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ORIGINAL ARTICLE

# Genetic transformation of grape varieties and rootstocks via organogenesis

**Xiaoqing Xie[1,2,3] · Cecilia B. Agüero[4] · Yuejin Wang[1,2,3] · M. Andrew Walker[4]**

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**Abstract** A protocol was standardized to regenerate six grape cultivars through meristematic bulk (MB) induction, which was used for genetic transformation. Meristematic bulk induction worked best with *Vitis vinifera* 'Thompson Seedless' (98.4 %), followed by 'Chardonnay' (97.6 %), 'Redglobe' (90.2 %) and 'Cabernet Sauvignon' (86.2 %), and was less successful with *Vitis rupestris* 'St. George' (85.4 %) and '101-14 Millardet et de Grasset (*Vitis riparia* × *V. rupestris*)' (79.6 %). Benzylaminopurine and naphthaleneacetic acid was the most effective combination of cytokinin and auxin for MB formation. 100 µg/ml kanamycin was a better antibiotic selection agent than 2.0 µg/ml hygromycin during transformation. The expression of green fluorescent protein was evaluated with in vitro leaves and roots. Transformation efficiency using meristematic slices was a function of the genotype. Transformation efficiency was greatest in Chardonnay (51.7 %), followed by Thompson Seedless (42.3 %), St. George (41.6 %), Redglobe (40 %), Cabernet Sauvignon (35.6 %) and 101-14 Mgt (29.9 %). This study found that MB induction was a fast and simple alternative for genetic transformation of grape cultivars.

**Keywords** Grape · Meristematic bulk · Regeneration · Genetic transformation

**Abbreviations**

| | |
|---|---|
| BA | 6-Benzylaminopurine |
| NAA | α-Naphthaleneacetic acid |
| TDZ | Thidiazuron |
| MB | Meristematic bulk |

## Introduction

The use of genetic transformation for molecular breeding in grape permits the insertion of specific useful genes without inducing significant genetic rearrangement in other areas of the genome. The application of tissue culture methods for grape genetic transformation depends on the availability of highly reproducible and efficient in vitro regeneration systems (Gray et al. 2014). To date, the regeneration of grape plants has been achieved from different explant types via both embryogenesis and organogenesis.

Mullins and Srinivasan (1976) were the first to regenerate complete plants in *Vitis* using embryogenesis from unfertilized ovules. Embryonic cultures obtained from zygotic or somatic embryos (Martinelli and Mandolino

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✉ Yuejin Wang
wangyj@nwsuaf.edu.cn

✉ M. Andrew Walker
awalker@ucdavis.edu

[1] College of Horticulture, Northwest A&F University, No. 3, Taicheng Road, Yangling 712100, Shaanxi, People's Republic of China

[2] Key Laboratory of Horticultural Plant Biology and Germplasm Innovation in Northwest China, Ministry of Agriculture, Yangling 712100, Shaanxi, People's Republic of China

[3] State Key Laboratory of Crop Stress Biology in Arid Areas, Northwest A&F University, No. 3, Taicheng Road, Yangling 712100, Shaanxi, People's Republic of China

[4] Department of Viticulture and Enology, University of California, Davis, 595 Hilgard Lane, Davis, CA 95616-3014, USA

1994; Mullins et al. 1990), petioles and leaves (Martinelli and Mandolino 2001), anthers and ovaries (Franks et al. 1998; Gray 1995; Iocco et al. 2001) have been extensively used as target materials for genetic transformation. However, regeneration and transformation achieved through embryogenic cultures are difficult and restricted to a few genotypes. The process demands the continuous induction and maintenance of embryonic cultures, requiring intensive labor, time, and space, as well as patience and skill.

Grape plants can also be regenerated via organogenesis. To our knowledge, in vitro adventitious bud formation in grapevine was first reported by Favre (1977). Adventitious shoot organogenesis has since been achieved from fragmented shoot apices (Barlass and Skene 1980; Dutt et al. 2007), inter-node segments (Favre 1977; Kurmi et al. 2011; Rajasekaran and Mullins 1981), leaves (Favre 1977; Nicholson et al. 2012; Stamp et al. 1990a, b; Torregrosa and Bouquet 1996; Zhang et al. 2011) and petioles (Reisch et al. 1990). However, there are reports of limited success when using shoot organogenesis in genetic transformation. An early study of direct shoot organogenesis suggests that transformed leaf lamina cells exhibiting GUS protein (beta-glucuronidase gene) expression are never involved in shoot regeneration (Colby and Meredith 1990). Moreover, Péros et al. (1998) found that efficiency of in vitro techniques is strongly genotype dependent, with 'Pinot Noir' and 'Pinot Meunier' exhibiting the lowest capabilities for leaf organogenesis when compared to 20 other grape cultivars.

In 2002, Mezzetti et al. reported on the genetic transformation of *Vitis vinifera* via organogenesis through meristematic bulks (MB) produced in the presence of α-naphtaleneacetic acid (NAA) and with increasing concentrations of benzylaminopurine (BA). They found that slices from MB tissue resulted in high regeneration-efficiency, and they used this technique to genetically engineer the table grape cultivars 'Silcora' and Thompson Seedless. In addition, non-transformed cells could be killed during three subcultures with increasing concentrations of kanamycin at 25, 50 and 75 mg/l in the selection medium. Bertsch et al. (2005) followed the Mezzetti et al. (2002) protocol to study genetic chimerism in Chardonnay. Their results suggested that plants obtained via organogenesis in the absence of antibiotics through MB were derived from both L1 and L2 cell layers of the Chardonnay tissues from which they grew, as opposed to separate regeneration events from the L1 and L2 cell layers as seen by others (Franks et al. 2002). Plants regenerated from MB might also overcome the juvenile phase exhibited by plants regenerated from embryogenesis (Ibáñez et al. 2011).

The objective of this study was to investigate the induction and maintenance of MB in six grape genotypes (*V. vinifera* cultivars Thompson Seedless, Chardonnay, Redglobe and Cabernet Sauvignon, and rootstocks *V. rupestris* St. George, and 101-14 Millardet et de Grasset (*V. riparia* × *V. rupestris*), to establish an effective protocol for genetic transformation via *Agrobacterium tumefaciens*. This technique has great potential to expedite genetic transformation and regeneration of grape.

## Materials and methods

### Plant materials

Herbaceous green cuttings of *V. vinifera* cvs. Chardonnay, Thompson Seedless, Redglobe, Cabernet Sauvignon, and rootstocks *V. rupestris* cv. St. George, and 101-14 Millardet et de Grasset (*V. riparia* × *V. rupestris*) (101-14 Mgt) collected from field-grown mothervines, were introduced to in vitro conditions and micropropagated on solid MS medium (Murashige and Skoog 1962) supplemented with 0.025 μM naphthaleneacetic acid (NAA).

### Initiation and maintenance of meristematic bulks

$IM_0$ medium, consisting of solid MS medium and 4.4 μM BA (Mezzetti et al. 2002), was used to multiply shoots. The shoot apices from proliferating shoots were gently sliced twice and then subcultured every 4 weeks onto media $IM_1$, $IM_2$ and $IM_3$ to induce the formation of meristematic bulks. A letter, A–L, was assigned to 12 $IM_1$–$IM_2$–$IM_3$ sequences which included combinations of NAA and increasing doses of BA or Thidiazuron (TDZ), a cytokinin-like compound that can stimulate the formation of adventitious shoots and somatic embryos (Hanson et al. 1999; Reisch et al. 1990). TDZ was also tested in combination with BA in 6 additional treatments designated M–R (Table 1). Meristematic bulks were maintained on $IM_3$ medium and sub-cultured every 4 weeks. Each of these media contained 3 % sucrose and 0.8 % agar, with the pH adjusted to 5.8–5.9 before autoclaving at 121 °C for 25 min. All cultures were maintained in growth chambers at 25 ± 1 °C under a 16 h photoperiod regime (cool white fluorescent light, 60 μmol $m^{-2}$ $s^{-1}$). MB induction rate was calculated as [(number of shoot tips developing MB/number of treated shoot tips) × 100].

### Genetic transformation

#### *Vectors and Agrobacterium tumefaciens strains*

Transformation experiments were carried out using *A. tumefaciens* strain EHA105 pCH32 harboring pCAMBIA 1303 or pCAMBIA 2303 binary vectors, which contain the *hygII* and *nptII* coding regions that confer hygromycin

**Table 1** Composition of the media tested for meristematic bulk induction in *Vitis*

| Treatment | Growth regulators (μM) | | | Treatment | Growth regulators (μM) | | |
|---|---|---|---|---|---|---|---|
| | BA | NAA | TDZ | | BA | NAA | TDZ |
| A | | | | J | | | |
| $IM_1$ | 4.40 | 0.05 | | $IM_1$ | | 0.10 | 1.00 |
| $IM_2$ | 6.60 | 0.05 | | $IM_2$ | | 0.10 | 2.00 |
| $IM_3$ | 8.80 | 0.05 | | $IM_3$ | | 0.10 | 4.00 |
| B | | | | K | | | |
| $IM_1$ | 8.80 | 0.05 | | $IM_1$ | | 0.50 | 0.25 |
| $IM_2$ | 13.2 | 0.05 | | $IM_2$ | | 0.50 | 0.50 |
| $IM_3$ | 17.6 | 0.05 | | $IM_3$ | | 0.50 | 1.00 |
| C | | | | L | | | |
| $IM_1$ | 4.40 | 0.10 | | $IM_1$ | | 0.50 | 1.00 |
| $IM_2$ | 6.60 | 0.10 | | $IM_2$ | | 0.50 | 2.00 |
| $IM_3$ | 8.80 | 0.10 | | $IM_3$ | | 0.50 | 4.00 |
| D | | | | M | | | |
| $IM_1$ | 8.80 | 0.10 | | $IM_1$ | 4.40 | | 0.25 |
| $IM_2$ | 13.2 | 0.10 | | $IM_2$ | 6.60 | | 0.50 |
| $IM_3$ | 17.6 | 0.10 | | $IM_3$ | 8.80 | | 1.00 |
| E | | | | N | | | |
| $IM_1$ | 4.40 | 0.50 | | $IM_1$ | 8.80 | | 0.25 |
| $IM_2$ | 6.60 | 0.50 | | $IM_2$ | 13.2 | | 0.50 |
| $IM_3$ | 8.80 | 0.50 | | $IM_3$ | 17.6 | | 1.00 |
| F | | | | O | | | |
| $IM_1$ | 8.80 | 0.50 | | $IM_1$ | 4.40 | | 1.00 |
| $IM_2$ | 13.2 | 0.50 | | $IM_2$ | 6.60 | | 2.00 |
| $IM_3$ | 17.6 | 0.50 | | $IM_3$ | 8.80 | | 4.00 |
| G | | | | P | | | |
| $IM_1$ | | 0.05 | 0.25 | $IM_1$ | 8.80 | | 1.00 |
| $IM_2$ | | 0.05 | 0.50 | $IM_2$ | 13.2 | | 2.00 |
| $IM_3$ | | 0.05 | 1.00 | $IM_3$ | 17.6 | | 4.00 |
| H | | | | Q | | | |
| $IM_1$ | | 0.05 | 1.00 | $IM_1$ | 4.40 | | 0.25 |
| $IM_2$ | | 0.05 | 2.00 | $IM_2$ | 8.80 | | 0.50 |
| $IM_3$ | | 0.05 | 4.00 | $IM_3$ | 13.2 | | 1.00 |
| I | | | | R | | | |
| $IM_1$ | | 0.10 | 0.25 | $IM_1$ | 4.40 | | 1.00 |
| $IM_2$ | | 0.10 | 0.50 | $IM_2$ | 8.80 | | 2.00 |
| $IM_3$ | | 0.10 | 1.00 | $IM_3$ | 13.2 | | 4.00 |

Proliferating shoots in $IM_0$ were subsequently subcultured onto media $IM_1$ (first subculture medium), $IM_2$ (second subculture medium) and $IM_3$ (third subculture medium) containing solid MS medium supplemented with different combinations of plant growth regulators

(pCAMBIA 1303) and kanamycin (pCAMBIA 2303) resistance as plant selectable marker (www.cambia.org).

*Transformation*

Bacterial cultures ($A_{600}$ = 0.5–1.0) were grown overnight (28 °C–150 rpm) in LB medium with 50 μg/ml rifampicin, 10 μg/ml tetracycline and 50 μg/ml kanamycin, centrifuged, and resuspended in MS salts supplemented with 2 % sucrose and 100 μM acetosyringone (pH 5.2) for 5 h at 25 °C. Slices (1 $cm^2$, 2 mm thick) obtained from the MB were dipped in the bacterial suspension for 15 min. After immersion, the slices were blotted onto sterile filter paper and placed on $IM_3$ medium with 100 μM acetosyringone for co-cultivation.

**Table 2** Influence of genotype and medium type on the induction of meristematic bulks (MB)

| Genotype | A | | B | | C | | D | | E | | F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| *Treatment* | | | | | | | | | | | | |
| Chardonnay | 255 (137) | 53.7$^{c,d,e}$ | 255 (236) | 92.5$^{a}$ | 255 (249) | 97.6$^{a}$ | 255 (112) | 43.9$^{e,f,g,h}$ | 255 (248) | 97.3$^{a}$ | 255 (148) | 58.0$^{c,d}$ |
| Thompson Seedless | 255 (231) | 90.6$^{a}$ | 255 (240) | 94.1$^{a}$ | 255 (195) | 76.5$^{b}$ | 255 (244) | 95.7$^{a}$ | 255 (251) | 98.4$^{a}$ | 255 (130) | 51.0$^{d,e}$ |
| Redglobe | 255 (209) | 82.0$^{a}$ | 255 (215) | 84.3$^{a}$ | 255 (180) | 70.6$^{b}$ | 255 (223) | 87.5$^{a}$ | 255 (230) | 90.2$^{a}$ | 2555 (135) | 52.9$^{c,d}$ |
| Cabernet Sauvignon | 255 (139) | 54.5$^{d,e,f}$ | 255 (209) | 82.0$^{a}$ | 255 (220) | 86.3$^{a}$ | 255 (120) | 47.1$^{e,f,g,h}$ | 255 (200) | 78.4$^{a,b}$ | 255 (145) | 56.9$^{d,e}$ |
| St. George | 255 (48) | 18.8$^{h,i,j}$ | 255 (55) | 21.7$^{h,i}$ | 255 (15) | 5.9$^{k}$ | 255 (44) | 17.3$^{i,j}$ | 255 (47) | 18.4$^{h,i,j}$ | 255 (48) | 18.8$^{h,i,j}$ |
| 101-14 Mgt | 255 (33) | 12.9$^{g}$ | 255 (41) | 16.1$^{g}$ | 255 (49) | 19.2$^{f,g}$ | 255 (11) | 4.3$^{h}$ | 255 (50) | 19.6$^{f,g}$ | 255 (49) | 19.2$^{f,g}$ |

| Genotype | G | | H | | I | | J | | K | | L | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Chardonnay | 180 (78) | 43.3$^{e,f,g,h}$ | 180 (70) | 38.9$^{g,h}$ | 180 (81) | 45.0$^{e,f,g,h}$ | 180 (90) | 50.0$^{d,e,f,g}$ | 180 (75) | 41.7$^{e,f,g,h}$ | 180 (71) | 39.4$^{f,g,h}$ |
| Thompson Seedless | 180 (90) | 50.0$^{d,e}$ | 180 (73) | 40.6$^{e,f,g}$ | 180 (64) | 35.6$^{g}$ | 180 (82) | 45.6$^{e,f,g}$ | 180 (74) | 41.1$^{e,f,g}$ | 180 (68) | 37.8$^{f,g}$ |
| Redglobe | 180 (82) | 45.6$^{d,e,f}$ | 180 (72) | 40.0$^{e,f,g}$ | 180 (59) | 32.8$^{g}$ | 180 (80) | 44.4$^{d,e,f}$ | 180 (73) | 40.6$^{e,f,g}$ | 180 (64) | 35.6$^{f,g}$ |
| Cabernet Sauvignon | 180 (72) | 40.0$^{g,h}$ | 180 (71) | 39.4$^{g,h}$ | 180 (80) | 44.4$^{f,g,h}$ | 180 (86) | 47.8$^{e,f,g,h}$ | 180 (72) | 40.0$^{g,h}$ | 180 (67) | 37.2$^{h}$ |
| St. George | 180 (55) | 30.6$^{f,g}$ | 180 (15) | 8.3$^{k}$ | 180 (61) | 33.9$^{e,f,g}$ | 180 (21) | 11.7$^{j,k}$ | 180 (49) | 27.2$^{g,h}$ | 180 (48) | 26.7$^{g,h}$ |
| 101-14 Mgt | 180 (8) | 4.4$^{h}$ | 180 (32) | 17.8$^{g}$ | 180 (35) | 19.4$^{f,g}$ | 180 (49) | 27.2$^{e,f}$ | 180 (53) | 29.4$^{e}$ | 180 (40) | 22.2$^{e,f,g}$ |

| Genotype | M | | N | | O | | P | | Q | | R | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Chardonnay | 260 (96) | 36.9$^{h}$ | 260 (136) | 52.3$^{d,e,f}$ | 260 (113) | 43.5$^{e,f,g,h}$ | 260 (121) | 46.5$^{d,e,f,g,h}$ | 260 (169) | 65.0b$^{c}$ | 260 (186) | 71.5$^{b}$ |
| Thompson Seedless | 260 (105) | 40.4$^{e,f,g}$ | 260 (155) | 59.6$^{c,d}$ | 260 (111) | 42.7$^{e,f,g}$ | 260 (124) | 47.7$^{e,f}$ | 260 (179) | 68.8$^{b,c}$ | 260 (232) | 89.2$^{a}$ |
| Redglobe | 260 (102) | 39.2$^{e,f,g}$ | 260 (151) | 58.1$^{c}$ | 260 (108) | 41.5$^{e,f,g}$ | 260 (122) | 46.9$^{e,d}$ | 260 (161) | 61.9$^{b,c}$ | 260 (225) | 86.5$^{a}$ |
| Cabernet Sauvignon | 260 (94) | 36.2$^{h}$ | 260 (132) | 50.8$^{d,e,f,g}$ | 260 (112) | 43.1$^{f,g,h}$ | 260 (119) | 45.8$^{e,f,g,h}$ | 260 (157) | 60.4$^{c,d}$ | 260 (183) | 70.4$^{b,c}$ |
| St. George | 260 (101) | 38.8$^{d,e,f}$ | 260 (148) | 56.9$^{c}$ | 260 (104) | 40.0$^{d,e}$ | 260 (114) | 43.8$^{d}$ | 260 (170) | 65.4$^{b}$ | 260 (222) | 85.4$^{a}$ |
| 101-14 Mgt | 260 (103) | 39.6$^{d}$ | 260 (151) | 58.1$^{c}$ | 260 (109) | 41.9$^{d}$ | 260 (122) | 46.9$^{d}$ | 260 (174) | 66.9$^{b}$ | 260 (207) | 79.6$^{a}$ |

No., number of treated shoot tips; %, MB induction rate; in parentheses, shoot tips developing MB

Means of the same genotype with different letters are significantly different at the 0.05 level

### *Selection*

After 48 h at 25 °C in dark, the slices were transferred to the same medium containing 300 µg/ml cefotaxime. Kanamycin or hygromycin were added to the medium 0, 1 or 2 weeks after co-cultivation at 100 µg/ml and 1–2.5 µg/ml respectively. Explants were subcultured on a monthly basis on the same medium. Survival rate was calculated as [(number of MB slices with regenerated shoots in selection medium/total number of treated MB slices) × 100].

### *Acclimatization of plant*

When new shoots were visible, they were transferred to MS medium supplemented with 2 µM BA without antibiotics for shoot elongation. After 4 weeks, plantlets were transferred to flasks containing modified WPM medium (Agüero et al. 2006). In vitro rooted transgenic plants were acclimatized and grown under greenhouse conditions in 1000 cc pots containing commercial potting soil mix.

### *DNA extraction and PCR analysis*

Grapevine DNA was isolated from 0.5 to 1.0 g of plants regenerated in selection medium (Thomas et al. 1993). Primer pairs used in PCR analysis were: 5′-ACTTCTA CACAGCCATCGGT-3′ and 5′-CGCAAGGAATCGGT CAATACA-3′ for *hptII* (GenBank accession No. AF234299); and 5′-ACCGTAAAGCACGAGGAAGC-3′ and 5′-ATGAACTCCAGGACGAGGCA-3′ for *nptII* (Genebank accession No. AF485783). PCR reactions were performed using the PCR Amplification Kit (Takara, Dalian, China) with the following cycle parameters: 3 min at 94 °C, followed by 1 min at 94 °C, 1.5 min at 65 °C, and 2 min at 72 °C for 35 cycles, with a 4-min extension at 72 °C in the last cycle. After amplification, PCR products were analyzed by GelRed$^{TM}$ (Biotium, Inc.)-stained agarose gel electrophoresis. Transformation efficiency was calculated as [(number of MB slices with PCR positive plant/total number of treated MB slices) × 100]. If more than one plant regenerated from a single slice, they were

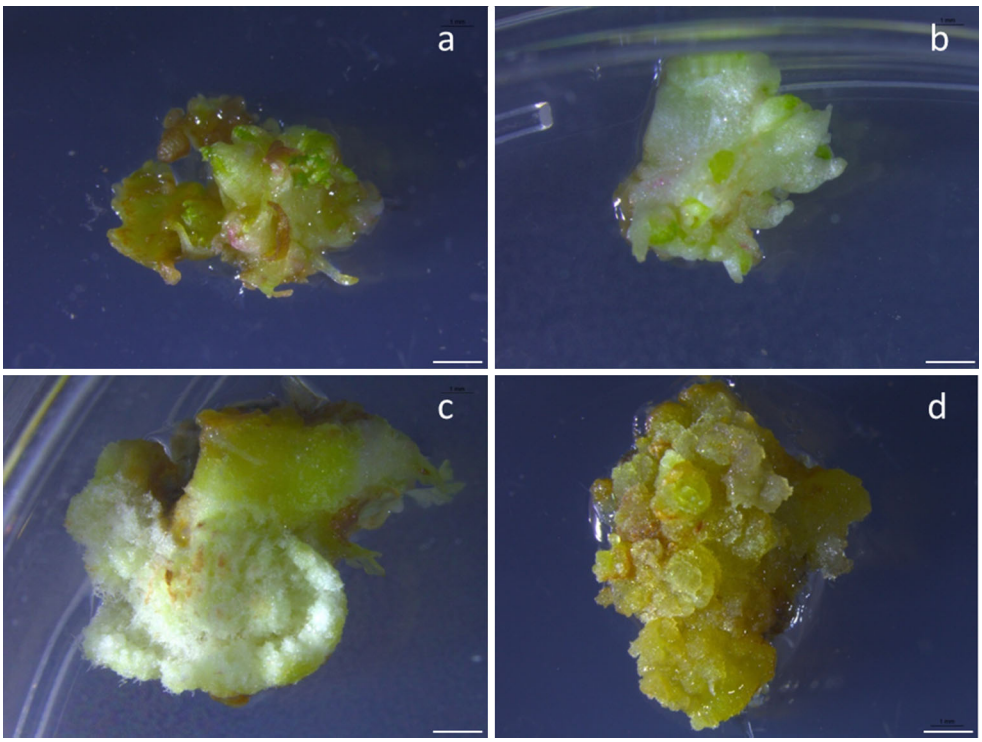


**Fig. 1** Different types of callus induced from explants of Thompson Seedless. **a**, **b** Compact, translucent, expanded callus induced on media B-IM$_3$ (*bar* = 1.0 mm); **c**, **d** Dark, soft callus induced on media R-IM$_3$ (*bar* = 1.0 mm)

considered to originate from the same transformation event, although they might not have.

*GFP detection*

Expression of green fluorescent protein in inoculated MB and developing plantlets was observed under an Olympus FluoView FV1000 Confocal Microscope (Olympus Corp., Tokyo, Japan) at an excitation wavelength of 480 ± 20 nm and emission wavelength of 510 ± 20 nm. Photographs were taken with an Olympus digital camera attached to the microscope.

### Statistical analysis

Data were analyzed by one-way ANOVA followed by post hoc comparison test (Student Newman–Keuls) at $P = 0.05$ with SPSS 21.0 for Windows (SPSS Inc., Chicago, IL).

## Results

### Influence of genotype and medium type on initiation of MB

Three different sets of plant growth regulators were added to MS basal media to initiate and maintain MB. The influence of genotype and medium type on the induction of MB was assessed 4 months after cultivation. The combined effects of cytokinin and auxin in different concentrations on the various culture phases are presented in Tables 1 and 2. Maximum MB induction frequency was produced in treatments that combined BA and NAA. BA + TDZ combinations exhibited moderate to high MB induction rates in all 6 cultivars, while shoots produced on the NAA + TDZ combination media were stunted and distorted.

Interaction between genotype and culture medium resulted in different optimal hormonal treatments for MB induction in the cultivars assayed (Table 2). The highest MB frequency was obtained with Thompson Seedless which exhibited 90.6, 94.1, 95.7, 98.4, and 89.2 % on media A, B, D, E and R, respectively. The other table grape cultivar, Redglobe, exhibited 82, 84.3, 87.5, 90.2, and 86.6 % on the same media. Wine grape cultivars Chardonnay and Cabernet Sauvignon produced the highest percentages on media B (92.5 and 82 %), C (97.6 and 86.2 %), and E (97.3 and 78.4 %). Medium R was most suitable for MB induction of rootstocks St. George (85.4 %) and 101-14 Mgt (79.6 %). In terms of genotypic response, Thompson Seedless was the most successful cultivar.

Shoot proliferation was successfully induced on the IM$_0$ medium in all the genotypes tested. By the end of first

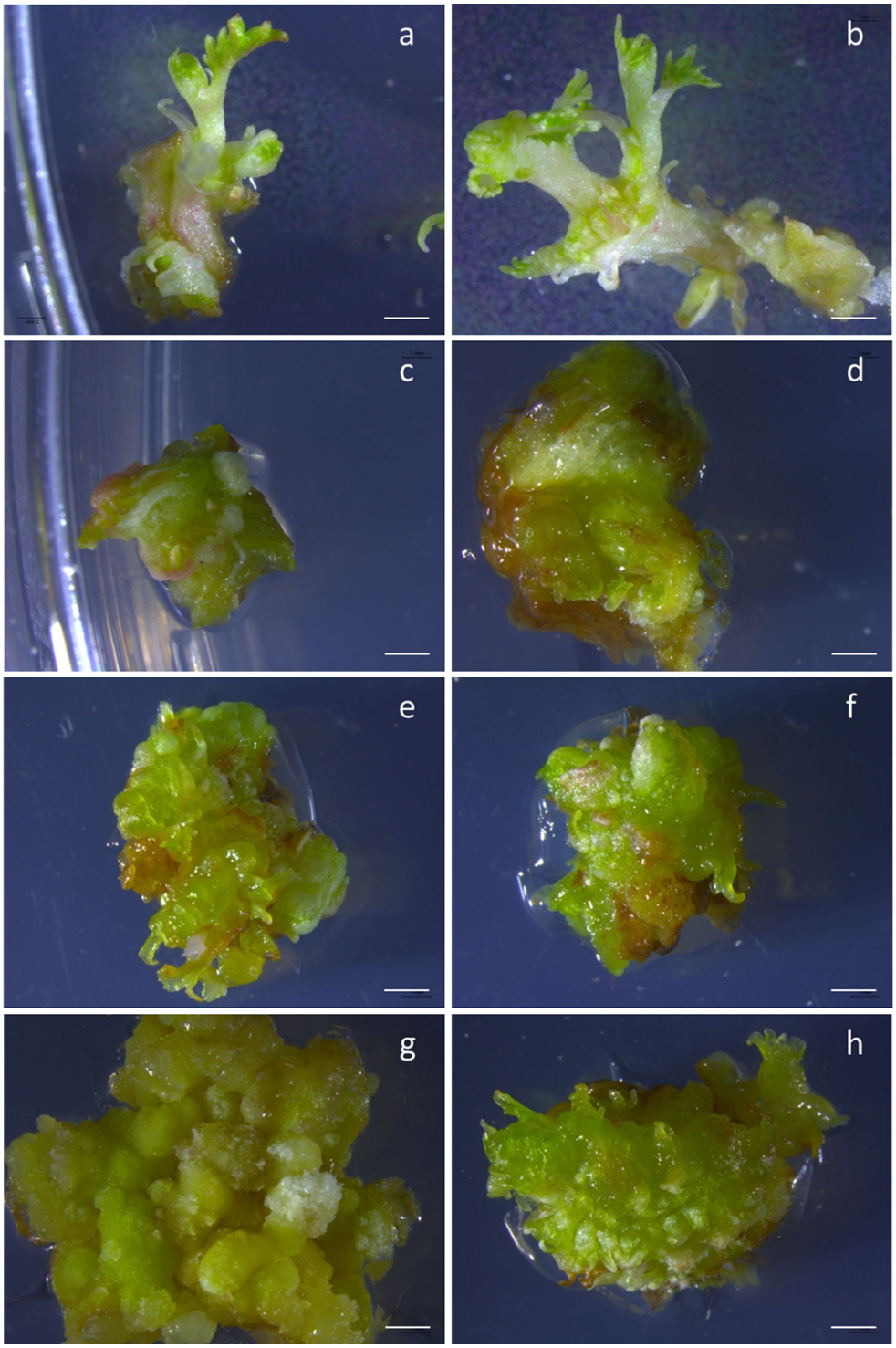


**Fig. 2** Organogenic culture system in *V. vinifera* cv. Chardonnay (*column 1*) and rootstock 101-14 Mgt (*column 2*). **a**, **b** Cultured shoot tips on C-IM$_1$ (**a**) and R-IM$_1$ (**b**) after the first 2 weeks; **c**, **d** Meristematic tissue on C-IM$_1$ (**c**) and R-IM$_1$ (**d**); **e**, **f** Meristematic tissue on C-IM$_2$ (**e**) and R-IM$_2$ (**f**); **g**, **h** Meristematic bulks on C-IM$_3$ (**g**) and R-IM$_3$ (*bars* = 1.0 mm)

4 weeks on IM$_0$, new shoots had swollen bases, a characteristic usually associated with shoot multiplication. However, different types of callus were obtained over subsequent subcultures. We found that the type of the callus produced during subculturing determined whether it would develop into MB. Compact, translucent, expanded

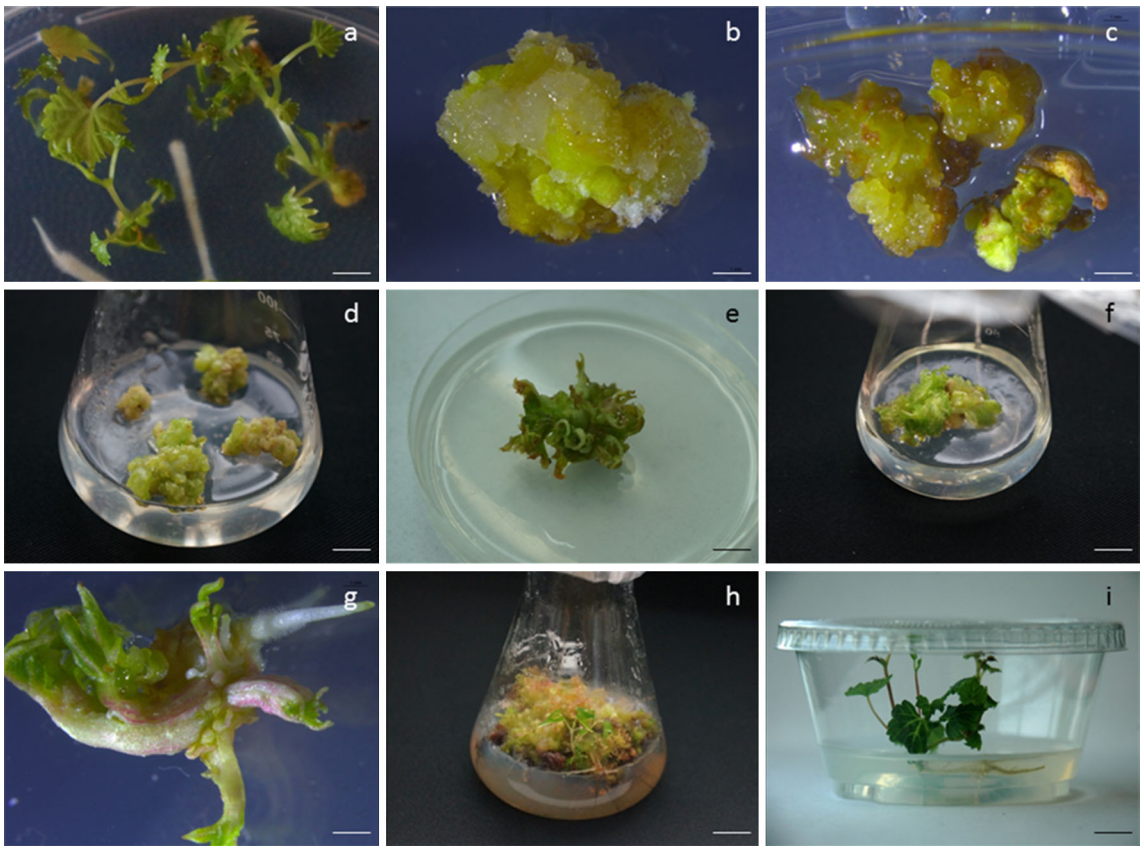


**Fig. 3** Genetic transformation system in *V. vinifera* cv. Thompson Seedless through organogenic regeneration. **a** Shoot cluster on $IM_0$ (*bar* = 5 mm); **b** Unorganized callus on media $IM_0$ (*bar* = 1.0 mm); **c** *Dark-brown* callus on media E-$IM_3$ (*bar* = 1.0 mm); **d** Transgenic meristematic bulk induced from transgenic meristematic slice on media E-$IM_3$ (*bar* = 5 mm); **e**, **f** Development of new shoots from transgenic meristematic bulks on media E-$IM_3$ (*bar* = 5 mm); **g** Formation of new roots on media E-$IM_3$ (*bar* = 4.5 mm); **h** Elongation of transgenic shoots on MS medium supplemented with 2 μM BA (*bar* = 4 mm); **i** Formation of transgenic grape plantlet on modified WPM medium (*bar* = 1.2 cm). (Color figure online)

callus (Fig. 1a, b) successfully differentiated into MB, while dark, soft callus (Fig. 1c, d) rarely showed meristematic competence. On $IM_1$, callus proliferation started from the portion in contact with the medium and spread upward after 2 weeks of culture (Fig. 2a, b). Green callus masses with many enlarged buds after 3–4 weeks of culture on $IM_1$ were then transferred to $IM_2$ (Fig. 2c, d). Adventitious buds developed directly from the surface of the callus masses (Fig. 2e, f). Adventitious buds were observed after the first subculture, and meristematic bulks were developed during each subculture. The majority of those adventitious structures and MB were generated by the third subculture on $IM_3$. At this time, MB were a mass of adventitious buds, less than 0.5 mm, some exhibiting leaf primordia differentiation (Fig. 2g, h). Observation under the microscope showed that several meristemoids developed on the surface of proliferating nodular compact MB (Fig. 2; Figs. S1, S2). Each meristemoid was connected with well-developed vascular tissues and was adjacent to shoot apical meristems surrounded by reduced leaf primordia. Meristematic bulks were maintained and multiplied by subculture every 4 weeks on $IM_3$.

Plantlet regeneration happened gradually after organogenic calli were subcultured onto $IM_0$. Developing shootlets were subsequently transferred to elongation medium (MS medium + 2 μM BA), where the length of shoots was significantly increased. Root initiation started 10–15 days after transfer of shootlets to modified WPM medium. Although the traits were not scored quantitatively, regenerated plants appeared phenotypically normal and true-to-type.

## Effects of selection on transformation efficiency

Transgenic plants of the six cultivars tested were regenerated from slices of MB inoculated with *Agrobacterium* and transformed with two different plasmids (Fig. 3). Effect of kanamycin and hygromycin on survival rate and transformation efficiency was assessed 8 weeks after selection.

**Table 3** Survival rate and transformation efficiency obtained from Chardonnay and Thompson Seedless using different concentrations of hygromycin for selection starting immediately after co-cultivation

| Antibiotics (μg/ml) | Number of treated MB slices (Surviving) | Survival rate (%) | Number of MB slices with PCR positive plants | Transformation efficiency (%) |
|---|---|---|---|---|
| Chardonnay | | | | |
| HYG 1.0 | 48 (19) | 39.06 ± 4.42[a] | 4 | 8.33 ± 2.55[b] |
| HYG 1.5 | 48 (24) | 48.44 ± 4.42[a] | 5 | 10.42 ± 2.55[a,b] |
| HYG 2.0 | 48 (8) | 18.75 ± 6.63[b] | 7 | 14.58 ± 5.1[a] |
| HYG 2.5 | 48 (7) | 13.75 ± 0.44[b] | 6 | 12.5 ± 5.1[a,b] |
| Thompson Seedless | | | | |
| HYG 1.0 | 69 (50) | 71.31 ± 3.65[a] | 11 | 11.74 ± 0.73[a] |
| HYG 1.5 | 56 (20) | 36.67 ± 12.25[b] | 11 | 14.17 ± 1.02[a] |
| HYG 2.0 | 48 (12) | 25 ± 12.76[b] | 7 | 14.58 ± 2.55[a] |
| HYG 2.5 | 48 (7) | 14.58 ± 7.65[b] | 6 | 12.5 ± 5.1[a] |

*HYG* hygromycin

Each value represents the mean ± SE of three different experiments. Means with different letters are significantly different at the 0.05 level

**Table 4** Survival rate and transformation efficiency obtained from Chardonnay and Thompson Seedless using delayed selection with different concentrations of hygromycin and kanamycin

| Antibiotics (μg/ml) | Number of treated MB slices (Surviving) | Survival rate (%) | Number of MB slices with PCR positive plants | Transformation efficiency (%) |
|---|---|---|---|---|
| Chardonnay | | | | |
| HYG 2.0—1 wk | 175 (106) | 60.57 ± 7.48[c] | 19 | 13.33 ± 1.63[b] |
| KAN 100—1 wk | 225 (190) | 84.44 ± 11.31[b] | 93 | 48 ± 1.63[a] |
| HYG 2.0—2 wk | 161 (149) | 91.69 ± 4.86[a,b] | 15 | 10.67 ± 1.63[b] |
| KAN 100—2 wk | 175 (170) | 97.14 ± 2.14[a] | 63 | 37.33 ± 3.27[a] |
| Thompson Seedless | | | | |
| HYG 2.0—1 wk | 175 (135) | 77.14 ± 4.28[c] | 23 | 10.67 ± 1.63[b] |
| KAN 100—1 wk | 225 (204) | 90.67 ± 1.89[b] | 108 | 41.33 ± 2.67[a] |
| HYG 2.0—2 wk | 175 (161) | 92.8 ± 6.32[a,b] | 17 | 9.33 ± 1.63[b] |
| KAN 100—2 wk | 150 (146) | 97.33 ± 1.15[a] | 56 | 36 ± 1.63[a] |

*KAN* Kanamycin, *HYG* hygromycin, *wk* weeks after co-cultivation

Each value represents the mean ± SE of three different experiments. Means with different letters are significantly different at the 0.0

During selection, tissues became yellow and brown due to antibiotic stress. Only the transformed cells maintained a high regenerative capacity while the other parts of the meristematic tissue became progressively necrotic. Since hygromycin severely inhibited the development of plant tissue, we compared the relationship of survival rate with transformation efficiency under different concentrations of hygromycin (1–2.5 μg/ml). The survival rate of Chardonnay and Thompson Seedless explants declined with increasing hygromycin concentration, and about 85 % of the tissues were damaged at 2–2.5 μg/ml (Table 3). However, transformation efficiency was promoted with an increase in hygromycin concentration, with 2 μg/ml of hygromycin producing the best results.

The effect of two delayed selection procedures on survival rate and transformation efficiency is summarized in Table 4. Results found that starting selection 1 or 2 weeks later allowed for higher survival rates (Tables 3, and 4). In Chardonnay, the survival rate with 2 μg/ml hygromycin selection immediately after co-cultivation was 18.75 % successful (Table 3), compared with success rates of 60.57 % (1 week delay) and 91.69 % (2 week delay) (Table 4). Similar effects were seen with Thompson Seedless, where the survival rate after immediate co-cultivation with 2 μg/ml hygromycin was 25 % (Table 3), while delayed selection produced success rates of 77.14 % (1 week delay) and 92.8 % (2 week delayed) (Table 4). However, delayed selection did not increase transformation

**Table 5** Efficiency of genetic transformation upon immediate selection treatment of tested cultivars

| Antibiotics (μg/ml) | Chardonnay | | Thompson Seedless | | Redglobe | | Cabernet Sauvignon | | St. George | | 101-14 Mgt | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| HYG 2.0 | 65 (11) | 16.26 ± 1.25$^{b,c}$ | 39 (6) | 15.15 ± 0.61$^{c}$ | 45 (14) | 31.11 ± 7.7$^{a}$ | 45 (12) | 26.67 ± 6.67$^{a,b}$ | 41 (6) | 14.95 ± 0.74$^{c}$ | 15 (2) | 14.44 ± 1.36$^{c}$ |
| KAN 100 | 60 (30) | 51.67 ± 18.37$^{a}$ | 111 (47) | 42.34 ± 2.2$^{a}$ | 45 (18) | 40 ± 5.45$^{a}$ | 45 (16) | 35.56 ± 7.7$^{a}$ | 46 (19) | 41.56 ± 0.56$^{a}$ | 41 (12) | 29.9 ± 6.68$^{a}$ |

*KAN* Kanamycin, *HYG* hygromycin

No., number of treated MB slices in three different experiments; %, Transformation efficiency; in parentheses, number of MB slices with PCR positive plants in selection medium in three different experiments

Each value represents the mean ± SE of three different experiments. Means with different letters are significantly different at the 0.05 level for each antibiotic selection

efficiencies (Tables 4 and 5). In Chardonnay, transformation efficiencies after immediate selection with 100 μg/ml kanamycin and 2 μg/ml hygromycin were 51.67 and 16.26 % (Table 5), compared with 48–37.33 % (1 or 2 weeks delay) for kanamycin and 13.33–10.67 % (1 or 2 weeks delay) for hygromycin (Table 4). In addition, transformation efficiencies of immediate selection using Thompson Seedless with 100 μg/ml kanamycin and 2 μg/ml hygromycin were 42.34 and 15.15 %, (Table 5) whereas delayed selection with kanamycin was 41.33–36 (1–2 weeks delay), and 10.67–9.33 % (1–2 weeks delay) with hygromycin (Table 4). In summary, the results found that delaying the start of selection by 1 or 2 weeks allowed higher survival rate, but did not increase transformation efficiency.

When comparing kanamycin and hygromycin as selectable markers, it is clear that at the concentrations tested in our study, kanamycin exhibited a lower impact on MB regeneration. Both survival and transformation efficiency were higher when 100 μg/ml kanamycin was used as the selection marker, compared with efficiencies using 2 μg/ml hygromycin for the six cultivars tested (Table 5).

### Molecular analyses

Putative transgenic shoots obtained from MB transformation with pCAMBIA1303 and pCAMBIA2303, were screened through PCR using primers for the *hygII* and *nptII* genes (Fig. S3). Transformants of Thompson Seedless successfully amplified bands at 559 and 563 bp for *hygII* and *nptII*, respectively, in almost all the samples tested. Putative transgenic plants were transferred to the greenhouse for further analysis.

### GFP expression

Fluorescence localization in transformed tissues was visualized in transgenic plants in order to confirm transformation and detect the presence of chimeras. Uniform GFP expression was visualized in leaves and roots (Fig. 4). Non-transformed cells should have been distinguished as bright red color compared to the bright green color of transformed cells under UV light. However, no such differences were detected in this research.

## Discussion

The experiments described in this study were prompted by the need for a simple, fast and efficient regeneration system available for genetic transformation of wine grapes, table grapes and rootstocks. Direct or indirect shoot organogenesis has been reported using leaves, petioles,

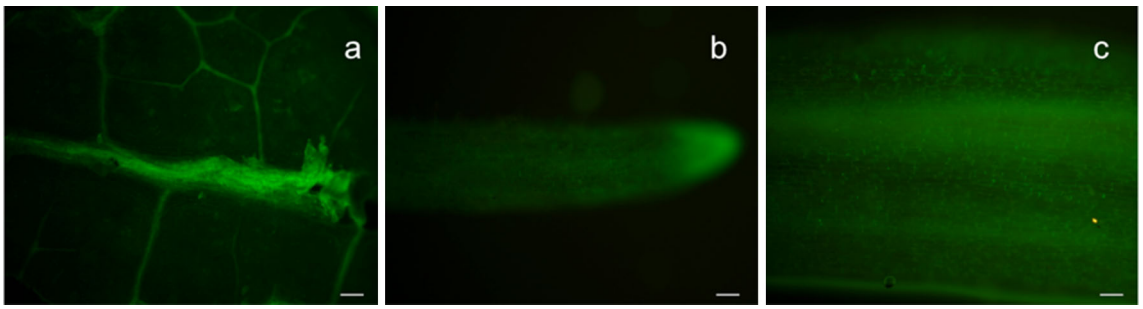


**Fig. 4** Expression of GFP in transformed Thompson Seedless. Uniform fluorescence with *bright green color* was observed in transformed tissue under UV light. **a** Leaf; **b** Root and **c** Root tip. *Scale bar* = 50 μm. (Color figure online)

nodal segments, and apices. However, only apices have shown real potential for application in genetic transformation. Dutt et al. (2007) reported successful transformation of wounded etiolated shoot tips of Thompson Seedless, but it is unclear if other varieties are as readily transformed. Shoot apices can also be used to initiate the production of MB. Shoot organogenesis from MB was first described in genetic transformation of the table grape cultivars Thompson Seedless and Silcora (Mezzetti et al. 2002). Meristematic bulks have been generated in 90 days and are valuable for both plant regeneration and genetic transformation. Bertsch et al. (2005) used MB to regenerate Chardonnay wine grapes. In this study, we found that this MB protocol can also be used to optimize the genetic transformation of other *V. vinifera* cultivars and rootstocks. The great advantage of this protocol is its simple implementation, with the only special requirement that apices be wounded in the first steps of MB induction, which can even be done with the naked eye. Conversely, transformation of pre-embryogenic cultures requires anther culture, embryogenic callus initiation, embryogenic callus maintenance, transformation and transgenic plant regeneration, a sequence that can take more than 1 year (Bouquet et al. 2007; Chaïb et al. 2010; Iocco et al. 2001). In addition, the period from transformation to plant regeneration can be at least 2 months shorter than transformation of somatic pre-embryogenic callus. Finally, transformation efficiency is high. In our research, transformation efficiency, calculated as number of MB slices producing transgenic plants relative to the number of treated MB, ranged between 30 and 50 %, depending on the genotype. These numbers are close to the best results obtained through embryogenesis, where efficiency is usually measured as the number of transformed cell clusters/mg of callus (Li et al. 2006; Maqsood et al. 2015; Torregrosa et al. 2002; Wang et al. 2005).

Transformation efficiency is strongly affected by the antibiotics used during selection and the concentrations at which they are implemented. Sensitivity to different antibiotics also depends on explants and their stage of development. Kanamycin, used in a wide range of concentrations, has been the most common antibiotic used in transformation of grape calli (Kiselev et al. 2007; López-Pérez et al. 2008; Mezzetti et al. 2002; Mulwa et al. 2015; Scorza et al. 1995, 1996; Zhou et al. 2014) followed by hygromycin, which is more potent and hence used at lower concentrations (Maqsood et al. 2015; Perl et al. 1996). In MB transformation, meristematic slices were subjected to stepwise selection by increasing concentrations of kanamycin on selection medium containing 25, 50 and 75 mg/l kanamycin (Mezzetti et al. 2002). In preliminary experiments with Thompson Seedless, we found that 100 mg/l kanamycin was well tolerated while increasing selection pressure, hence our study included the use of 100 μg/ml kanamycin and 1–2.5 μg/ml hygromycin as a selection agent. We demonstrated that hygromycin was able to quickly kill most non-transformed MB cells at a concentration of 2.0 μg/ml when applied immediately after inoculation without inhibiting the growth of transgenic tissues. Nevertheless, 100 μg/ml kanamycin was more effective than hygromycin as a selection marker because it allowed higher regeneration of transgenic plants. Delayed selection using either antibiotic did not increase transformation efficiency when compared to the immediate selection system.

Production of chimeras is a special concern in organogenic systems and the use of a more stringent selection agent like hygromycin would decrease their occurrence. Visual screening and detection of chimeras can be monitored after regeneration using reporter genes. In our research, GFP reporter genes were driven by the CaMV35S promoter. Abnormal GFP expression patterns, which would indicate the absence of introduced gene expression in some cells, were not found in regenerated plants using either kanamycin or hygromycin suggesting that no chimeric transgenic plants were produced in this research. Nevertheless, transgenic plants will be maintained in a greenhouse for another 2 years to continue monitoring GFP expression.

This study demonstrated for the first time highly efficient initiation and maintenance of MB, as well as the use of MB in the regeneration of Chardonnay, Thompson Seedless, Redglobe, Cabernet Sauvignon, St. George and 101-14 Mgt. It also demonstrated the potential for MB cultures to be used as target tissues for the genetic transformation of favorable traits such as disease resistance and seedlessness in *Vitis* cultivars. In addition, the high plant regeneration rate will greatly improve the efficiency of genetic transformation. The transformation system for grape cultivars described in this research has the potential to greatly optimize the application of gene technology to the improvement of established premium grape cultivars.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Author contributions** Conceived and designed the experiments: CBA MAW. Performed the experiments: XX CBA. Analyzed the data: XX. Contributed reagents/materials/analysis tools: YW MAW. Wrote the paper: XX CBA YW MAW. All authors read and approved the final manuscript.

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