



Harnessing embryogenic cell suspension culture for *Agrobacterium*-mediated transformation of microvine

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Abstract

Microvine 04C023V0004 (V4) is a model *Vitis vinifera* genotype that carries a heterozygous *gibberellin insensitive 1* (*Vvigail*) mutation making the plants compact in stature and constantly fruiting, yet its utility is hindered by long regeneration times and modest transformation efficiency levels. To improve microvine V4 transformation, we developed a method utilizing embryogenic cell suspension (ECS) cultures and a novel protocol for *Agrobacterium*-mediated transformation. Friable globular (translucent or cream-colored) somatic embryos dissected from microvine embryogenic calli were used to initiate suspension cultures. ECS cultures require a modest amount of time and effort to maintain and produce abundant, rapidly growing explant material within several months. For transformation, the ECS cells were heat shocked at 45 °C and co-cultivated with *Agrobacterium tumefaciens* carrying a binary vector with the microvine *Ubiquitin 7* (*VviUbi7*) promoter controlling *mCherry* expression allowing the use of red fluorescence as a visible marker. Development of transgenic microvine plants was improved with the addition of gibberellic acid to the shooting medium. Eighteen independent transgenic plants were characterized using droplet digital PCR (ddPCR) demonstrating that nine (50%) had one or two copies of the introduced selection marker transgene. This method produced approximately 30 transgenic plants per 100 mg fresh weight of ECS culture five months after co-cultivation. Use of microvine V4 ECS cultures and a modified transformation protocol can efficiently generate transgenic plants advancing grapevine biotechnology research.

Key message

An *Agrobacterium*-mediated transformation protocol using embryogenic cell suspension cultures was developed for efficiently and rapidly generating microvine transgenic plants.

Keywords *Agrobacterium tumefaciens* · Embryogenic cell suspension · Grapevine · Transgenic plants · mCherry · Droplet digital PCR

Introduction

Grapevine is typically genetically engineered using somatic embryos or embryogenic calli derived from reproductive tissues. However, its large size, long juvenile period, and biannual flowering impede research progress. The ‘Pinot Meunier’ cultivar of grapevine carries a *Vvigail* (*gibberellic*

acid-insensitive 1) gene mutation in the epidermal cell L1 layer of its apical meristem (Boss and Thomas 2002). *VviGAIL* is involved in gibberellin signaling and a single nucleotide mutation converts leucine to a histidine in the DELLA domain of the protein (Boss and Thomas 2002; Pellegrino et al. 2019; Torregrosa et al. 2019), resulting in a tomentose (densely covered with trichomes) phenotype. Plants somatically derived from ‘Pinot Meunier’ that are heterozygous for the *VviGAIL/Vvigail* mutation are called “microvine” and have a dwarf and hairy phenotype along with the conversion of tendrils into inflorescences, producing constantly but with physiologically normal flowering and fruiting. Microvine therefore serves as a model system for grape research and functional genomic studies. The original microvine mutant was not amenable to

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Agrobacterium-mediated transformation, so to overcome this limitation, crosses were performed and the hybrid progeny was tested for embryogenic callus development and transgenic plant regeneration. The microvine genotype 04C023V0004 (V4), resulting from a cross between the L1 mutant and the black-berried variety ‘Grenache’, showed improved transformation efficiency (Chaïb et al. 2010) and has been used to create transgenic plants in previous studies (Chaïb et al. 2010; Gouthu and Deluc 2019). However, despite the advantages of microvine as an amenable model, its transformation efficiency is low, transgenic shoot regeneration and development is inefficient and slow, and establishment of the regenerated plants in soil is challenging.

Previously, embryogenic cell suspension (ECS) cultures were used to significantly improve plant regeneration and transformation efficiency in multiple crop species (Ondzighi-Assoume et al. 2019; Shivani and Tiwari 2019; Tripathi et al. 2015; Woo et al. 2021). ECS cultures rapidly proliferate, are more easily sub-cultured than callus, and efficiently regenerate plants (Finer 1994; Jayasankar et al. 1999). Use of ECS cultures has also been shown to enhance *Agrobacterium*-mediated transformation due to higher explant tissue uniformity, improved accessibility to infection, and better selection efficiency for transformed tissues (Finer 1994). Despite these advantages, ECS cultures are easily contaminated with microorganisms, have shorter subculture intervals, and can exhibit early senescence if not sub-cultured on time. In grapevine, cell suspension cultures were previously shown to improve regeneration in ‘Thompson Seedless’, ‘Chardonnay’, ‘Richter 110’, ‘Cabernet Sauvignon’, ‘Chancellor’, and ‘Mencía’ (Acanda et al. 2015; Amar et al. 2007; Forgács et al. 2017; Jayasankar et al. 1999; Jiao et al. 2012; Torregrosa et al. 2002). Plant regeneration from somatic embryos of grapevine interspecific hybrids was also previously reported from liquid cultures (Zlenko et al. 2005). A ‘Chardonnay’ suspension culture has also been used for transformation in gene editing studies (Park et al. 2014; Ren et al. 2016), but use of ECS cultures in microvine has not been reported. Liquid medium derived somatic embryos can develop a distinct embryonic structure known as the suspensor, a cellular connection between the embryo and surrounding tissue that transfers nutrients and growth regulators for embryo development (Jayasankar et al. 2003; Kawashima and Goldberg 2010) and smaller cotyledons than counterpart proembryogenic masses grown on solid media. These structures correlated with more advanced shoot apical meristem development and minimal dormancy during germination relative to solid media grown somatic embryos and produce plants more efficiently (Jayasankar et al. 2003; Kawashima and Goldberg 2010).

The objective of this study was to establish microvine V4 suspension cultures from embryogenic calli and to rapidly

and efficiently generate transgenic plants using *Agrobacterium*-mediated transformation. The reported protocol successfully created high-viability suspension cultures, ample embryogenic explant material for transformation, and produced numerous transgenic microvine plants.

Materials and methods

Callus induction and proliferation

Microvine V4 plants (provided by Dr. Paul Boss and Dr. Ian Dry, CSIRO, via Dr. Laurent Deluc) were grown in a USDA greenhouse in Albany, California. Inflorescences were collected as an explant source at stage 57 (Lorenz et al. 1995) just after the immature flower clusters had begun to separate from each other (Wang et al. 2023). The flowers were washed under running water, divided into small subclusters, and were surface sterilized with 100 mL of fresh 20% bleach solution containing a drop (~50 µL) of Tween-20 in a conical flask for 1 min. The flowers were washed three times with sterile water to remove residual bleach, then drained and either used directly for dissection or stored at 4 °C for up to three days before dissection. Flowers were dissected as previously described (Torregrosa et al. 2015) and cultured on callus initiation medium (PIV: Online Resource 1 A) (Franks et al. 1998). The culture plates were incubated in the dark at 28 °C and the resulting callus was transferred onto callus proliferation and maintenance medium (CIM: Online Resource 1B) after three weeks for further callus proliferation. Embryogenic calli were transferred every three weeks onto fresh CIM media and incubated at 28 °C in the dark until used for ECS initiation, plant regeneration or plant transformation. The microvine suspension cultures were maintained for up to two years.

ECS culture establishment

Individual immature somatic embryos separated from ~4 month-old embryogenic calli were used to initiate a suspension culture. Approximately 50 mg (fresh weight) of friable globular somatic embryos (translucent or cream-colored) dissected from embryogenic callus, were transferred to a 50 mL Erlenmeyer flask containing 7 mL of suspension medium (Online Resource 1 C) and incubated at 28 °C with 90 rotations per minute (rpm) in the dark on a rotary shaker. ECS cultures were refreshed once a week by replacing 6 mL of medium with fresh suspension medium. As the suspension cells proliferate, fine cells (size < 0.6 mm) were transferred to a sterile flask (120 or 250 mL) maintaining a suspension cells/medium volume ratio of 5–10% (approximately 15 mL suspension medium in 120 mL flask, measured using settled

cell volume of the suspension cells in a 50 mL sterile falcon tube) and the residual bigger cell clusters in initial 50 mL flask were maintained as mentioned above. Sub-culturing large flasks of ECS cultures was performed every 10 days. After transferring to a 120 mL flask, the suspension culture was used for transformation or grown for future use. Bigger cell clusters within large flasks were filtered out using a 3.3-inch diameter 0.6 mm mesh sieve to synchronize the cultures' developmental stage every 1–2 months (Fig. S2). Initial small flask cultures were not filtered, they were maintained in a 50 mL flask for continuous proliferation. Suspension cells along with the medium were placed on a glass slide, covered with a coverslip, and observed directly under an Olympus microscope BX51 (Olympus Corporation, Tokyo, Japan) to assess their status and produce the images shown in Fig. 2.

Regeneration and plant development

For checking regeneration, settled fine suspension cells (<0.6 mm) along with 200–300 μ L suspension medium were pipetted from the bottom of the flask onto somatic embryo development and maturation (SEDM) medium (Online Resource 1D). The plate was gently shaken to ensure even distribution of the cells. Plates were incubated at 28 °C in the dark in a growth chamber (Percival Scientific, Iowa, US). After 30–50 days on SEDM, cells developed into mature somatic embryos, and at the torpedo/cotyledon stage were transferred to suspension shooting medium (SSM) (Online Resource 1E) for growth and maintained at 28 °C under a 16-hour light/8-hour dark cycle ($40\text{--}70 \mu\text{mol m}^{-2} \text{s}^{-1}$). When plantlets regenerated, they were transferred onto SSM-I (Online Resource 1F) under the same light and temperature conditions for further development. Once plants had grown to 4–5 cm in height, they were transferred to soil in pots, covered with polythene bags or a plastic dome to retain humidity for acclimatization, and maintained at 28 °C under a 16-hour light ($40\text{--}70 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8-hour dark cycle in a growth chamber (Conviron, North Dakota, US).

Construct design

The *VviUbi7* promoter (Genbank Accession number: PV929291), including its 5' intron (2,151 bp), which was previously shown to confer strong expression in grapevine tissues (Li et al. 2012), was amplified from microvine V4 genomic DNA and cloned into a Level 0 Golden Gate acceptor backbone from the MoClo Golden Gate library (Engler et al. 2008). PCR amplification was performed using Q5 high fidelity DNA Polymerase (New England Biolabs, MA, US) with sequence-specific primers (Online Resource 2_Table S1) and microvine V4 genomic DNA under the following

cycling conditions: 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 67 °C for 10 s, 72 °C for 1.10 min, and a final extension of 72 °C for 2 min. The Level 0 vector carrying the *VviUbi7* promoter was confirmed with Sanger DNA sequencing and cloned into a Level 2 acceptor vector driving the expression of the red fluorescence gene *mCherry* with a CaMV35S terminator and a kanamycin resistance plant selection cassette. This construct exhibited strong red fluorescence in transgenic grapevine tissues and thus provided an easily detectable visible phenotype in transgenic events. A diagram of the vector construct T-DNA is shown in Fig. 4A.

Agrobacterium-mediated transformation

Suspension cells were transformed using the *Agrobacterium tumefaciens* strain AGL1 containing the *VviUbi7-mCherry* binary vector plasmid. *Agrobacterium* starter culture (5 mL) was set up using a single colony from a streaked plate with selection antibiotic (kanamycin 50 mg/L) and grown overnight at 28 °C. One milliliter of the starter culture was then inoculated into 50 mL of LB medium containing the selection antibiotic (kanamycin 50 mg/L) and grown at 28 °C with 250 rpm orbital shaking for 3–5 h until it became turbid. The culture was then transferred to a sterile falcon tube and spun down at $4,000 \times g$ for 10 min at 18 °C and resuspended at an $\text{OD}_{600}=0.2$ in co-cultivation medium (CCM) (Online Resource 1G) with 400 μ M aceto-syringone. ECS cells (500 mg fresh weight, measured after removing supernatant with a pipette from settled cells in falcon tube) were collected into a 50 mL falcon tube and the original medium replaced with fresh media. The ECS cells were then heat shocked in a water bath at 45 °C for 5 min just prior to *Agrobacterium* co-cultivation. Ten milliliters of CCM containing the *Agrobacterium* were added to 500 mg fresh weight of suspension cells and mixed gently at room temperature for 10 min on a platform rocker (Crystal Technology & Industries, Texas, US) at 50 rpm. After mixing, suspension cells were allowed to settle to the bottom of the tube. Five milliliters of medium were removed, and the remaining solution and cells were transferred with a sterile serological pipette onto sterile filter paper (a stack of 2–3 sterile filter paper disks placed in a sterile petri dish) and was dried in a sterile flow hood for ~10 min. Once the cells no longer appeared wet, the topmost filter paper layer with cells was transferred to CIM plates and co-cultured at 22 °C in dark for 2 days. After co-cultivation, suspension cells were transferred to a falcon tube containing 10 mL suspension medium with 400 mg/L cefotaxime using a cell scraper (Alkali Scientific, Florida, US) and mixed on a platform rocker (50 rpm) for 15–30 min at room temperature. The cells were washed 3–4 times with suspension medium to remove the *Agrobacterium* and then transferred

onto stacked filter papers in a sterile petri dish to air dry (until no longer visibly wet, carefully without over drying the cells) in the laminar flow hood. The dried cells were then transferred with a cell scraper to semi-solid SEDM medium containing cefotaxime (250 mg/L) and divided into small groups of cells (Fig. 4C) to track individual transformation events. Cultures were incubated in the dark at 28 °C for 20 days and observed under a microscope for red fluorescence to confirm transgene expression. The transformed cells were subcultured onto SEDM media containing 250 mg/L cefotaxime and plant selection antibiotic (50 mg/L kanamycin) once a month until they develop mature somatic embryos. Mature somatic embryos exhibiting nonchimeric, uniform red fluorescence were then transferred to SSM containing 250 mg/L cefotaxime and gibberellic acid (GA₃, 5 mg/L) and maintained at 28 °C in light (16 h/8 h light/dark at 40–70 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity) in a growth chamber. Regenerated plantlets were transferred onto SSM-I with GA₃ (10 mg/L) for further development and later transferred to soil for acclimatization under the same conditions

before being moved to the greenhouse. A flow chart of the procedure is shown in Fig. 1.

Molecular analysis

Genomic DNA was extracted using a modified Puregene kit protocol (Qiagen, Redwood City, CA, US). Leaf samples (~0.5 g) were snap-frozen in liquid nitrogen in 1.5 mL centrifuge tubes and ground into a fine powder using a plastic pestle. 400 μL of cell lysis solution and 25 μL of β -mercaptoethanol were added to the ground sample, vortexed, and incubated at 65 °C for 1 h, with additional mixing at 30 and 60 min. 5 μL of RNase A (10 mg/mL) solution was added, mixed gently, and incubated at 37 °C for 30 min. The sample was placed on ice for 1 min, and 133 μL of protein precipitation buffer was added. The mixture was vortexed for 20 s and put on ice for 20 min. The samples were then centrifuged for 3 min at 13,000 $\times$ g, and the supernatant was transferred into a new 1.5 mL microcentrifuge tube. Equal amounts of chloroform: isoamyl alcohol were added, inverted 50 times, and centrifuged for 5 min at 13,000 $\times$ g.

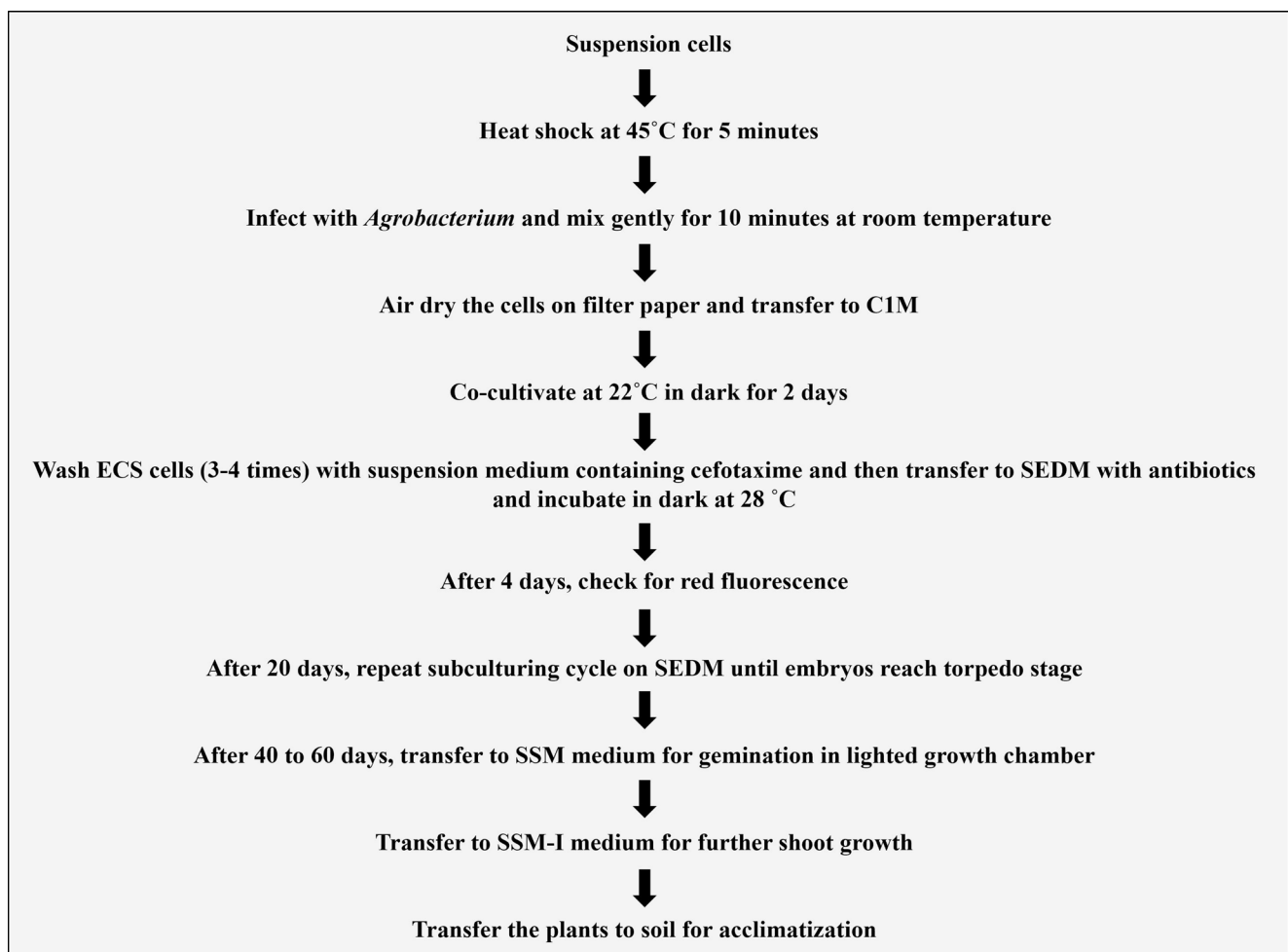


Fig. 1 *Agrobacterium*-mediated transformation protocol of ECS

The supernatant was transferred into a new 1.5 mL microcentrifuge tube, and an equal amount of 100% isopropanol was added and mixed by inverting the tube 50 times. The samples were then incubated on ice for 5 min and then centrifuged for 1 min at 13,000 $\times$ g. The supernatant was discarded, and the DNA pellet was washed with 300 μ L of 70% ethanol, mixed by inverting, and centrifuged at 13,000 $\times$ g for 1 min. The supernatant was discarded, and the genomic DNA pellet was air dried and then dissolved in 30 μ L of sterile ultra-pure water. Genomic DNA was quantified with a DeNovix microvolume spectrophotometer (DeNovix, Davis, CA) and its integrity evaluated by separation on a 1% agarose gel prior to use.

Droplet digital PCR (ddPCR) was used to measure the copy number of *nptII* in the T_0 transgenic events once they were established in the greenhouse (Collier et al. 2017). The grapevine *chalcone synthase* (*VviCHI*) gene (Costa et al. 2022; Harris et al. 2013) was used as a single copy reference gene. Primers and probes (Online Resource 2_Table S1) were designed for the reference gene and used as described previously for *nptII* (Thilmony et al. 2022) following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, US). A FAMTM-labeled probe was used to detect the reference gene amplicon, and a HEXTM-labeled probe was used for *nptII* amplicon detection. Genomic DNA (200 ng) of transgenic and control plants was digested with *EcoRI*-HF restriction enzyme (New England Biolabs, MA, US) prior to ddPCR. Each 20 μ L reaction contained 10 μ L of 2 $\times$ ddPCR supermix for probes (no dUTP) (Bio-Rad), 0.5 μ L of each primer (22.5 μ M), 0.25 μ L of reference and target probe (25 μ M), 3 μ L of digested DNA (60–100 ng), and 5.5 μ L water for a total reaction volume of 20 μ L. Droplets were generated in the QX200 droplet generator for each sample and PCR was carried out at: 95 $^{\circ}$ C for 10 min, 40 cycles (94 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 1 min), 98 $^{\circ}$ C for 10 min, 12 $^{\circ}$ C hold. After PCR, the droplets were read in the Bio-Rad QX200 droplet reader, and the data was analyzed using QuantaSoft software (Bio-Rad). PCR amplification was also used to test for potential *Agrobacterium* contamination in the T_0 transgenic events used for transgene copy number measurement. Primers designed for the *Agrobacterium tumefaciens* C58 *flhG* sequence (Table S1) and Taq DNA Polymerase (New England Biolabs, MA, US) was used for amplification with the following cycling conditions: 95 $^{\circ}$ C for 30 s, 35 cycles of 95 $^{\circ}$ C for 15 s, 62 $^{\circ}$ C for 20 s, 68 $^{\circ}$ C for 20 s, and a final extension at 68 $^{\circ}$ C for 5 min. Approximately 100 ng of genomic DNA from each of the microvine samples, and genomic DNA from lysed *A. tumefaciens* AGL1 cells (used as a positive control) were used as template in the PCR amplification reactions. After amplification, the samples were evaluated by separation on a 1.5% agarose

gel at 90 volts for 40 min, staining with ethidium bromide and visualizing the results on a UV transilluminator.

Results

Development of microvine ECS

The development of callus and suspension cultures using stamen explants is illustrated in Fig. 2. An early stage microvine inflorescence is shown in Fig. 2A. Immature flowers that are the most suitable for initiating embryogenic callus are at the stage when they begin to separate from each other within the inflorescence (stage 57 as in Lorenze et al. 1995; Fig. 2B). At this stage, the anthers are pale-yellow and appear translucent (Fig. 2C). We tested stamens, filaments, anthers, pistils, and whole flowers with the calyptra and without the calyptra as tissue types for callus induction and found that intact flowers without the calyptra produced the greatest number of embryogenic calli, but stamens and dissected filaments also generate embryogenic calli (data not shown). Within 20 days of dissection, anthers turn brown, and the filament tips attached to the ovary swell (Fig. 2D). Induced calli start to proliferate within two rounds of subculturing on CIM and were further maintained by transferring to fresh CIM medium every 2–4 weeks. Embryogenic callus generation efficiency in microvine V4 was 2–6% (number of embryogenic calli/total number of stamens*100) from healthy, greenhouse-grown plants.

After approximately four months of sub-culturing on CIM, friable, translucent or cream-colored globular somatic embryos (Fig. 2E and F) were selected to initiate ECS culture in 50 mL flasks (Fig. 2G). Selection of globular somatic embryos as the source material, rather than using whole embryogenic callus pieces, is the most critical step in establishing an ECS culture. Compact callus pieces fail to develop ECS and eventually turn brown within 2–3 weeks in liquid medium (Online Resource 3_Fig. S1 A-C). Approximately 50 mg fresh weight of somatic embryos were used to initiate the cell suspension culture in 7 mL of suspension medium in a 50 mL Erlenmeyer flask. During subculturings, removal of brown cell clusters (if any) helps to maintain a healthy ECS culture. Embryogenic cultures typically proliferate within 2 months (Fig. 2H). After proliferation, the small cell clusters (<0.6 mm) from culture were transferred into larger flasks (125 or 250 mL), leaving behind the bigger cell clusters in the original flask for further proliferation, maintaining cells and suspension medium ratio of 5–10% as shown in Online Resource 3_Fig. S2. After 1–2 months in the larger flask (the time required varies in different cell lines), bigger cell clusters in suspension cultures were strained through a 0.6 mm mesh sieve and removed. This process aids in maintaining a

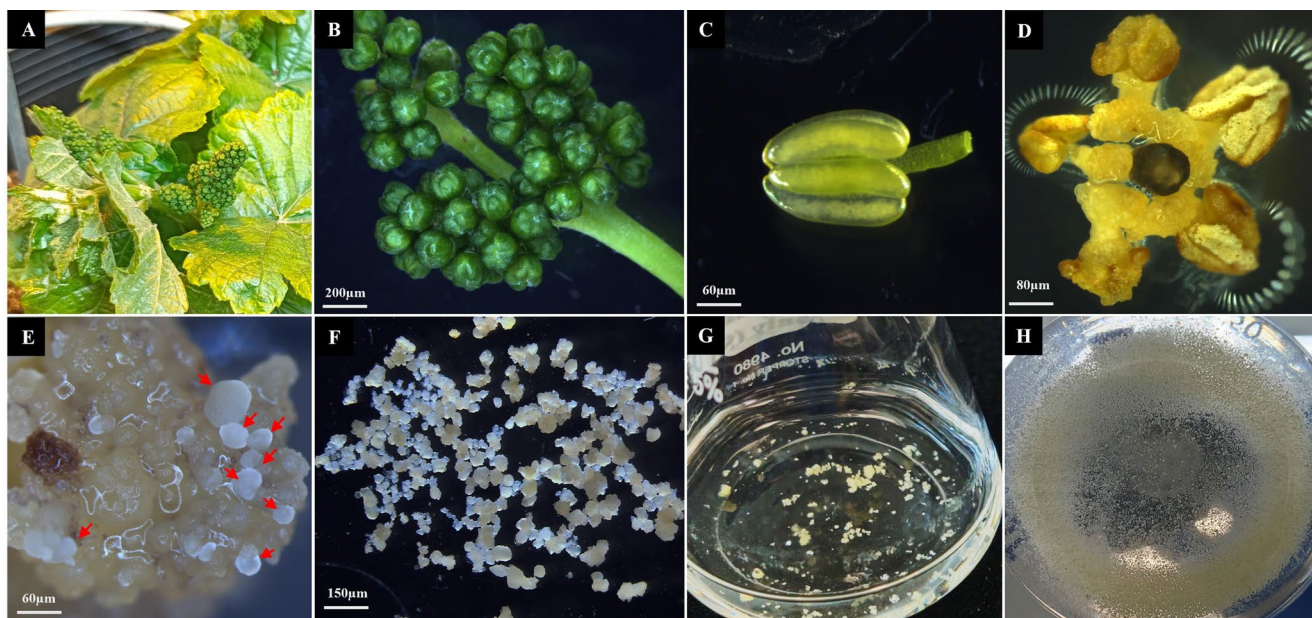


Fig. 2 Microvine Callus and ECS development. **A** Flowering microvine plant. **B** Immature inflorescence used for dissection (with flowers that have just begun to separate from each other). **C** Slightly transparent and yellow stage of stamen used for callus inoculation **D** Reproductive floral tissues (after removing calyptra) on PIV medium producing callus. **E** Friable, transparent somatic embryos emerging from embryogenic callus after ~4 months on CIM. Desirable friable

somatic embryos marked with red arrows. **F** Cream/translucent globular somatic embryos (~50 mg fresh weight) used for initiating ECS. **G** Inoculation of somatic embryos in suspension medium for ECS culture initiation. **H** Proliferation of suspension cells after two months with continuous shaking under dark conditions at 28°C (view from the bottom of the flask)

culture with uniform smaller sized cell clusters (<0.6 mm) within the ECS culture (Online Resource 3_Fig. S2).

Microscopic observations of ECS cultures show small clusters of cells (Fig. 3A). An embryogenic suspension cell culture that can efficiently generate shoots will typically contain small spherical cell clusters where the cells are filled with dense cytoplasm (starch granules) as shown in Fig. 3A–C. Non-embryogenic cultures contain cells and cell clusters with irregular shapes, a large vacuole, and noticeably less starch granules (Fig. 3D). These non-embryogenic cells and cell clusters were frequently observed in older ECS cultures and became more abundant as they aged. When these non-embryogenic cells predominate in a culture, it is no longer useful for plant regeneration. Embryogenic microvine ECS cultures that can efficiently regenerate plants were maintained for up to two years using this method, but there is variation between separately established cultures, and some started to lose their embryogenic capacity after one year. Assessment of the status of an established ECS culture should be completed regularly to ensure its utility for transformation and shoot regeneration.

Plant regeneration from microvine ECS cultures

Suspension cells (along with a 200–300 µL of the suspension medium) were transferred onto SEDM plates to monitor

their shoot regeneration capacity (Online Resource 3_Fig. S3A). The suspension cells developed into mature somatic embryos on SEDM after approximately 30–50 days of sub-culturing on the semi-solid medium (Online Resource 3_Fig. S3B), although the exact timeframe can vary between independently derived ECS cultures. Mature globular and torpedo staged somatic embryos were observed under the microscope (Online Resource 3_Fig. S3C) for cultures with the capacity to regenerate shoots. These explants turned green and grew into small shoots with roots on SSM after 15–30 days (Online Resource 3_Fig. S3D). These shoots then developed into plantlets after 1–2 months of subculturing on SSM-I containing gibberellin hormone (Online Resource 3_Fig. S4). Subsequently, 4–5 cm tall plantlets were transferred to soil for acclimatization and further growth (Online Resource 3_Fig. S3F).

Agrobacterium-mediated transformation of microvine ECS

To evaluate the efficiency of transformation in microvine ECS cultures, we used an *Agrobacterium*-mediated transformation approach. Schematic diagram of the transformation construct is shown in Fig. 4A. Co-cultivation of suspension cells with *Agrobacterium* carrying the binary vector construct is shown in Fig. 4B. After co-cultivation,

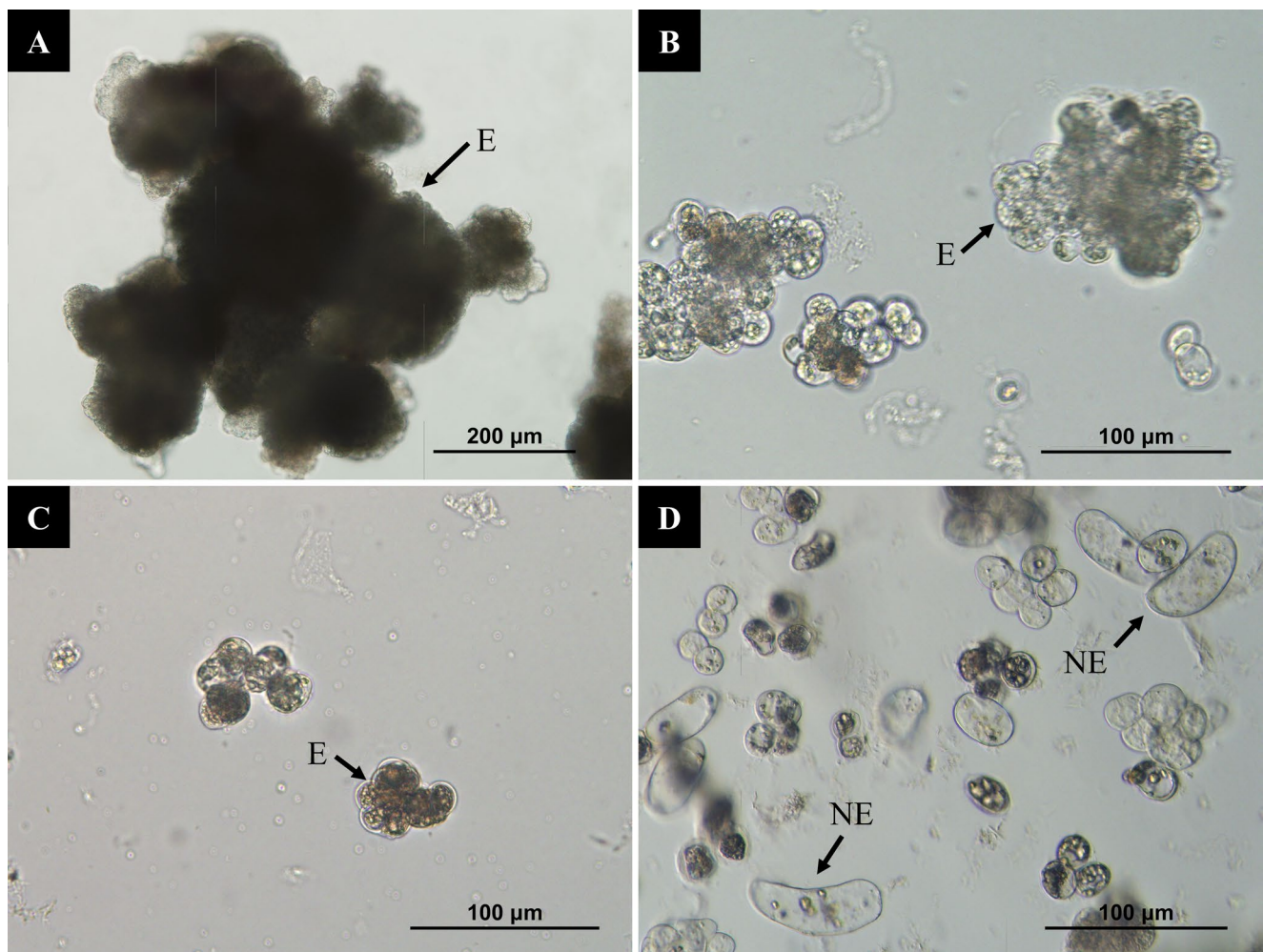


Fig. 3 Microscopic observation of ECS. **A–C** are of the same ECS cell line culture. **A** Large cell cluster of embryogenic cells (E, with arrow) with dense cytoplasm (filled with starch granules). **B & C** Different sized embryogenic cell clusters showing a compact and gener-

ally spherical shape. **D** Non-embryogenic cells (NE, with arrow) with irregular shapes and large vacuoles without starch granules from an old cell suspension culture that cannot efficiently regenerate shoots

the plant cells were washed and transferred as small groups of cell clusters to semi-solid SEDM containing kanamycin and cefotaxime (Fig. 4C). Transformed cell foci exhibited red fluorescence four days post co-cultivation (Fig. 4D). The transformed cells matured into the torpedo and cotyledon staged somatic embryos on SEDM selection medium within 2–3 months (Fig. 4E). These mature somatic embryos exhibited uniform bright red fluorescence confirming expression of the *mCherry* transgene (Fig. 4F). The transformed somatic embryos were then transferred to SSM medium and developed roots and shoots (Fig. 4G), but some of the emerging shoots exhibited slow and/or abnormal growth where the shoots failed to thrive, elongate and produce new stems and leaves (Online Resource 3_Fig. S5). To enhance shoot growth, we supplemented the SSM medium with four different concentrations of GA₃: 5 mg/L, 8 mg/L, 10 mg/L and 15 mg/L. Microvine shoots developed into healthy

plantlets on SSM-I containing GA₃ after an additional 60 days (Fig. 4H). We observed a greater number of normal shoots with the addition of GA₃, and the highest percentage of normal shoots were observed with 10 mg/L GA₃ (Online Resource 3_Fig. S4, Online Resource 2_Table S2). Plants grown with 15 mg/L GA₃ showed some undesirable curling of shoot tips. After two months, well-established rooted plants were transferred to soil and exhibited normal growth (Fig. 4I). This approach generated approximately 150 red-fluorescent transgenic plants from 500 mg fresh weight of starting ECS culture. Eighteen randomly selected plants were grown in soil for further characterization. The overall timeline for the generation of transgenic microvine V4 plants from ECS cultures is illustrated in Fig. 5.

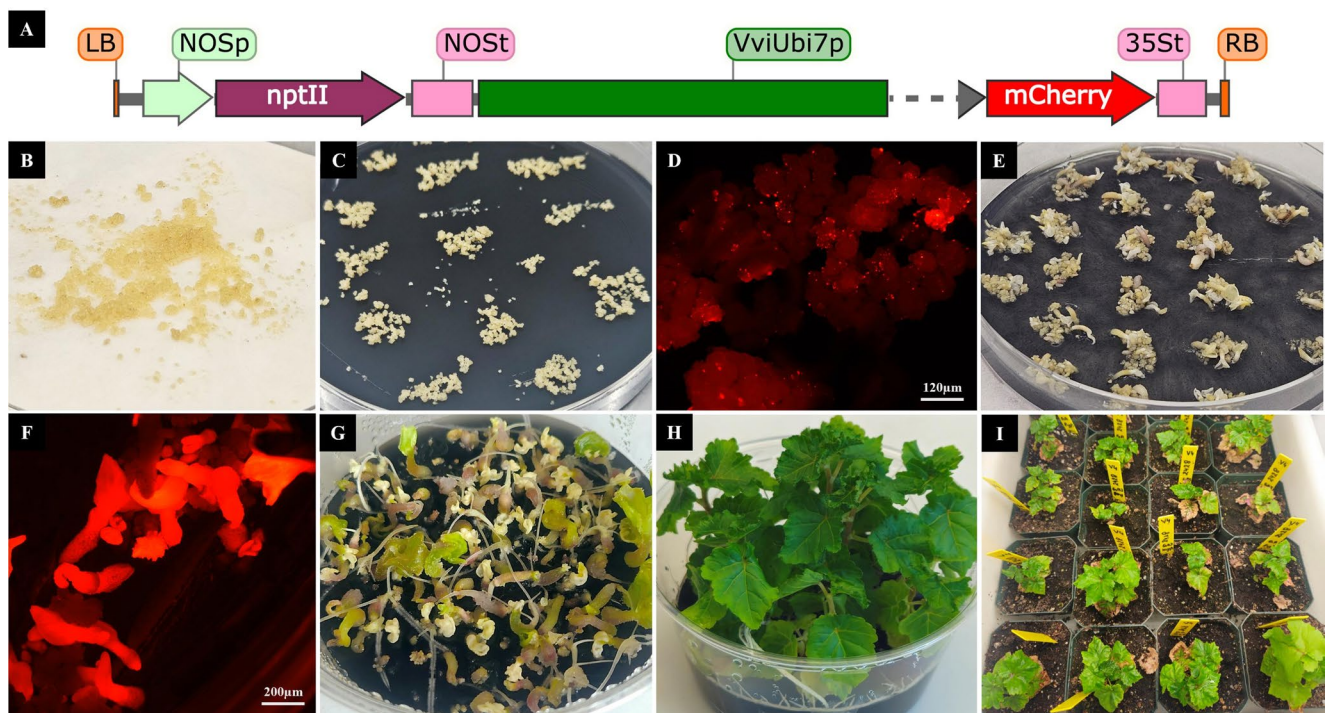


Fig. 4 *Agrobacterium*-mediated transformation of ECS cultures and the recovery of microvine transgenic plants. **A** Schematic diagram of the T-DNA construct used for ECS transformation. The *nopaline synthase* promoter (NOSp) and terminator (NOST) controls expression of *nptII* (to confer plant resistance to kanamycin) and the microvine *ubiquitin 7* promoter (*VviUbi7p*) with 5' intron (dashed arrow) and CaMV35S terminator (35St) controls *mCherry* reporter gene expression, *Agrobacterium* T-DNA left border (LB) and right border (RB). **B** Suspension cells after infection with *Agrobacterium*. **C** The suspension cells on SEDM and divided into groups after washing with cefo-

taxime. **D** Transformed cells showing red fluorescence (3s exposure) four days after co-cultivation. **E** Somatic embryo development on SEDM with cefotaxime (250 mg/L) and kanamycin (50 mg/L) three months after co-cultivation. **F** Transformed suspension cells exhibiting red fluorescence (1s exposure) three months after co-cultivation. **G** Regenerating shoots and roots on SSM medium (without selection antibiotic) 20 days after subculturing. **H** Regenerated plants growing on SSM-I medium containing GA₃ (10 mg/L) 60 days after subculturing. **I** Transgenic microvine plants growing in soil

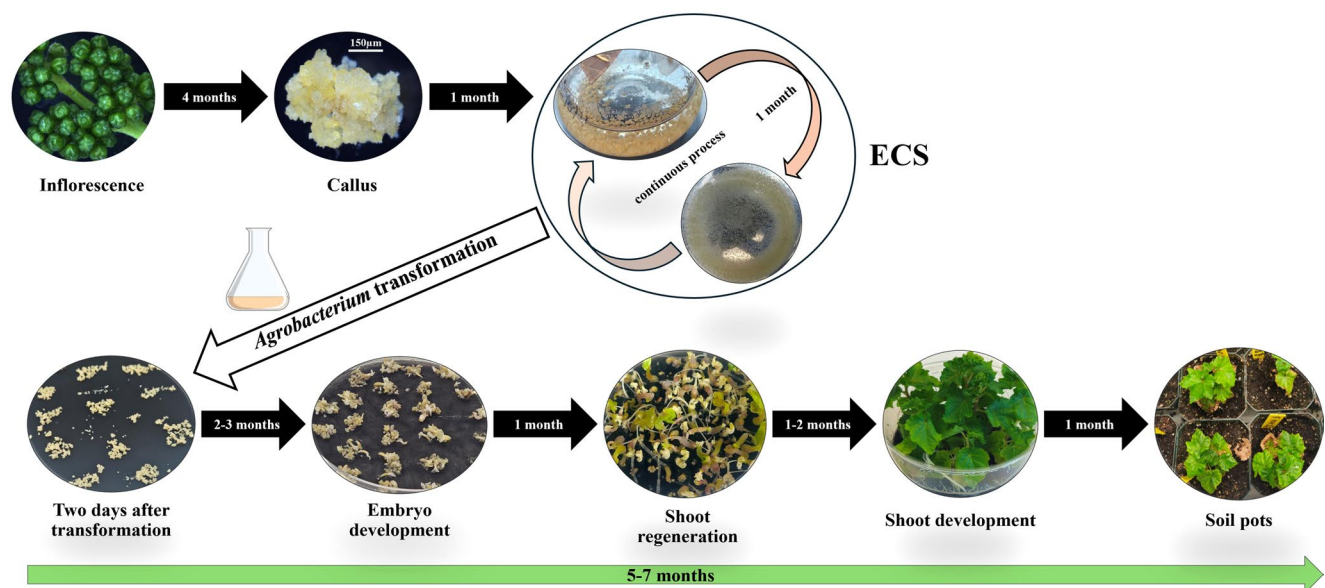


Fig. 5 Timeline to develop plants from ECS after *Agrobacterium*-mediated transformation

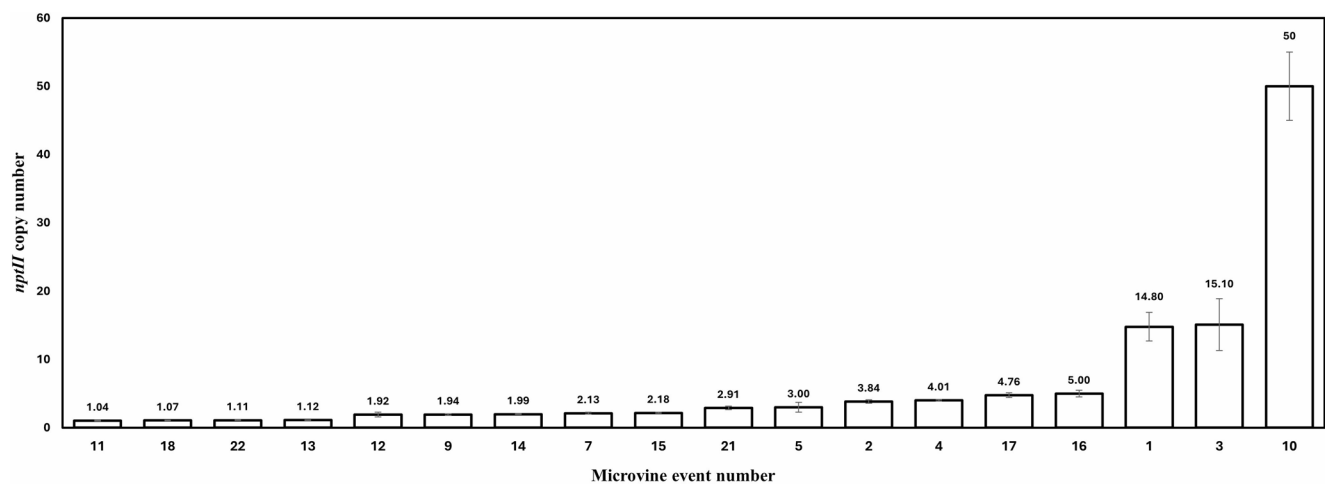


Fig. 6 Copy number of *nptII* in eighteen independent microvine transgenic plants derived from ECS

Molecular confirmation of transgenic plants

Eighteen independent putative transgenic plants were analyzed with ddPCR to measure *nptII* copy number. The transgene quantification was calculated using the endogenous single-copy grapevine gene *VviChi* as a reference (Costa et al. 2022; Harris et al. 2013). Among the tested plants, four carried a single-copy integration, five had two copies of *nptII*, and nine plants contained three or more transgene copies (Fig. 6, Online Resource 2_Table S3). These results demonstrate that the use of ECS cultures with *Agrobacterium* transformation generates stably transformed microvine V4 plants carrying one or two copies of an introduced transformation construct about half of the time. Similar results were observed when ddPCR was used to measure copy number in transgenic events from several other plant species (Collier et al. 2017). We confirmed that the tested 18 microvine samples were free of contaminating *Agrobacterium* by performing PCR amplification with primers that detect the native *Agrobacterium fliG* sequence (see Methods). All 18 tested plant samples were negative, while a positive control reaction that used *Agrobacterium* genomic DNA as a template amplified the expected product. These results confirm that the ddPCR results reflect the transgene copy numbers of each of those transgenic events.

Discussion

A major bottleneck in grapevine biotechnology is the rapid regeneration of transgenic plants carrying constructs of interest. Microvine is a desirable grapevine research model system because of several favorable attributes, but it is somewhat recalcitrant for shoot regeneration and grows relatively slowly in culture in part due to its gibberellin

insensitivity (Chaïb et al. 2010; Pellegrino et al. 2019; Torregrosa et al. 2019). In this study, we report a novel and efficient method for the genetic transformation of microvine V4 that addresses these issues. This improved protocol features several unique technical variations that distinguish it from currently available grapevine and microvine transformation protocols. First, for ECS culture initiation, we specifically select friable, translucent or cream-colored somatic embryos, not whole callus pieces, to establish high quality proliferative suspension cultures. Second, zeatin hormone is used in the suspension medium to accelerate cell proliferation. Third, a 5-minute, 45 °C heat shock treatment is used on the ECS culture prior to co-cultivation with a low density *Agrobacterium* culture ($OD_{600} \sim 0.2$) that is induced with a high acetosyringone concentration (400 μ M). Fourth, following co-cultivation, the cultures are washed with a liquid medium containing 400 mg/L cefotaxime to ensure the recovery of transgenic grapevine cells without *Agrobacterium* overgrowth. Fifth, a two phase, selection-free shoot regeneration process is used on new medium, first with and then without plant hormones to promote shoot regeneration and development. Additionally, early visual selection of transgenic somatic embryos using red fluorescence is performed to recover non-chimeric transgenic plants and permits the removal of antibiotic selection reagent from the shoot regeneration media that can slow plant growth. Sixth, GA₃ was used to promote shoot regeneration and growth, complementing the partial gibberellin insensitivity of the microvine explants and improving healthy shoot development. Finally, for molecular characterization, a single-reaction ddPCR assay with labeled probes was utilized to precisely measure *nptII* copy number for the efficient identification of single copy transgenic events.

The successful establishment of this protocol begins with the selection of suitable explant starting material for

generating embryogenic calli. Previous grapevine studies used stamens, pistils and whole flowers to produce embryogenic calli on PIV medium (Capriotti et al. 2022; Dhekney et al. 2009; Gambino et al. 2007). In the current study, among tested explants, microvine filament tissues from stamens as well as flowers without the calyptra induced callus. We recovered more embryogenic callus with whole flowers without calyptra, compared to cultures of stamens alone, as has been observed for other grapevine cultivars (Capriotti et al. 2022; Dhekney et al. 2009; Gambino et al. 2007). This may be due to the presence of endogenous hormone levels within these reproductive structures that contribute to more efficient embryogenic callus induction (Jiménez 2001; Jiménez and Bangerth 2000; Mostafa et al. 2020). The second most important consideration for establishing ECS is the selection of good quality starting material; friable globular immature somatic embryos derived from the embryogenic calli. Inoculation of whole embryogenic callus, even carrying globular somatic embryos may result in a mixed suspension culture that fails to thrive and subsequently turns brown. The selection of globular somatic embryos for ECS initiation significantly improves the quality and uniformity of the resulting suspension culture. An earlier study also reported that somatic embryos from ‘Chardonnay’ callus produced a rapidly growing suspension culture (Jayasankar et al. 1999). However, specificity of starting material used for establishing ‘Chardonnay’ ECS is not mentioned in other studies (Ren et al. 2016; Torregrosa et al. 2002; Vidal et al. 2003). Some articles reported the use of embryogenic cells, embryo masses and pro-embryonic masses in ‘Chardonnay’, ‘Cabernet Sauvignon’, rootstocks and ‘Mencia’ grapevine varieties (Acanda et al. 2015; Amar et al. 2007; Villette et al. 2024). Our study demonstrated that immature, friable, and translucent/cream colored somatic embryos at the globular stage exhibit the highest embryogenic potential in liquid culture, consistent with previous studies in other crop species (Abohatem et al. 2011; Shivani and Tiwari 2019). For maintaining ECS, the ratio of suspension cells to suspension medium also plays an important role, as an excessive number of cells in the culture can lead to browning and cell death as observed previously in grapevine and palm (Abohatem et al. 2011; Jayasankar et al. 1999). Frequently subdividing cells into new flasks reduces cell browning in microvine, as reported earlier in ‘Chardonnay’ and ‘Thompson Seedless’ (Jayasankar et al. 1999). Cell debris sticking to the inner wall of the flask may also contribute to the browning of the cells and the removal of these dead cells by transferring the culture to a new flask is necessary to maintain healthy suspension cultures. Using the reported protocol, we were able to maintain high quality microvine ECS cultures for up to two years before they lost their capacity to efficiently regenerate plants. Suspension cells have also been shown to be a

good source material for protoplast isolation (Li et al. 2021; Masani et al. 2013), so this may be a useful approach to produce microvine protoplasts as well.

Grapevine cell suspension cultures have been used for biolistic and *Agrobacterium*-mediated transformation previously, but the process is somewhat inefficient for producing plants compared to other species (Hebert et al. 1993; Martinelli and Mandolino 1994; Ren et al. 2016; Torregrosa et al. 2002; Villette et al. 2024). One report produced 240 plants per 10 mL settled cell volume with a Chardonnay suspension culture, and another report generated 37 positive plants with Chancellor cell suspension using a biolistic approach (Kikkert et al. 1996; Vidal et al. 2003). Another study of Chardonnay *Agrobacterium*-mediated transformation that used liquid selection medium to generate resistant cell masses reported the generation of four transgenic plants (Ren et al. 2016). However, microvine transformation has only been reported using calli explants with *Agrobacterium*-mediated transformation, and no reports describe the development of microvine cell suspension cultures. One of those previous studies reported the recovery of nine microvine transgenic plants (Costa et al. 2018), and other studies have reported the time for the production of microvine transgenic plants was 6–10 months (Gouthu and Deluc 2019; Torregrosa et al. 2019). Comparatively, ECS cultures have been reported to be highly responsive to *Agrobacterium* infection and amenable to efficient shoot regeneration in crops like banana, citrus, switchgrass, and medicinal plants (Babich et al. 2020; Moniruzzaman et al. 2021; Ondzighi-Assoume et al. 2019; Shivani and Tiwari 2019). The protocol reported in this study generated approximately 150 transgenic microvine plants from 500 mg fresh weight of ECS in 5–7 months from the start of *Agrobacterium* co-cultivation, and all eighteen candidate transgenic plants selected for molecular characterization were red-fluorescent and transgene positive. For better recovery of transgenic plants, we heat-shocked suspension cells prior to *Agrobacterium* infection as reported previously to improve transformation efficiency (Gurel et al. 2009; Hiei et al. 2006; Khanna et al. 2004; Patel et al. 2013) and discontinued the use of antibiotic selection at the shooting stage since kanamycin selection has been shown to negatively impact shoot growth and regeneration in grapevine (Li et al. 2006; Saporta et al. 2014; Torregrosa et al. 2016). A heat shock treatment has been hypothesized to promote the survival of transformed cells due to the prevention of programmed cell death (Khanna et al. 2004) and induces the production of heat shock proteins that can stabilize *Agrobacterium* virulence proteins like VirE2 promoting *Agrobacterium*-mediated transformation (Hwang et al. 2015; Park et al. 2014; Tsai et al. 2009).

In the present study, a method for producing rapidly growing microvine ECS cultures is described. These ECS

cultures were demonstrated to be a viable source material for *Agrobacterium*-mediated transformation generating fluorescent transgenic shoots and transgenic plants (150 transgenic plants/500 mg fresh weight of ECS) within approximately 5–7 months. An advantage of using microvine ECS cultures is that they grow rapidly and provide ample source material for transformation and other experiments, while requiring a modest amount of time and effort to maintain. The protocol presented can be used to efficiently engineer novel traits, or to deploy CRISPR genome editing reagents to better understand gene function in microvine. This approach might also be adapted for other grapevine genotypes, enabling more efficient biotechnology research in this important crop.

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Author contributions RT and S designed the study. S, TM and JTP performed experiments. S wrote the manuscript and designed the figures. All authors contributed to editing and revising the manuscript. RT provided supervisory guidance and funding support.

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Data availability All relevant data are within the paper and its Supporting Information files, and are available on reasonable request from the corresponding author. The microvine VviUbi7 promoter sequence used in the study is available from GenBank (accession number PV929291).

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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