 0	R 0	R 0	R 1395766	R 0	R 0	R1395766
2021	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0
TOTAL	R 0	R 0	R 0	R 0	R4001516	R 0	R 0	R 4001516