AJEV | American Journal of Enology and Viticulture

Research Report
DOI: 10.5344/ajev.2024.24012

Efficient Genetic Transformation of *Vitis vinifera* L. Mencía using a Hypervirulent Strain of *Agrobacterium tumefaciens* and qPCR Determination of Transgene Copy Number

Óscar Martínez [1,4], Elena Palomo-Ríos [2], Manuel Rey [1], and María Victoria González [3*]

Abstract

Background and goals

Agrobacterium-mediated transformation efficiency is generally low in grapevine and varies significantly among cultivars, making it necessary to optimize the protocol for each grapevine cultivar.

Methods and key findings

Different strategies to select potentially transformed material were tested by combining different kanamycin concentrations at different time points in solid medium supplemented with 250 mg/L timentin. The additional selection step of 50 mg/L kanamycin in liquid medium allowed recovery of completely transformed plant material with up to 19.8% transformation efficiency.

qPCR copy number estimates of the transgenes *nptII* and *uidA* using the ΔCq method were inconsistent and depended on the endogenous single-copy control gene (*NCED2* or *chi*) used to perform the calculations. Modifying the analysis by including the geometric average of the two control genes resulted in equivalent estimated copy numbers in all but two lines. The highest qPCR expression level of the *uidA* gene was observed in the line with the most integrated transgene copies, although there was no correlation between these two measures in lines with a high transgene copy number.

Conclusions and significance

An efficient transformation protocol for the grapevine cultivar Mencía was established for the first time, using somatic embryo aggregates cocultured with the hypervirulent *Agrobacterium tumefaciens* strain AGL1 harboring the binary vector pBINUbiGUSInt. A robust qPCR analysis of transgene copy number allowed early and easy selection of the most interesting transgenic lines using very little plant material.

Key words: AGL1 *Agrobacterium tumefaciens*, grapevine, Mencía, qPCR, somatic embryogenesis, transgene copy number

Introduction

Galicia is an important wine-producing region in northwestern Spain that includes five Denominations of Origin. Wine production is based on the quality and typicity of cultivars such as Mencía or Albariño. This industry is dealing with unforeseen problems due to the effects of climate change on water availability and the expansion of pests and diseases. Hence, it seems appropriate to develop and provide the wine industry with grapevine cultivars adapted to local climate conditions to maintain production and quality standards. The development of biotechnology tools speeds production of better-adapted plants that will maintain agronomic standards required by the wine industry.

Massive sequencing has led to great advances in knowledge of the grapevine (*Vitis vinifera* L.) genome (Nuzzo et al. 2022). Grapevine characteristics such as a long life cycle make improvements through traditional breeding difficult; therefore, genetic transformation is the most useful technology to create improved varieties and to study functional genomics (Saporta et al. 2016). Grapevine is considered a recalcitrant species in the application of this technology due to the difficulty of obtaining in vitro regeneration systems compatible with the transformation process (Campos et al. 2021, Butiuc-Keul and Coste 2023), resulting in low transformation efficiency

[1]Departamento de Biología Vegetal y Ciencia del Suelo, Campus Universitario, Universidade de Vigo, 36310 Vigo, Spain; [2]Departamento de Botánica y Fisiología Vegetal, Instituto de Hortofruticultura Subtropical y Mediterránea La Mayora (IHSM-UMA-CSIC), Universidad de Málaga, 29010 Málaga, Spain; [3]Departamento de Biología Funcional, Universidad de Santiago de Compostela, Campus Sur, 15872 Santiago de Compostela, Spain; and [4]present address, Departamento de Biología Funcional, Universidad de Santiago de Compostela, Campus Sur, 15872 Santiago de Compostela, Spain.

*Corresponding author (mvictoria.gonzalez@usc.es)

Manuscript submitted March 2024, accepted July 2024, published Sept 2024

This is an open access article distributed under the CC BY 4.0 license.

By downloading and/or receiving this article, you agree to the Disclaimer of Warranties and Liability. If you do not agree to the Disclaimers, do not download and/or accept this article.

with significant variation among cultivars (Torregrosa et al. 2002, Bouquet et al. 2008, Li et al. 2008). Because of low transformation efficiency, most *Agrobacterium*-mediated transformation has used a few grapevine cultivars like Chardonnay and Thompson Seedless (reviewed in Saporta et al. [2016] and Campos et al. [2021]). These features make it necessary to optimize genetic transformation for each particular cultivar.

An essential factor for successful transformation is using a suitable combination of *Agrobacterium* strain and transformation vector (Gelvin 2003). The first *Agrobacterium* transformation protocols developed for grapevine were made using strain LBA4404 (Bouquet et al. 2008). Due to the low transformation efficiencies obtained, more virulent *Agrobacterium* strains were tested and EHA105 became the most widely used strain for grapevine transformation (Wang et al. 2005, Li et al. 2008, Kandel et al. 2016). The search for better transformation efficiencies has led to the use of hypervirulent strains such as AGL1, used with good results in other woody species recalcitrant to this technology (Ramesh et al. 2006, Álvarez et al. 2009, Torreblanca et al. 2010, Palomo-Ríos et al. 2012). Strain AGL1 has also been used in grapevine (Torregrosa et al. 2002, Urso et al. 2013) and although these authors have not provided a detailed analysis of its influence on transformation efficiency, their data suggest that strain AGL1 may be a viable alternative to improve grapevine transformation.

In addition to stable insertion of the transgene into the host plant, an efficient transformation protocol requires that regeneration occur only from transformed cells (Wang et al. 2005). With this objective, somatic embryogenesis is a very useful tool in grapevine due to the unicellular origin of somatic embryos (Jayasankar et al. 2003), which reduces the possibility of obtaining chimeric plants. Transformation and selection of transformed cells affect the regeneration potential of the tissue, which ultimately leads to a decrease in plantlet conversion rates (Iocco et al. 2001). For this reason, optimizing selection of potentially transformed cells is another critical point to improve transformation efficiency (Wang et al. 2005). Our group has established very efficient somatic embryogenesis protocols for several interesting grapevine cultivars from Galicia in northwestern Spain (Prado et al. 2010, Acanda et al. 2013). These cultivars include Mencía, the most important autochthonous cultivar for red wine production in Galicia, which makes it an interesting candidate for genetic improvement by *Agrobacterium*.

Transgene expression in the transformed tissue depends on multiple factors such as the number of integrated copies, their orientation, and the genomic context of the insertion (Iglesias et al. 1997). Detecting transgene copy number early in the transformation process saves time and plant material (Chu et al. 2013). Southern blots, often used for this purpose, are costly, laborious, and require large quantities of plant material (Mallón et al. 2013, Biricolti et al. 2016), which is incompatible with early analysis of transgene copy number (Song et al. 2002). Moreover, Southern blots are not always conclusive (Biricolti et al. 2016).

Quantitative PCR (qPCR) has been used successfully to estimate the number of integrated gene copies in different species. Most analyses have been based on TaqMan probes with various mathematical approaches (Wen et al. 2012, Dalla Costa et al. 2022). In several of these cases, the analyses required a previously available homozygous transformed line with a single copy of the transgene and an amplification efficiency similar to that of the test samples to be quantified (Bubner and Baldwin 2004), a requirement that is not always met. A simpler and more reliable method estimates the number of integrated copies using SYBR Green and an endogenous gene with known copy number as a reference (Yuan et al. 2007, Zhang et al. 2015, Biricolti et al. 2016).

In this work, the first efficient transformation protocol for the grapevine cultivar Mencía was established using somatic embryo aggregates cocultured with the hypervirulent *Agrobacterium tumefaciens* strain AGL1, followed by different selection strategies to recover transformed plant material. In addition, early analysis of the number of integrated copies of the neomycin phosphotransferase (*nptII*) and β-glucuronidase (*uid*A) transgenes by qPCR was optimized using two single-copy endogenous genes as controls. CRISPR/Cas9 technology is becoming the primary tool for gene editing in plants, as it efficiently generates targeted mutations. Genome editing protocols using *Agrobacterium*-mediated transformation are already available for Chardonnay (Ren et al. 2016, Osakabe et al. 2018); this work extends the CRISPR/Cas9 technology to other important grapevine cultivars such as Mencía.

Materials and Methods

Plant material and somatic embryogenesis

Adult field-grown vines of *V. vinifera* L. Mencía were selected from the grapevine collection at the Centro de Formación y Experimentación de Viticultura y Enología de Ribadumia (Galicia, northwestern Spain). Inflorescences at stage H of the Baggiolini phenological scale (Baggiolini 1952), corresponding to separated clusters, were collected. Flowers were at developmental stage R3 (corresponding to a late binucleate microspore stage, Prado et al. 2010), as determined by squashing the anthers in the presence of 4′,6-diamidino-2-phenylindole and viewing the microspores using an MZ10F fluorescence stereomicroscope (Leica Microsystems GmbH). Flower clusters were collected and washed twice with distilled water containing 20 µL detergent (Mistol, Henkel Ibérica, S.A.) per 200 mL water for 5 min and chilled at 4°C for 4 to 6 days. Clusters were then sterilized as described (Kikkert et al. 2005), prior to dissection of the immature stamens (anther plus filament) to be used as explants for somatic embryogenesis induction. Embryogenic cultures were induced (Acanda et al. 2020) on a medium containing Nitsch and Nitsch (1969) salts supplemented with 0.1 µM cobalt(II) chloride, Murashige and Skoog (1962) vitamins, 0.1% casein hydrolysate, 6% sucrose, 1 µM 2,4-dichlorophenoxyacetic acid (Duchefa Biochemie), and 4.5 µM thidiazuron (Duchefa). pH was adjusted to 5.8

prior to autoclaving at 98 kPa and 121°C, and the media were solidified using 0.3% Gelrite (Duchefa). Cultures were maintained at 24 ± 1°C in continuous darkness and subcultured onto fresh medium every 30 days.

Transformation vector and *A. tumefaciens* strain

The transformation experiments used the binary vector pBINUbiGUSInt (Humara et al. 1999). This plasmid contains genes encoding the enzymes *nptII* and *uid*A, under the control of the nopaline synthase and maize polyubiquitin promoters, respectively. In addition, *uid*A contains intron PIV2 of the ST-L1 gene of *Solanum tuberosum* L. in its coding sequence, which prevents its expression in *Agrobacterium* (Vancanneyt et al. 1990).

A. *tumefaciens* strain AGL1 containing the plasmid pBINUbiGUSInt was used as the bacterial vector and was supplied for this work by Prof. Ricardo Ordás (University of Oviedo, Spain). Bacterial suspensions were cultured in LB medium (Bertani 1951) supplemented with 10 mg/L rifampicin and 50 mg/L kanamycin for 24 hr at 28°C in a roller mixer. Prior to infection of the somatic embryo aggregates for the transformation experiments, bacterial suspensions were centrifuged at 4000 × g, then the precipitate was washed with 10 mM magnesium sulfate and resuspended in liquid embryogenesis induction medium until reaching an optical density (600 nm) of 0.5.

Effect of kanamycin on the growth of grapevine somatic embryo aggregates

To establish the minimum dose of kanamycin necessary to inhibit growth of non-transformed somatic embryo aggregates, a dose-response curve was made against this antibiotic. Five petri dishes (biological replicates), each containing 0.5 g of somatic embryo aggregates, were cultured on induction medium supplemented with 0, 50, 100, 150, or 200 mg/L kanamycin, respectively. Aggregates were transferred to fresh media every 4 wk and their fresh weight was recorded at 4 and 8 wk of culture. Then, somatic embryo aggregates from the different kanamycin treatments were grown on induction medium without the antibiotic for eight additional weeks to determine whether the kanamycin produced permanent effects on their embryogenic capacity.

Transformation and selection strategies

Due to the small size of the proembryos developed in induction medium and their friable nature, somatic embryo aggregates of ~16 mm^2 were inoculated into the suspension of *Agrobacterium* cells in liquid induction medium and incubated at room temperature for 20 min on a roller mixer. Then, somatic embryo aggregates were collected, briefly dried on sterile filter paper, placed in 90-mm diameter petri dishes containing 25 mL solid induction medium (eight aggregates in each), and cocultured at 24 ± 1°C in the dark for 48 hr. Control somatic embryo aggregates were treated in the same way, but were incubated under agitation in liquid induction medium without *Agrobacterium*. After coculture, aggregates were washed with liquid induction medium supplemented with 50 mg/L kanamycin and 300 mg/L timentin for 2 hr. After washing, they were briefly dried on sterile filter paper and transferred to selection medium in 90-mm diameter petri dishes. Selection was carried out on induction medium supplemented with 250 mg/L timentin.

Four different selection strategies were tested (Figure 1): a standard selection strategy, where aggregates were cultured in induction medium supplemented with 50 mg/L kanamycin for 5 wk (strategy 1); an incremental selection strategy with 25 mg/L kanamycin for 4 wk followed by 50 mg/L for 1 wk (strategy 2); a progressively increasing kanamycin concentration strategy (6, 12, 25, 50, and 50 mg/L) for 1 wk each (strategy 3); and no initial selection for 2 wk followed by 50 mg/L for 3 wk (strategy 4). After 5 wk, somatic embryo aggregates were maintained in the presence of 50 mg/L kanamycin in all strategies. Somatic embryo aggregates were incubated at 24 ± 1°C in the dark and transferred weekly to fresh medium which corresponded to each selection strategy during the first 4 wk, to medium with 50 mg/L kanamycin biweekly the following 4 wk, and monthly from the eighth week of culture. Six replicates consisting of eight somatic embryo aggregates were made for each strategy.

Transformation efficiency was defined as the percentage of aggregates that showed growth due to secondary embryogenesis. Each aggregate that showed growth after selection was isolated and treated as a potentially transformed independent line. Once independent lines were established, they were transferred to induction medium supplemented with 100 mg/L kanamycin, where they were cultured for at least 2 mo. Embryogenic material was collected from each independent line to perform β-glucuronidase (GUS) histochemical assays.

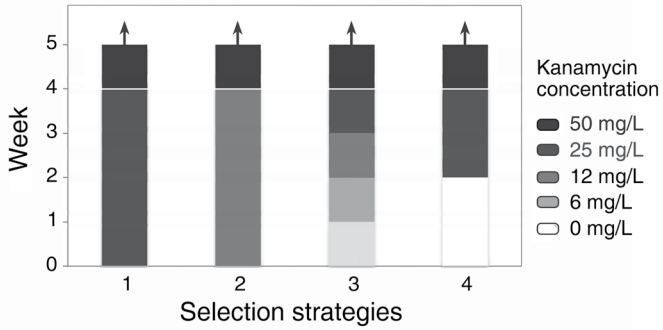

Figure 1 Kanamycin selection strategies used in this study. Transformed somatic embryo aggregates were cultured in selection media composed of induction medium supplemented with 250 mg/L timentin and the indicated kanamycin concentrations (mg/L). The selection strategies were a standard strategy (strategy 1), where aggregates were cultured in selection medium with 50 mg/L kanamycin for 5 wk; an incremental strategy (strategy 2), with 25 mg/L kanamycin for 4 wk followed by 50 mg/L kanamycin for 1 wk; a progressively increasing kanamycin concentration strategy (strategy 3), with 6, 12, 25, 50, and 50 mg/L kanamycin for 1 wk each; and no initial selection strategy (strategy 4), with no kanamycin for 2 wk followed by 50 mg/L kanamycin for 3 wk. After 5 wk, treated somatic embryo aggregates were maintained in the presence of 50 mg/L kanamycin in all strategies.

Additional selection in liquid induction medium

Due to the probable existence of escapes during selection of transformed plant material, an additional selection in liquid medium was tested. For this, 0.4 g (fresh weight; FW) of somatic embryo aggregates from the selected lines (at least 15 lines of each strategy) were transferred to 100 mL Erlenmeyer flasks containing 50 mL liquid induction medium supplemented with 50 mg/L kanamycin. Suspensions were incubated on an orbital shaker (140 rpm) at 24 ± 1°C in the dark, replacing weekly 75% of the culture medium with fresh medium.

After 5 wk of culture, suspensions were passed through a 500-µm nylon mesh and 0.5 mL of the filtrate was transferred to a sterilized WhatmanTM (GE Healthcare) filter paper, then cultured on 90-mm diameter petri dishes containing solid induction medium supplemented with 50 mg/L kanamycin. The filter paper was transferred to a new plate with the same selection medium every 4 wk. After 12 wk of culture in selection medium, the percentage of embryogenic response was quantified. The final transformation efficiency was calculated by multiplying this value by the transformation efficiency after the first selection in solid medium. In addition, samples of embryogenic material from each independent line were used to perform GUS histochemical assays.

Recovery of transformed plant material

Differentiation of potentially transformed proembryos was carried out on 90-mm diameter polystyrene petri plates containing 25 mL DM1 medium (Acanda et al. 2020) consisting of the induction medium described above, without casein hydrolysate or phytohormones, and supplemented with 0.25% activated charcoal (Duchefa). The pH was adjusted to 5.8 before autoclaving at 98 kPa and 121°C and the medium was solidified using 0.3% Gelrite (Duchefa). In addition, a semi-permeable cellulose acetate membrane (Sigma) was placed between the culture medium and the somatic embryo aggregates as described (Acanda et al. 2020). Cultures were maintained for 5 wk at 24 ± 1°C in continuous darkness.

Somatic embryos were germinated by culturing cotyledonary embryos in DM1 differentiation medium supplemented with 0.25% activated charcoal, 3% sucrose, pH 5.8, and 0.3% gelrite as a gelling agent. After 4 wk, germinated somatic embryos were transferred to a conversion medium consisting of MS medium with the concentration of macronutrients reduced by half, 1.5% sucrose, pH 5.8, and 0.8% agar as a gelling agent. Regenerated plantlets were multiplied by cultivating their apices and nodal segments in the same culture medium. Cultures for somatic embryo germination, plantlet conversion, and multiplication were maintained at 25 ± 1°C (20 ± 1°C night temperature) under a 16-hr photoperiod, with a photon flux density of 45 µmol/$m^2 \cdot$sec provided by cool-white, fluorescent tubes. During different phases of development, samples of plant material were selected for GUS histochemical assays.

GUS histochemical analysis of transformed plant material

GUS activity was analyzed by histochemical assay (Jefferson 1987). Tissue samples were immersed in a solution consisting of 100 mM phosphate buffer at pH 7, 10 mM ethylenediaminetetraacetic acid disodium salt dihydrate at pH 8, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc). After a brief vacuum, the tissue samples were incubated overnight at 37°C in the dark. When necessary, chlorophyll was eliminated by washing the tissues twice with 70% ethanol for 24 hr each. Samples were observed with a Leica MZ10F stereomicroscope (Leica Microsystems GmbH) equipped with a Canon PowerShot G6 camera.

Extraction of genomic DNA (gDNA) and RNA and synthesis of complementary DNA (cDNA)

For gDNA extraction, samples of at least 100 mg (FW) somatic embryo aggregates were collected from 10 randomly selected, potentially transformed lines out of the 47 that grew after selection in liquid medium. Samples were frozen with liquid nitrogen and gDNA was extracted using the NucleoSpin Plant II Kit (Macherey-Nagel Gmbh & Co. KG) following the manufacturer's instructions with small modifications. PL2 buffer was used for extraction, column filtration was avoided during filtration, and clarification of the crude extract and the washing step with PW1 buffer was omitted. For RNA extraction, three independent samples of at least 65 mg (FW) were collected from somatic embryo aggregates from the same 10 potentially transformed lines that were also used for gDNA extraction. Samples were frozen with liquid nitrogen prior to total RNA extraction using the Aurum Total RNA Mini Kit (Bio-Rad), following the manufacturer's instructions.

DNA and RNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) and their purities were based on the absorbance ratios at 260/280 nm and 260/230 nm. An Agilent 2100 Bioanalyzer RNA 6000 Nano Lab-Chip was used to assess RNA quality. cDNA for analysis of *uid*A expression was synthesized from total RNA at a ratio of 1 µg/20 µL reaction volume using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Reactions were performed on an iCycler thermal cycler (Bio-Rad).

Design of qPCR primers

All qPCR primer sequences used, their sources, and qPCR efficiencies are shown (Table 1). Primers for the chalcone isomerase gene (*chi*) were designed based on the 12X grapevine reference genome in the Ensembl Plants database (https://plants.ensembl.org/Vitis_vinifera/Info/Index). The presence of *chi* in the Mencía genome was verified using conventional PCR. Primers were designed using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). In addition, Gene Runner software (v3.01, Hasting Software Inc.) was used to verify the potential interactions between primers.

qPCR

qPCR reactions (20 µL) comprising 1X SsoFast EvaGreen Supermix (Bio-Rad), 0.4 µM of each primer, and 3 µL diluted gDNA (9 to 48 ng) or cDNA (1.66 ng) were carried out in 96-well plates in an iCycler iQ real-time thermal cycler (Bio-Rad). Reactions were performed as follows: 1 min at 98°C, 40 cycles of 5 sec at 98°C, and 20 sec at 58°C for annealing and extension. Dissociation curves to verify the specificity of each amplification reaction were obtained by heating the amplicons from 65 to 90°C with a ramp setting at 0.5°C/10 sec. Duplicate nontemplate controls were included for each plate. For the analysis of *uid*A gene expression, qPCR data were normalized with two reference genes (*Ef1-α (m)* and *GAPDH (m)*), previously determined as the most appropriate for our experimental system (Acanda et al. 2020).

Data analysis

All experiments were independently repeated at least twice to ensure reproducibility. Data for the dose-response curve of cultures against kanamycin and for the percentage of embryogenic response after selection were analyzed using a Mann-Whitney U test. Statistical tests ($p < 0.05$) were performed using PASW Statistics 18 software (IBM). Data from qPCR were analyzed using iCycler iQ software (Real-Time Detection System Software, Windows ver. 3.0, Bio-Rad). Raw fluorescence data were analyzed using LinRegPCR software (Ruijter et al. 2009) to obtain the mean PCR efficiency for each primer pair (Table 1).

The estimated number of integrated copies of the transgenes *nptII* and *uid*A was obtained using Equation 1 (Zhang et al. 2015), in which the ΔCq value of the transgene is calculated with reference to an endogenous control whose number of copies is known.

$$\Delta Cq\ transgene = 2^{(Cq\ endogenous - Cq\ transgene)} \quad \text{(Eq. 1)}$$

To achieve better normalization, we used both 9-cis-epoxycarotenoid dioxygenase 2 (*NCED2*) and *chi* as control genes to estimate the number of integrated copies of *nptII* and *uid*A. Both control genes are present in a single copy in the grapevine genome (Dalla Costa et al. 2009) and have qPCR efficiencies similar to the target transgenes (Table 1; Yuan et al. 2007). Thus, Equation 1 was modified (Equation 2) by introducing a second endogenous single-copy grapevine gene following the algebraic pattern used to normalize qPCR relative expression stability as described (Vandesompele et al. 2002):

$$\Delta Cq(m)\ transgene = 2^{(G - Cq\ transgene)} \quad \text{(Eq. 2)}$$

where: G = Geometric average (Cq endogenous genes)

The estimated number of transgene copies integrated into the genome was calculated as twice the value (ΔCq(m) transgene), since lines generated after transformation are usually heterozygous for the transgene. Ten randomly selected, independent transformed lines were analyzed after selection in liquid medium. Analysis of each line consisted of two biological replicates, each analyzed in quadruplicate (technical replicates). Analyses were repeated twice independently.

Relative *uid*A expression in different transgenic lines and its statistical significance ($p < 0.05$) were determined using REST-2009 (Relative Expression Software Tool, ver. 2009; Pfaffl et al. 2002), with PCR efficiency correction and normalization to the reference genes *GAPDH(m)* and *EF1-α(m)* as described (Acanda et al. 2020) and compared with the $2^{-\Delta\Delta Cq}$ method. Each different transgenic line was tested in triplicate (biological replicates) with two experimental (technical) replicates. The transformed line with the lowest transcript level was considered the calibrator group.

Results

Effect of kanamycin on the growth of grapevine somatic embryo aggregates

We determined the sensitivity of Mencía somatic embryo aggregates to kanamycin, measured as the increase in fresh weight (final weight - initial weight) after 4 and 8 wk of culture, at different antibiotic concentrations (Figure 2A). Growth was reduced even at the lowest kanamycin dose (50 mg/L) after 4 wk. Although fresh weight of the aggregates

Table 1 Primer sources and sequences, amplicon lengths, and efficiency for qPCR assays. Efficiency was determined with LinRegPCR software and the indicated value represents the average of all qPCRs performed.

Gene	Sequence source	Primer sequence (forward / reverse)	Amplicon length (bp)	qPCR efficiency
nptII	Casu et al. 2012	GCCGAATATCATGGTGGAA / AATATCACGGGTAGCCAACG	106	1.95
uidA	Álvarez et al. 2004	CCAAAAGCCAGACAGAGTGTG / CCATCAGCACGTTATCGAATC	168	1.94
NCED2	Dalla Costa et al. 2009	CGGAGCAACCTGTCTGTCACTC / CGCCGTTTCGCAGATAAACTC	81	1.93
chi	AM430986.2, X75963.1	CCACCAACGACCTCTTCCTC / CAAACCTCTCACCCCTGACC	118	1.95
EF1-α (m)	Reid et al. 2006	CGCCTGTCAATCTTGGTCAGTAT / AATGGCTATGCCCCTGTTCTG	83	1.92
GAPDH (m)	Reid et al. 2006	TTCTCGTTGAGGGCTATTCCA / CCACAGACTTCATCGGTGACA	70	1.92

increased in all kanamycin treatments, there was significantly more growth in the control medium without kanamycin (0.39 ± 0.04). Growth at concentrations of 100 (0.10 ± 0.02) and 200 (0.06 ± 0.02) mg/L kanamycin was significantly ($p < 0.05$) less than at 50 mg/L (0.21 ± 0.03).

Somatic embryo aggregate growth differences between control (without antibiotic) and kanamycin treatments increased after 8 wk of culture (Figure 2A). The lowest growth was obtained with 200 mg/L kanamycin, although differences with 100 and 150 mg/L kanamycin were not significant ($p < 0.05$).

Despite the negative effect of kanamycin on the growth of somatic embryo aggregates, complete necrosis of the cultures was not observed. To determine whether kanamycin produced permanent effects on proliferation capacity by secondary embryogenesis, aggregates cultured with different kanamycin concentrations were transferred to induction medium without kanamycin. After two subcultures of 4 wk, it was observed that only the control cultures maintained their proliferation capacity, while widespread necrosis was observed in all somatic embryo aggregates previously cultured in the presence of kanamycin (Figure 2B).

Effect of the selection strategy on transformation efficiency

Four different strategies (Figure 1) were tested for selection of potentially transformed material. All strategies resulted in embryogenic responses 2 mo after transformation, when development of small somatic embryos was observed on the surface of some aggregates, despite their necrotic appearance (Figure 3A). These neoformed somatic embryos showed a good rate of proliferation by secondary embryogenesis, which resulted in the formation of large groups of somatic embryo aggregates after one additional month of culture (Figure 3B). Since the origin of somatic embryos in grapevine is unicellular (Jayasankar et al. 2003), each aggregate was separated and treated as an independent line.

Transformation efficiency for each selection strategy was calculated as a function of the embryogenic response of the treated somatic embryo aggregates. The best embryogenic response was obtained with selection strategy 2 (26.7%, Figure 4A), although it was not statistically different from that obtained with strategies 1 and 3 (18.8 and 19.6%, respectively). The worst response was obtained with strategy 4, with only 12.7%. Aggregates of control plates not co-cultured with *Agrobacterium* became necrotic during the first 2 mo of selection, with no subsequent embryogenic response.

Potentially transformed lines were identified by GUS histochemical assays on somatic embryo aggregates with active proliferation resulting from different selection strategies. The results (Figure 4B) were defined as positive (GUS+) if an intense blue color was present in the whole somatic embryo aggregate. Non-uniform color or no color were defined as a negative result (GUS-). The lowest GUS+ percentage was obtained with strategy 1 (40%), indicating the existence of escapes from the selection process, while the highest GUS+ percentage was observed with strategy 4 (79%). The GUS histochemical assay was repeated after 2 mo of culture of the different embryogenic lines in induction medium supplemented with 100 mg/L kanamycin. The results of this second GUS assay did not vary from the first, nor was there necrosis or reduction of growth in any line.

Selection in liquid medium of potentially transformed embryogenic lines

An additional selection process was performed in liquid medium to try to minimize the number of escapes observed with the tested selection strategies (Figure 4B). Potentially transformed lines generated after selection on solid media were cultured in liquid induction medium supplemented with 50 mg/L kanamycin. After 5 wk, treated somatic embryo aggregates were transferred to solid induction medium supplemented with 50 mg/L kanamycin. Selection in liquid medium produced a general necrosis of aggregates such that secondary embryogenesis was not observed until 1 mo after the transfer of the aggregates from liquid to solid medium. Hence, the percentage of embryogenic response was quantified 3 mo after transfer to solid selection medium, when non-responding lines became totally necrotic. Only 55.6% of the lines generated with strategy 1 showed an embryogenic response after selection in liquid medium (Table 2). The opposite result was obtained with the lines from strategy 4,

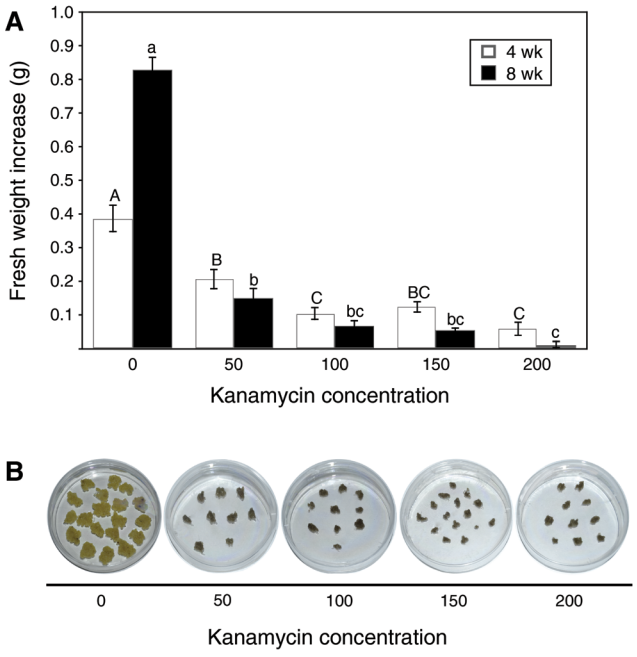

Figure 2 Effect of kanamycin concentration on growth of Mencía grapevine somatic embryo aggregates in induction medium. **A**) Variation in fresh weight (final weight - initial weight; mean ± standard error) of somatic embryo aggregates after 4 (white) and 8 (black) wk of culture in induction medium with different concentrations of kanamycin. **B**) Growth of somatic embryo aggregates in induction medium for 8 wk after treatment with different concentrations of kanamycin. In (**A**), different letters within the same time of culture (uppercase for data collected after 4 wk of culture, lowercase for data after 8 wk) indicate statistically significant differences between kanamycin concentrations ($p < 0.05$).

where the percentage of lines with secondary embryogenesis reached 92.9%. The lines regenerated after selection in liquid medium were analyzed using GUS histochemical assays, and all lines tested positive. With these results, the final transformation efficiency (Table 2) was calculated as the percentage of initial embryogenic response (Figure 4A) multiplied by the response percentage after selection in liquid medium. All procedures reached a final transformation efficiency higher than 10%, but the most efficient procedure was that which used the initial incremental selection (strategy 3) combined with the additional selection in liquid medium (19.8% efficiency).

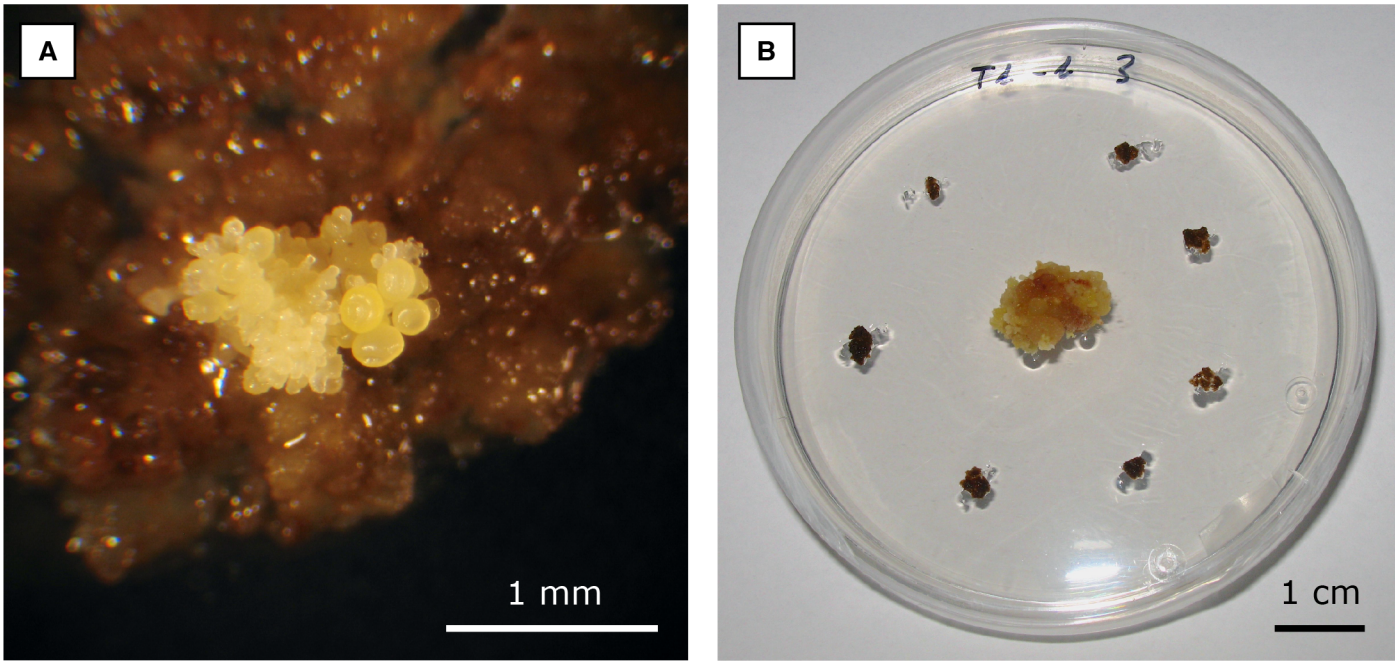

Figure 3 Selection and recovery of transformed Mencía grapevine embryogenic material from somatic embryo aggregates cultured in induction medium supplemented with 50 mg/L kanamycin (selection strategy 1, Figure 1). **A**) Formation of potentially transformed grapevine Mencía somatic embryos after 2 mo of culture in the presence of kanamycin. **B**) Growth of Mencía grapevine somatic embryo aggregates after 3 mo of culture in the presence of kanamycin. Bars: 1 mm (**A**); 1 cm (**B**).

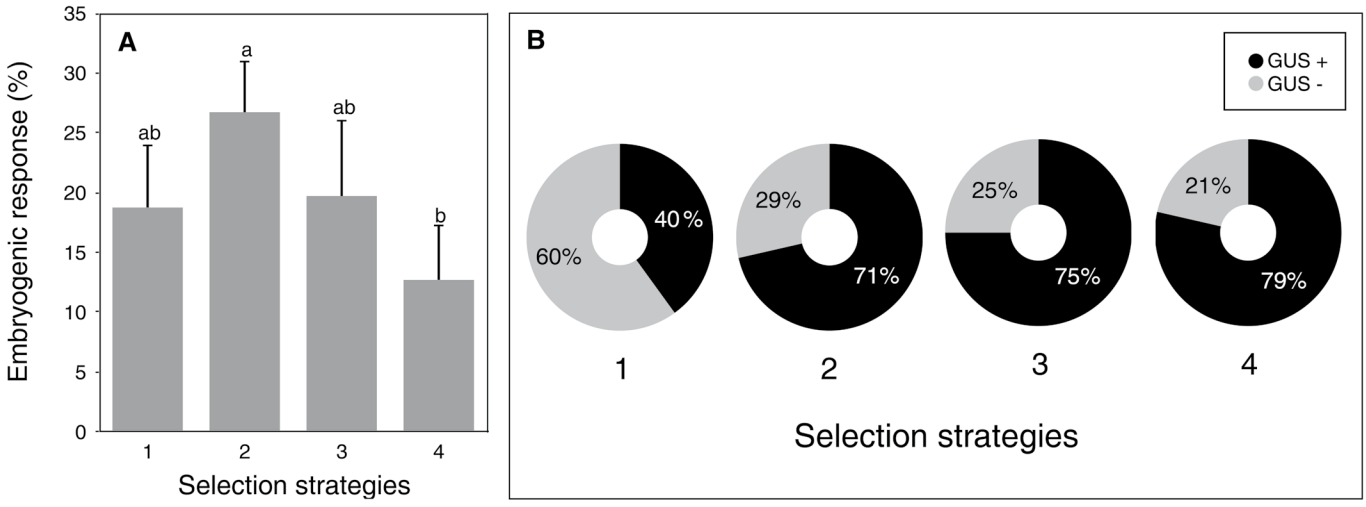

Figure 4 Effect of selection strategy on the transformation efficiency of Mencía grapevine somatic embryo aggregates, indicated as the percentage of embryos showing response after selection treatment. The selection strategies tested were a standard strategy (strategy 1); an incremental strategy (strategy 2); a progressively increasing kanamycin concentration strategy (strategy 3); and no initial selection strategy (strategy 4). After 5 wk, treated somatic embryo aggregates were maintained in the presence of 50 mg/L kanamycin in all strategies. (**A**) Percentage of aggregates with embryogenic response (mean ± standard error) after each selection strategy. Different letters indicate statistically significant differences ($p < 0.05$). (**B**) Percentage of positive (black; GUS+) or negative (grey; GUS-) for the β-glucuronidase (GUS) histochemical analysis of the transformed embryogenic lines generated in each selection strategy.

Lines established after selection in liquid medium developed according to the usual response in non-transformed embryogenic cultures. Transfer to DM1 differentiation medium of somatic embryo aggregates from the different lines induced the development of somatic embryos as described previously (Acanda et al. 2013). These somatic embryos maintained positive GUS staining (Figure 5A). Cotyledonary somatic embryos were selected for germination and subsequent conversion to plantlets. Regenerated plantlets were analyzed by GUS histochemical assay with positive results (Figure 5B).

qPCR to determine copy number of the *nptII* and *uidA* genes integrated into the potentially transgenic lines

To determine the transgene copy number for *nptII* and *uid*A in the genome of grapevine somatic embryos after transformation, somatic embryo aggregates from 10 independent transformed lines generated after selection in liquid medium were randomly collected and analyzed by qPCR as described (Zhang et al. 2015). *NCED2* and *chi* were used as alternative endogenous controls, since they are present in a single copy in the grapevine genome (Dalla Costa et al. 2009). When the *chi* gene was used as the control, the estimated transgene copy numbers of *nptII* and *uid*A were different in up to six transformed lines: T13, T27, T29, T35, T41, and T44 (Table 3). However, when *NCED2* was used as the control, the estimated number of copies of both transgenes was the same in all lines except T29 and T30. In addition, results for the same transgene differed with the control gene used. Thus, depending on the control gene used, a different number of *uid*A copies was estimated in lines T13, T35, and T44, and of *nptII* copies in lines T27, T30, and T41 (Table 3).

In view of these inconsistent results, we increased the robustness of the analysis by modifying the formula to use the geometric average of both single-copy reference genes. With this new approach, the estimated copy number varied between one and nine, with most lines having one or two copies (Table 3). Estimated copy number was the same for the two genes included in the transformation vector, with only two exceptions: line T29 had four copies of *uid*A and five of *nptII*, while line T41 had eight copies of *uid*A and nine of *nptII*.

Expression of *uidA* in potentially transgenic lines

Expression of *uid*A was studied by qPCR in the same 10 randomly selected independent lines used to determine gene copy number. Gene expression was calculated in relation to line T23 (Figure 6), which had the lowest transcript levels (determined preliminarily by the Cq value, data not shown).

The highest level of expression of *uid*A was obtained in line T41, which contained the most transgene copies (eight copies; Table 3). Expression of *uid*A in the other lines did not show a clear correlation to the number of transgene copies. Thus, line T27, with three integrated copies, had the second highest expression value, while line T29 (four copies) had lower expression levels than even line T13, which had only one copy of *uid*A.

Table 2 Transformation efficiency after additional selection in liquid medium. Transformed Mencía somatic embryo aggregates treated with each selection strategy in solid media were cultured in liquid induction medium supplemented with 50 mg/L kanamycin for 5 wk. The final transformation efficiency was calculated as the percentage of embryogenic response observed in each of the initial selection strategies, multiplied by the percentage embryogenic response after selection in liquid medium.

Initial selection strategy	Selection in liquid medium	
	Embryogenic response (%)	Final transformation efficiency (%)
1 (Standard selection)	55.6	10.5
2 (Incremental selection)	74.2	19.8
3 (Progressive selection)	75.0	14.7
4 (No initial selection)	92.9	11.8

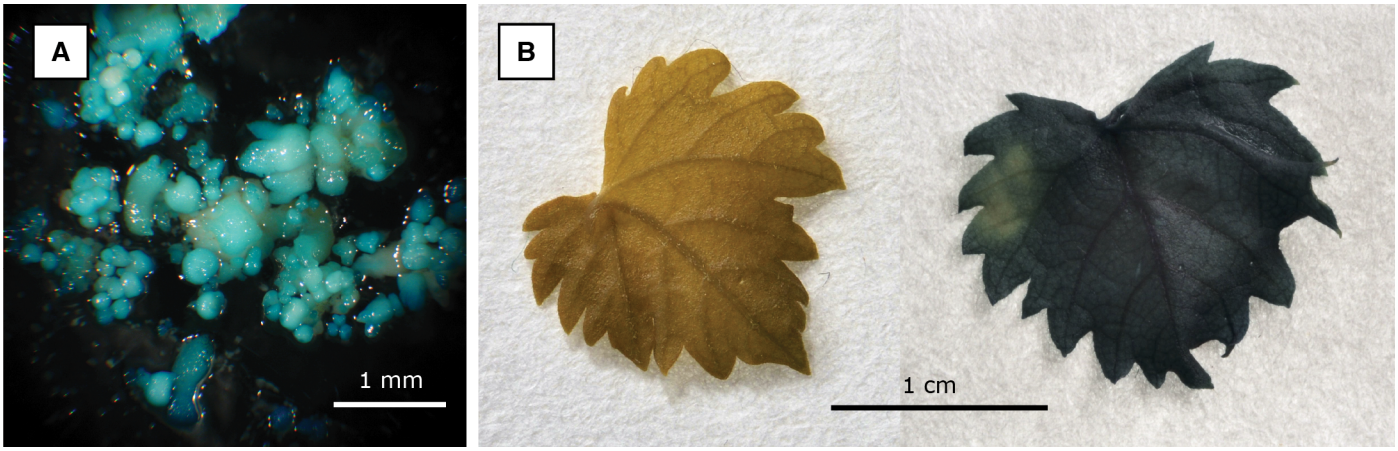

Figure 5 β-glucuronidase (GUS) histochemical assay in Mencía grapevine somatic embryo transformed lines and regenerated plantlets. (**A**) Asynchronic somatic embryo aggregates cultured in DM1 differentiation medium. (**B**) Leaves of plantlets not transformed (left) and transformed (right) grown in conversion medium. Bars: 1 mm (**A**); 1 cm (**B**).

Discussion

Effect of kanamycin on the growth of grapevine somatic embryo aggregates

Selection of potentially transformed cells is a crucial step in any transformation protocol and is a primary obstacle to application of this technology in the genus *Vitis* (Bouquet et al. 2008). To obtain a correct regeneration of transformed material, there must be a balance between selection efficiency and the inhibitory effects of the selective agent on plant tissues (Saporta et al. 2016, Campos et al. 2021). This balance is especially important when embryogenic cultures that are not tolerant to stress are transformed, such as in grapevine, where both contact with *Agrobacterium* and experimental manipulation promote tissue necrosis. In addition, this necrosis frequently produces phenolic compounds that inhibit the growth of nearby cells (Torregrosa et al. 2000, Bouquet et al. 2008), especially in woody species.

Kanamycin is one of the most widely used and successful antibiotics for selection of transformed plant material (Campos et al. 2021). However, its optimal concentration must be established for each species and even plant explant (Saporta et al. 2014, 2016, Campos et al. 2021). Here, growth of grapevine somatic embryo aggregates was reduced significantly when induction medium was supplemented with kanamycin (Figure 2). The most common range of antibiotic concentration is between 15 and 100 mg/L, depending on the genotype and type of explant (Vidal et al. 2003, Wang et al. 2005, Li et al. 2008, Saporta et al. 2014). Based on the results obtained (Figure 2A), 50 mg/L kanamycin appears sufficient to inhibit embryogenic capacity in Mencía and was therefore used to select potentially transformed cultures.

Effect of the selection strategy on transformation efficiency

Correct selection of transformed cells is a critical part of every transformation protocol. However, the effect of the selection process on transformation efficiency depends not only on the type and concentration of antibiotic used, but also on how and when this selection is applied (Wang et al. 2005). Kanamycin interferes with regeneration of transformed material, possibly due to exudation of phenolic compounds during the necrosis of non-transformed tissue (Campos et al. 2021). The first month after co-cultivation with *Agrobacterium* is crucial for transformation (Zhou et al. 2014), as cell damage and necrosis are observed frequently during this phase (Li et al. 2008, Palomo-Ríos et al.

Table 3 Estimation of the number of copies of transgenes *nptII* and *uidA* based on the values (mean ± standard error) of ΔCq and modified ΔCq in lines of transformed grapevine somatic embryo aggregates. The grapevine single-copy genes *NCED2* and *chi* were used as the endogenous control genes.

Transformed lines	***uidA* gene**					
	NCED2		***chi***		***NCED2 / chi***	
	ΔCq	**Estimated no. copies**	**ΔCq**	**Estimated no. copies**	**Modified ΔCq**	**Estimated no. copies**
T13	0.57 ± 0.03	1	0.82 ± 0.08	2	0.68 ± 0.01	1
T23	0.47 ± 0.26	1	0.45 ± 0.26	1	0.46 ± 0.19	1
T27	1.34 ± 0.11	3	1.57 ± 0.29	3	1.55 ± 0.15	3
T28	0.34 ± 0.14	1	0.35 ± 0.06	1	0.34 ± 0.07	1
T29	1.93 ± 0.13	4	1.87 ± 0.05	4	1.90 ± 0.02	4
T30	1.05 ± 0.11	2	0.78 ± 0.11	2	0.90 ± 0.08	2
T35	0.94 ± 0.14	2	0.75 ± 0.03	1	0.84 ± 0.05	2
T41	3.85 ± 0.01	8	4.18 ± 0.22	8	4.01 ± 0.08	8
T42	0.50 ± 0.03	1	0.54 ± 0.03	1	0.52 ± 0.02	1
T44	1.21 ± 0.14	2	1.27 ± 0.06	3	1.23 ± 0.03	2
Transformed lines	***nptII* gene**					
	NCED2		***chi***		***NCED2 / chi***	
	ΔCq	**Estimated no. copies**	**ΔCq**	**Estimated no. copies**	**Modified ΔCq**	**Estimated no. copies**
T13	0.35 ± 0.07	1	0.50 ± 0.02	1	0.42 ± 0.03	1
T23	0.58 ± 0.11	1	0.53 ± 0.13	1	0.55 ± 0.08	1
T27	1.53 ± 0.09	3	1.78 ± 0.29	4	1.70 ± 0.08	3
T28	0.49 ± 0.00	1	0.56 ± 0.15	1	0.52 ± 0.05	1
T29	2.48 ± 0.20	5	2.40 ± 0.03	5	2.44 ± 0.06	5
T30	1.37 ± 0.39	3	1.02 ± 0.32	2	1.18 ± 0.25	2
T35	0.97 ± 0.07	2	0.78 ± 0.14	2	0.87 ± 0.08	2
T41	4.10 ± 0.18	8	4.44 ± 0.03	9	4.26 ± 0.05	9
T42	0.47 ± 0.05	1	0.51 ± 0.06	1	0.49 ± 0.04	1
T44	1.02 ± 0.13	2	1.06 ± 0.03	2	1.04 ± 0.04	2

2012). Based on this evidence, we tested several selection strategies in this work, based on the initial use of suboptimal concentrations, increasing concentrations, and even absence of selection during the first weeks after transformation, followed by transfer to the optimal concentration (50 mg/L, Figure 1).

The greatest embryogenic response was obtained with selection strategy 2 (Figure 4A), consisting of culture in 25 mg/L kanamycin during the first 4 wk and subsequent transfer to 50 mg/L kanamycin (Figure 1). Reduced selection pressure in the first stages after transformation has produced good results in other woody species, such as oak (Vidal et al. 2010) or avocado (Palomo-Ríos et al. 2012).

Progressive increases in kanamycin concentration during the first 4 wk (strategy 3, Figure 1) produced the second-best embryogenic response rate (Figure 4A), although the difference was not statistically significant with respect to the other strategies. A similar result was obtained when a progressive increase in the hygromycin concentration improved grapevine transformation efficiency over selection at a constant antibiotic concentration (Fan et al. 2007).

Strategy 4 provided the lowest response percentage (Figure 4A). In this strategy, somatic embryo aggregates were cultured in medium without kanamycin for the first 2 wk and then transferred to medium with 50 mg/L kanamycin (Figure 1). In grapevine, less than 3 or 4 wk in the absence of selection does not affect transformation efficiency (Zhou et al. 2014). The inclusion of an initial period without selection has given positive results in other woody species such as apricot (da Câmara Machado et al. 1992), apple (Yao et al. 1995), and almond (Ramesh et al. 2006). However, one of the greatest drawbacks in extending the period without a selection agent is the formation of numerous escapes and chimeras (Padilla and Burgos 2010, Zhou et al. 2014), so this strategy should be used with caution.

GUS histochemical analysis revealed the presence of untransformed somatic embryo aggregates in all selection strategies (Figure 4B). Unexpectedly, the most severe selection strategy, with a constant concentration of 50 mg/L kanamycin (strategy 1, Figure 1), resulted in the most escapes. These escapes indicate that the calculated transformation efficiency, measured as embryogenic response (Figure 4A), was overestimated to a different extent for each selection strategy. To eliminate these untransformed somatic embryo aggregates, a second selection was carried out in liquid induction medium supplemented with 50 mg/L kanamycin for 5 wk. This selection was very effective, since all lines that showed response after selection were GUS+, indicating the absence of escapes. Selection of potentially transformed cultures in liquid medium is an effective way to eliminate chimeric lines in mango (Mathews et al. 1992), avocado (Raharjo et al. 2008), American chestnut (Andrade et al. 2009), and olive (Torreblanca et al. 2010).

The percentage of embryogenic response after selection in liquid medium (Table 2) was consistent with the results obtained in the initial GUS analysis (Figure 4B). It should be noted that in this work, a GUS result was considered negative when the coloration of the aggregate was heterogeneous. For example, in the initial GUS assay, only 40% of the lines generated with selection strategy 1 had a homogeneous blue color (GUS+; Figure 4B). For this reason, it would be expected that after selection in liquid medium,

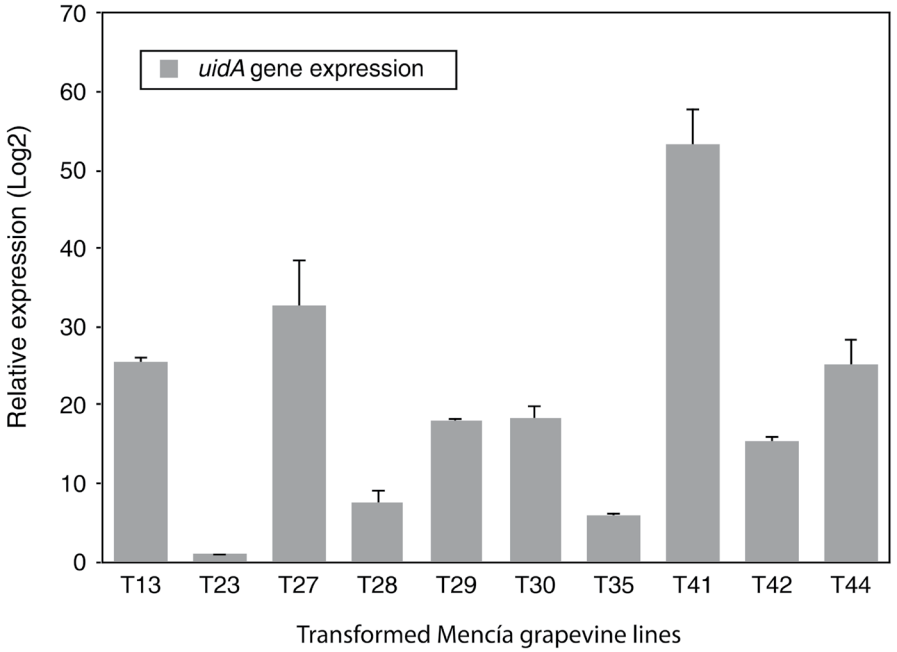

Figure 6 Relative expression of the *uidA* gene in Mencía grapevine somatic embryo transformed lines. qPCR data were determined and statistically analyzed ($p < 0.05$) using REST-2009 with PCR efficiency correction and normalization with two reference genes, then compared with the $2^{-\Delta\Delta Cq}$ method. *GAPDH(m)* and *EF1-α(m)* were used as reference genes for normalization and the transformed line with lowest transcript level (T23) was used as the calibrator. The average values of two independent experiments are represented ± the standard error.

the percentage of response obtained would have been 40% at most, while in fact it was 55.6% (Table 2). This improvement could be because the selection in liquid medium eliminated some untransformed cells in heterogeneous aggregates (GUS-), allowing regeneration only of those that were really transformed. This phenomenon was also observed in the somatic embryo aggregates coming from selection strategies 2 and 4.

With these results, final transformation efficiency was calculated by eliminating the escapes (Table 2), and varied between 10.4% and 19.8%, with selection strategy 2 yielding the most transformed lines and selection strategy 1 the fewest. Based on the few grapevine reports conducted under similar conditions (Zhou et al. 2014, Ahmed et al. 2015, Kandel et al. 2016), it can be inferred that our transformation efficiency was high for all strategies, but particularly for selection strategy 2. Ahmed et al. (2015) obtained much lower transformation efficiencies (2.83% in the best strategy) when transforming grapevine King's Ruby embryogenic cultures with the LBA4404 strain of *Agrobacterium*. Better results were obtained by Kandel et al. (2016) when transforming somatic embryos (cotyledonary stage) of grapevine Thompson Seedless and the hybrid Bronx Seedless with *Agrobacterium* strain EHA105, reaching a maximum transformation rate of 9.6%. Only Zhou et al. (2014) report greater efficiencies than our strategy 2, transforming Thompson Seedless proembryogenic masses with *Agrobacterium* strain GV3101. In addition, they tested different selection regimes of potentially transformed material, two of which had efficiencies of 32.3% and 28% (Zhou et al. 2014). Such strategies cultured the embryogenic material in medium without kanamycin during the first 3 or 4 wk after *Agrobacterium* co-cultivation, respectively. The same authors performed a selection regime equivalent to our strategy 4 (absence of kanamycin during the first 2 wk) and obtained a greater efficiency (17.3%) than found here (11.8%). Due to the differences between the protocol of Zhou et al. (2014) and the protocol developed in this work (grapevine genotype, explant type, bacterial strain, vector, culture media, etc.), it is difficult to determine whether the highest transformation efficiency was due to the protocol used or to a greater predisposition of Thompson Seedless to transformation.

In this work we used a hypervirulent strain of *Agrobacterium* AGL1 and this could explain the high transformation efficiency obtained. This strain has proven to be very efficient in transformation of a broad spectrum of species, both monocotyledons such as barley (Bartlett et al. 2008) and rice (Zhao et al. 2011), and woody species recalcitrant to transformation such as cork oak (Álvarez et al. 2009), olive (Torreblanca et al. 2010), and avocado (Palomo-Ríos et al. 2012). One study agroinfiltrated leaves of grapevine with *Agrobacterium* strains GV3101 and AGL1 (Urso et al. 2013). Although the objective was to analyze genes related to powdery mildew resistance, the authors observed that AGL1 was able to transform both grapevine genotypes used, while GV3101 only transformed one of them. We have found no other report in which the hypervirulent strain AGL1 was used for grapevine transformation.

Molecular analysis of the transgenic material

Estimates of transgene copy number using the ΔCq method (Zhang et al. 2015) in 10 transformed Mencía lines was inconsistent (Table 3), as it depended on which endogenous control gene was used to perform the calculations. In addition, for some lines the number of copies of the two genes was different when the calculations were made using a single control gene (*NCED2* or *chi*, Table 3), although both transgenes were in the same transformation vector. This could be due to the qPCR variability, or to DNA rearrangements during integration. This last phenomenon has been observed in trifoliate orange, where 15.4% of the lines analyzed showed a different copy number for the two genes included in the transformation vector (Wen et al. 2012). With the goal of adding robustness to the analysis, we modified the estimation formula (Equation 1) by including the geometric average of two single-copy grapevine endogenous genes, with the result that the estimated copy number of the *nptII* and *uid*A genes was similar in most of the lines analyzed (Table 3). Exceptions were line T29, with five *nptII* and four *uid*A copies, and line T41, with eight and nine copies, respectively. It must be noted that both lines had the highest number of estimated transgene copies.

The number of integrated copies found agreed with the range observed in other woody species. Integration of between one and three copies was observed in olive (Torreblanca et al. 2010), between one and four in avocado (Palomo-Ríos et al. 2012), four in cork oak (Álvarez et al. 2009), >10 in a hybrid between *Populus nigra* L. and *Populus maximowiczii* A. Henry (Yevtushenko and Misra 2010), and between one and 15 in oak (Mallón et al. 2013).

Determination of transgene copy number was complemented by expression analysis, also using qPCR, of the *uid*A gene for the 10 lines previously selected (Figure 6). The greatest expression levels were obtained with line T41, which also had the most integrated transgene copies, but there was no correlation between copy number and expression in the other lines. Although lines with only one copy of the transgene generally showed lower levels of *uid*A gene expression (Figure 6), in line T13 (also with a single copy), the expression was higher than in lines with two or even four copies of the transgene. This discordance between the number of integrated copies and the expression of the transgene has been observed in other species such as *Nicotiana tabacum* L. (Hobbs et al. 1993) and *Castanea sativa* Mill. (Corredoira et al. 2012). Several reasons have been suggested to explain these variations in transgene expression, such as rearrangements during integration (Iglesias et al. 1997), positional effects of the transgene within the plant genome (Kooter et al. 1999), or epigenetic alterations (Stam et al. 1997). It has also been shown that transgene expression in *Pinus strobus* L. was silenced when the integration of more than three copies occurred in the same chromosome (Tang et al. 2007), so this could be another reason for the low *uid*A expression in lines T29 and T41. These results highlight the importance of assessing both number of integrated copies and expression of transgenes before selecting lines of greatest interest.

Conclusion

A highly efficient protocol for transforming Mencía somatic embryo aggregates was developed, based on the use of AGL1, a hypervirulent strain of A. *tumefaciens*. This work constitutes the first transformation protocol developed for Mencía grapevine, with a transformation efficiency that largely exceeds the usual efficiency observed for grapevine. It was also demonstrated that it is possible to estimate transgene copy numbers in somatic embryo aggregates using qPCR and a modified ΔCq method that adds robustness to the analysis. Unlike previous estimates of copy number with qPCR, the strategy described here does not require a transformed homozygous line. For this reason, the developed protocol allows early selection of the most interesting transgenic lines with very little plant material and in a simple way, significantly saving resources and time.

Acknowledgments

This paper is a contribution of the Interuniversity Research Group in Biotechnology and Reproductive Biology of Woody Plants (group code 08IDI1705). The authors gratefully thank Prof. Fernando Pliego Alfaro (Univ. Málaga, Spain) for his generous advice and encouragement. The authors also thank Ma José Graña for her invaluable help during plant material collection at the Centro de Formación y Experimentación de Viticultura y Enología de Ribadumia (Pontevedra, Spain), a viticultural facility owned by the regional government of Galicia (Spain). The authors also thank Elene Valdivia for her English language revision of the manuscript. Óscar Martínez thanks the Spanish Ministry of Education, Culture and Sport for its support through an FPU fellowship. Partial financial support is acknowledged from the regional government of Galicia (Xunta de Galicia, Spain, grants ED431C2019/20 and ED431B2022/26). The authors declare no competing interests and that the funding bodies had no role in the design of the study; in the collection, analysis, and interpretation of data; or in writing the manuscript.

ORCID

Óscar Martínez 0000-0001-8170-0022
Elena Palomo-Ríos 0000-0002-4538-6037
Manuel Rey 0000-0002-1445-5538
María Victoria González 0000-0002-5673-0585

Citation

Martínez Ó, Palomo-Ríos E, Rey M and González MV. 2024. Efficient genetic transformation of *Vitis vinifera* L. Mencía using a hypervirulent strain of *Agrobacterium tumefaciens* and qPCR determination of transgene copy number. Am J Enol Vitic 75:0750020. DOI: 10.5344/ajev.2024.24012

Data Availability

All data underlying this study are included in the article.

References

Acanda Y, Prado MJ, González MV and Rey M. 2013. Somatic embryogenesis from stamen filaments in grapevine (*Vitis vinifera* L. cv. Mencía): Changes in ploidy level and nuclear DNA content. In Vitro Cell Dev Biol Plant 49:276-284. DOI: 10.1007/s11627-013-9499-7

Acanda Y, Martínez Ó, Prado MJ, González MV and Rey M. 2020. Changes in abscisic acid metabolism in relation to the maturation of grapevine (*Vitis vinifera*, cv. Mencía) somatic embryos. BMC Plant Biol 20:487. DOI: 10.1186/s12870-020-02701-z

Ahmed M, Khan N, Hafiz IA, Abbasi NA, Anjum MA and Hussain S. 2015. Effect of various factors on the efficiency of *Agrobacterium*-mediated transformation of grape (*Vitis vinifera* L.). Vegetos 28:171-178. DOI: 10.5958/2229-4473.2015.00024.5

Álvarez R, Alonso P, Cortizo M, Celestino C, Hernández I, Toribio M et al. 2004. Genetic transformation of selected mature cork oak (*Quercus suber* L.) trees. Plant Cell Rep 23:218-223. DOI: 10.1007/s00299-004-0810-2

Álvarez R, Álvarez JM, Humara JM, Revilla Á and Ordás RJ. 2009. Genetic transformation of cork oak (*Quercus suber* L.) for herbicide resistance. Biotechnol Lett 31:1477-1483. DOI: 10.1007/s10529-009-0033-2

Andrade GM, Nairn CJ, Le HT and Merkle SA. 2009. Sexually mature transgenic American chestnut trees via embryogenic suspension-based transformation. Plant Cell Rep 28:1385-1397. DOI: 10.1007/s00299-009-0738-7

Baggiolini M. 1952. Les stades repères dans le développment annuel de la vigne et leur utilisation pratique. Rev Rom Agric Vitic Arboric 8:4-6.

Bartlett JG, Alves SC, Smedley M, Snape JW and Harwood WA. 2008. High-throughput *Agrobacterium*-mediated barley transformation. Plant Methods 4:22. DOI: 10.1186/1746-4811-4-22

Bertani G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62:293-300. DOI: 10.1128/jb.62.3.293-300.1951

Biricolti S, Bogani P, Cerboneschi M and Gori M. 2016. Inverse PCR and quantitative PCR as alternative methods to southern blotting analysis to assess transgene copy number and characterize the integration site in transgenic woody plants. Biochem Genet 54:291-305. DOI: 10.1007/s10528-016-9719-z

Bouquet A, Torregrosa L, Iocco P and Thomas MR. 2008. Grapes. *In* Compendium of Transgenic Crop Plants. Part 4. Transgenic Temperate Fruits and Nuts. Kole C and Hall TC (eds.), pp. 189-232. Wiley-Blackwell, Oxford, UK. DOI: 10.1002/9781405181099.k0407

Bubner B and Baldwin IT. 2004. Use of real-time PCR for determining copy number and zygosity in transgenic plants. Plant Cell Rep 23:263-271. DOI: 10.1007/s00299-004-0859-y

Butiuc-Keul A and Coste A. 2023. Biotechnologies and strategies for grapevine improvement. Horticulturae 9:62. DOI: 10.3390/horticulturae9010062

Campos G, Chialva C, Miras S and Lijavetzky D. 2021. New technologies and strategies for grapevine breeding through genetic transformation. Front Plant Sci 12:767522. DOI: 10.3389/fpls.2021.767522

Casu RE, Selivanova A and Perroux JM. 2012. High-throughput assessment of transgene copy number in sugarcane using real-time quantitative PCR. Plant Cell Rep 31:167-177. DOI: 10.1007/s00299-011-1150-7

Chu Y, Bhattacharya A, Wu C, Knoll JE and Ozias-Akins P. 2013. Improvement of peanut (*Arachis hypogaea* L.) transformation efficiency and determination of transgene copy number by relative quantitative real-time PCR. In Vitro Cell Dev Biol Plant 49:266-275. DOI: 10.1007/s11627-013-9518-8

Corredoira E, Valladares S, Allona I, Aragoncillo C, Vieitez AM and Ballester A. 2012. Genetic transformation of European chestnut somatic embryos with a native thaumatin-like protein (CsTL1) gene isolated from *Castanea sativa* seeds. Tree Physiol 32:1389-1402. DOI: 10.1093/treephys/tps098

da Câmara Machado ML, da Câmara Machado A, Hanzer V, Weiss H, Regner F, Steinkellner H et al. 1992. Regeneration of transgenic plants of *Prunus armeniaca* containing the coat protein gene of Plum Pox Virus. Plant Cell Rep 11:25-29. DOI: 10.1007/BF00231834

Dalla Costa L, Vaccari I, Mandolini M and Martinelli L. 2009. Elaboration of a reliable strategy based on real-time PCR to characterize

genetically modified plantlets and to evaluate the efficiency of a marker gene removal in grape (*Vitis* spp.). J Agric Food Chem 57:2668-2677. DOI: 10.1021/jf802740m

Dalla Costa L, Vinciguerra D, Giacomelli L, Salvagnin U, Piazza S, Spinella K et al. 2022. Integrated approach for the molecular characterization of edited plants obtained via *Agrobacterium tumefaciens*-mediated gene transfer. Eur Food Res Technol 248:289-299. DOI: 10.1007/s00217-021-03881-0

Fan C, Pu N, Wang X, Wang Y, Fang L, Xu W et al. 2007. *Agrobacterium*-mediated genetic transformation of grapevine (*Vitis vinifera* L.) with a novel stilbene synthase gene from Chinese wild *Vitis pseudoreticulata*. Plant Cell Tissue Organ Cult 92:197-206. DOI: 10.1007/s11240-007-9324-2

Gelvin SB. 2003. *Agrobacterium*-mediated plant transformation: The biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev 67:16-37. DOI: 10.1128/MMBR.67.1.16-37.2003

Hobbs SLA, Warkentin TD and DeLong CMO. 1993. Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol Biol 21:17-26. DOI: 10.1007/BF00039614

Humara JM, Marín MS, Parra F and Ordás RJ. 1999. Improved efficiency of *uidA* gene transfer in stone pine (*Pinus pinea*) cotyledons using a modified binary vector. Can J Forest Res 29:1627-1632. DOI: 10.1139/x99-117

Iglesias VA, Moscone EA, Papp I, Neuhuber F, Michalowski S, Phelan T et al. 1997. Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell 9:1251-1264. DOI: 10.1105/tpc.9.8.1251

Iocco P, Franks T and Thomas M. 2001. Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. Transgenic Res 10:105-112. DOI: 10.1023/A:1008989610340

Jayasankar S, Bondada BR, Li Z and Gray DJ. 2003. Comparative anatomy and morphology of *Vitis vinifera* (Vitaceae) somatic embryos from solid- and liquid-culture-derived proembryogenic masses. Am J Bot 90:973-979. DOI: 10.3732/ajb.90.7.973

Jefferson RA. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep 5:387-405. DOI: 10.1007/BF02667740

Kandel R, Bergey DR, Dutt M, Sitther V, Li ZT, Gray DJ et al. 2016. Evaluation of a grapevine-derived reporter gene system for precision breeding of *Vitis*. Plant Cell Tissue Organ Cult 124:599-609. DOI: 10.1007/s11240-015-0918-9

Kikkert JR, Striem MJ, Vidal JR, Wallace PG, Barnard J and Reisch BI. 2005. Long-term study of somatic embryogenesis from anthers and ovaries of 12 grapevine (*Vitis* sp.) genotypes. In Vitro Cell Dev Biol Plant 41:232-239. DOI: 10.1079/IVP2004609

Kooter JM, Matzke MA and Meyer P. 1999. Listening to the silent genes: Transgene silencing, gene regulation and pathogen control. Trends Plant Sci 4:340-347. DOI: 10.1016/S1360-1385(99)01467-3

Li ZT, Dhekney SA, Dutt M and Gray DJ. 2008. An improved protocol for *Agrobacterium*-mediated transformation of grapevine (*Vitis vinifera* L.). Plant Cell Tissue Organ Cult 93:311-321. DOI: 10.1007/s11240-008-9378-9

Mallón R, Vieitez AM and Vidal N. 2013. High-efficiency *Agrobacterium*-mediated transformation in *Quercus robur*: Selection by use of a temporary immersion system and assessment by quantitative PCR. Plant Cell Tissue Organ Cult 114:171-185. DOI: 10.1007/s11240-013-0313-3

Mathews H, Litz RE, Wilde HD, Merkle SA and Wetzstein HY. 1992. Stable integration and expression of β-glucuronidase and NPT II genes in mango somatic embryos. In Vitro Cell Dev Biol Plant 28:172-178. DOI: 10.1007/BF02823312

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x

Nitsch JP and Nitsch C. 1969. Haploid plants from pollen grains. Science 163:85-87. DOI: 10.1126/science.163.3862.85

Nuzzo F, Gambino G and Perrone I. 2022. Unlocking grapevine *in vitro* regeneration: Issues and perspectives for genetic improvement and functional genomic studies. Plant Physiol Biochem 193:99-109. DOI: 10.1016/j.plaphy.2022.10.027

Osakabe Y, Liang Z, Ren C, Nishitani C, Osakabe K, Wada M et al. 2018. CRISPR-Cas9-mediated genome editing in apple and grapevine. Nat Protoc 13:2844-2863. DOI: 10.1038/s41596-018-0067-9

Padilla IMG and Burgos L. 2010. Aminoglycoside antibiotics: Structure, functions and effects on *in vitro* plant culture and genetic transformation protocols. Plant Cell Rep 29:1203-1213. DOI: 10.1007/s00299-010-0900-2

Palomo-Ríos E, Barceló-Muñoz A, Mercado JA and Pliego-Alfaro F. 2012. Evaluation of key factors influencing *Agrobacterium*-mediated transformation of somatic embryos of avocado (*Persea americana* Mill.). Plant Cell Tissue Organ Cult 109:201-211. DOI: 10.1007/s11240-011-0086-5

Pfaffl MW, Horgan GW and Dempfle L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36. DOI: 10.1093/nar/30.9.e36

Prado MJ, Grueiro MP, González MV, Testillano PS, Domínguez C, López M et al. 2010. Efficient plant regeneration through somatic embryogenesis from anthers and ovaries of six autochthonous grapevine cultivars from Galicia (Spain). Sci Hortic 125:342-352. DOI: 10.1016/j.scienta.2010.04.019

Raharjo SHT, Witjaksono, Gomez-Lim MA, Padilla G and Litz RE. 2008. Recovery of avocado (*Persea americana* Mill.) plants transformed with the antifungal plant defensin gene PDF1.2. In Vitro Cell Dev Biol Plant 44:254-262. DOI: 10.1007/s11627-008-9117-2

Ramesh SA, Kaiser BN, Franks T, Collins G and Sedgley M. 2006. Improved methods in *Agrobacterium*-mediated transformation of almond using positive (mannose/*pmi*) or negative (kanamycin resistance) selection-based protocols. Plant Cell Rep 25:821-828. DOI: 10.1007/s00299-006-0139-0

Reid KE, Olsson N, Schlosser J, Peng F and Lund ST. 2006. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol 6:27. DOI: 10.1186/1471-2229-6-27

Ren C, Liu XJ, Zhang Z, Wang Y, Duan W, Li SH et al. 2016. CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (*Vitis vinifera* L.). Sci Rep 6:32289. DOI: 10.1038/srep32289

Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, van den Hoff MJB et al. 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37:e45. DOI: 10.1093/nar/gkp045

Saporta R, de la Torre F, Segura A and Vidal JR. 2014. Toxic effect of antibiotics in grapevine (*Vitis vinifera* 'Albariño') for embryo emergence and transgenic plant regeneration from embryogenic cell suspension. Vitis 53:89-94. DOI: 10.5073/vitis.2014.53.89-94

Saporta R, San Pedro T and Gisbert C. 2016. Attempts at grapevine (*Vitis vinifera* L.) breeding through genetic transformation: The main limiting factors. Vitis 55:173-186. DOI: 10.5073/vitis.2016.55.173-186

Song P, Cai C, Skokut M, Kosegi B and Petolino J. 2002. Quantitative real-time PCR as a screening tool for estimating transgene copy number in WHISKERS™-derived transgenic maize. Plant Cell Rep 20:948-954. DOI: 10.1007/s00299-001-0432-x

Stam M, Mol JNM and Kooter JM. 1997. The silence of genes in transgenic plants. Ann Bot 79:3-12. DOI: 10.1006/anbo.1996.0295

Tang W, Newton RJ and Weidner DA. 2007. Genetic transformation and gene silencing mediated by multiple copies of a transgene in eastern white pine. J Exp Bot 58:545-554. DOI: 10.1093/jxb/erl228

Torreblanca R, Cerezo S, Palomo-Ríos E, Mercado JA and Pliego-Alfaro F. 2010. Development of a high throughput system for genetic transformation of olive (*Olea europaea* L.) plants. Plant Cell Tissue Organ Cult 103:61-69. DOI: 10.1007/s11240-010-9754-0

Torregrosa L, Lopez G and Bouquet A. 2000. Antibiotic sensitivity of grapevine: A comparison between the effect of hygromycin and kanamycin on shoot development of transgenic 110 Richter Rootstock (*Vitis Berlandieri* x *Vitis rupestris*). S Afr J Enol Vitic 21:32-39. DOI: 10.21548/21-1-2185

Torregrosa L, Iocco P and Thomas MR. 2002. Influence of *Agrobacterium* strain, culture medium, and cultivar on the transformation efficiency of *Vitis vinifera* L. Am J Enol Vitic 53:183-190. DOI: 10.5344/ajev.2002.53.3.183

Urso S, Zottini M, Ruberti C, Lo Schiavo F, Stanca AM, Cattivelli L et al. 2013. An *Agrobacterium tumefaciens*-mediated gene silencing system for functional analysis in grapevine. Plant Cell Tissue Organ Cult 114:49-60. DOI: 10.1007/s11240-013-0305-3

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L and Rocha-Sosa M. 1990. Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220:245-250. DOI: 10.1007/BF00260489

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A et al. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:research0034.1. DOI: 10.1186/gb-2002-3-7-research0034

Vidal JR, Kikkert JR, Wallace PG and Reisch BI. 2003. High-efficiency biolistic co-transformation and regeneration of 'Chardonnay' (*Vitis vinifera* L.) containing npt-II and antimicrobial peptide genes. Plant Cell Rep 22:252-260. DOI: 10.1007/s00299-003-0682-x

Vidal N, Mallón R, Valladares S, Meijomín AM and Vieitez AM. 2010. Regeneration of transgenic plants by *Agrobacterium*-mediated transformation of somatic embryos of juvenile and mature *Quercus* robur. Plant Cell Rep 29:1411-1422. DOI: 10.1007/s00299-010-0931-8

Wang Q, Li P, Hanania U, Sahar N, Mawassi M, Gafny R et al. 2005. Improvement of *Agrobacterium*-mediated transformation efficiency and transgenic plant regeneration of *Vitis vinifera* L. by optimizing selection regimes and utilizing cryopreserved cell suspensions. Plant Sci 168:565-571. DOI: 10.1016/j.plantsci.2004.09.033

Wen L, Tan B and Guo WW. 2012. Estimating transgene copy number in precocious trifoliate orange by TaqMan real-time PCR. Plant Cell Tissue Organ Cult 109:363-371. DOI: 10.1007/s11240-011-0101-x

Yao J-L, Cohen D, Atkinson R, Richardson K and Morris B. 1995. Regeneration of transgenic plants from the commercial apple cultivar Royal Gala. Plant Cell Rep 14:407-412. DOI: 10.1007/BF00234044

Yevtushenko DP and Misra S. 2010. Efficient *Agrobacterium*-mediated transformation of commercial hybrid poplar *Populus nigra* L. x *P. maximowiczii* A. Henry. Plant Cell Rep 29:211-221. DOI: 10.1007/s00299-009-0806-z

Yuan JS, Burris J, Stewart NR, Mentewab A and Stewart Jr CN. 2007. Statistical tools for transgene copy number estimation based on real-time PCR. BMC Bioinform 8:S6. DOI: 10.1186/1471-2105-8-S7-S6

Zhang Y, Liu Y, Zhang J, Wang G, Wang J and Liu Y. 2015. Assessment of transgene copy number and zygosity of transgenic maize overexpressing *Cry1Ie* gene with SYBR® Green qRT-PCR. In Vitro Cell Dev Biol Plant 51:125-134. DOI: 10.1007/s11627-014-9658-5

Zhao W, Zheng S and Ling HQ. 2011. An efficient regeneration system and *Agrobacterium*-mediated transformation of Chinese upland rice cultivar Handao297. Plant Cell Tissue Organ Cult 106:475-483. DOI: 10.1007/s11240-011-9946-2

Zhou Q, Dai L, Cheng S, He J, Wang D, Zhang J et al. 2014. A circulatory system useful both for long-term somatic embryogenesis and genetic transformation in *Vitis vinifera* L. cv. Thompson Seedless. Plant Cell Tissue Organ Cult 118:157-168. DOI: 10.1007/s11240-014-0471-y