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Transgene-free Genome Editing in Grapevine

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Abstract

CRISPR/Cas9 genome editing technology has revolutionized plant breeding by offering precise and rapid modifications. Traditional breeding methods are often slow and imprecise, whereas CRISPR/Cas9 allows for targeted genetic improvements. Previously, direct delivery of Cas9-single guide RNA (sgRNA) ribonucleoprotein (RNP) complexes to grapevine (*Vitis vinifera*) protoplasts has been demonstrated, but successful regeneration of edited protoplasts into whole plants has not been achieved. Here, we describe an efficient protocol for obtaining transgene/DNA-free edited grapevine plants by transfecting protoplasts isolated from embryogenic callus and subsequently regenerating them. The regenerated edited plants were comparable in morphology and growth habit to wild-type controls. This protocol provides a highly efficient method for DNA-free genome editing in grapevine, addressing regulatory concerns and potentially facilitating the genetic improvement of grapevine and other woody crop plants.

Key features

- Protoplasts are one of the most commonly used systems for the application of new breeding technologies, including DNA-free genome editing.
- Protoplasts are a highly accessible platform by CRISPR-Cas9 ribonucleoparticles through chemical or physical transfection.
- CRISPR-Cas9 ribonucleoparticles avoid the use of both *Agrobacterium tumefaciens* and plasmids; no stable integration of exogenous DNA occurs.
- The genetic background of DNA-free edited plants regenerated from protoplasts remains unchanged and identical to the original plant.

Keywords: CRISPR/Cas9, Genome editing, Grapevine, Protoplasts, DNA free, Ribonucleoprotein complexes, Embryogenic callus, Plant regeneration

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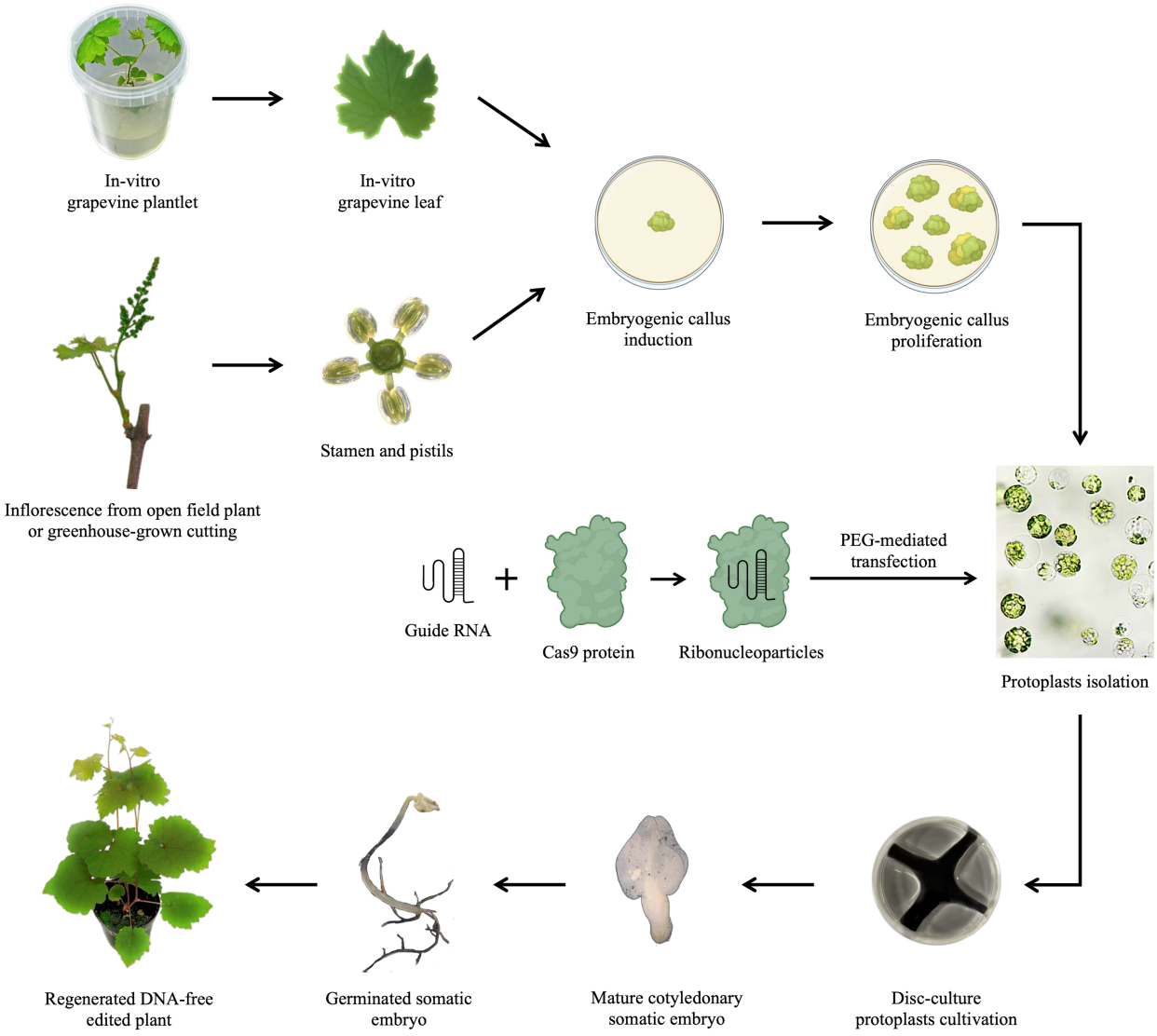
Graphical overview



Graphical overview of the workflow of DNA-free genome editing application in embryogenic calli-derived protoplasts and whole plant regeneration. Embryogenic calli are induced from leaves of in vitro plants or from stamens and pistils. After induction and proliferation, embryogenic calli are used for protoplast isolation. Pre-assembled CRISPR-Cas9 ribonucleoproteins (RNPs) are introduced into protoplasts by PEG-mediated transfection. Transfected protoplasts are cultivated using the disc-culture method. The regeneration of whole edited plants from transfected protoplasts occurs through somatic embryo formation.

Background

Grapevine (*Vitis vinifera* L.) is a significant global fruit crop, valued for its fresh produce and especially for wine. Genetic improvement of grapevine is crucial to address challenges such as climate change, higher demands for quality and quantity, and the need for product differentiation [1,2]. Breeding is a long, time-consuming process, often requiring several years to develop new varieties. Despite the quicker development potential of genetically modified (GM) crops, they encounter opposition from both the public and regulatory agencies due to concerns regarding health and environmental impacts [3]. Nowadays, new breeding technologies, particularly genome editing through the CRISPR/Cas9 system, provide a promising alternative by ensuring precise and targeted genetic modifications without altering the original genetic background. CRISPR/Cas9 uses the Cas9 endonuclease, guided to specific DNA sequences by a single guide RNA (sgRNA), to create site-specific double-stranded breaks. These breaks are typically repaired by non-homologous end joining, resulting in insertions or deletions, or by homology-directed repair if a donor template is available [4]. This method has been used to study and modify various genes in grapevine, enhancing traits such as disease resistance and plant architecture [5–9].

Most CRISPR/Cas9 applications in grapevine have involved stable integration of the editing machinery, thus creating GMOs. To comply with regulations requiring the absence of foreign DNA, two main strategies are used: removing CRISPR/Cas9 components after editing or directly delivering Cas9-sgRNA ribonucleoproteins (RNPs). The latter method avoids foreign DNA integration and has been demonstrated in grapevine protoplasts, but regenerating whole plants from these edited protoplasts has been challenging [10,11]. Recent advancements include both a procedure for regenerating whole plants from embryogenic callus-derived protoplasts in specific grapevine varieties [12] and a method for the regeneration of transgene-free edited grapevine plants from RNP-transfected protoplasts, confirming the feasibility of these approaches for producing normal and healthy transgene-free edited grapevine plants [13]. The advantages of this protocol include avoiding foreign DNA integration, thus addressing regulatory and public concerns associated with GMOs. Additionally, this method reduces the occurrence of genetic chimeras, which are common in organogenesis-based regeneration methods [14,15]. The approach is particularly suitable for varieties recalcitrant to traditional gene transfer methods, offering a versatile tool for grapevine breeding and functional genomics. Potential applications of this protocol extend beyond grapevine to other economically important woody and herbaceous crops. By refining the procedures for protoplast isolation, RNP transfection, and plant regeneration, this method could become a standard for developing transgene-free edited plants. This aligns with regulatory frameworks and facilitates broader acceptance of genome editing technologies in agriculture [16].

Materials and reagents

Biological materials

1. In vitro–grown grapevine plants
2. Inflorescences of open field plants or greenhouse-grown cuttings

Reagents

1. Enzymes

a. Cellulase R10 (Duchefa Biochemie, catalog number: C8001)

b. Macerozyme R10 (Duchefa Biochemie, catalog number: M8002)

c. Pectolyase Y-23 (Duchefa Biochemie, catalog number: P8004)

2. Fluorophores

a. Fluorescein diacetate (FDA) (Sigma-Aldrich, catalog number: 343209)

b. Fluorescent brightener 28 disodium salt (Chemcruz, catalog number: sc-218504)

3. Gelling agents

a. Agar TC (PhytoTech Labs, catalog number: A296)

b. Phytagel (Sigma-Aldrich, catalog number: P8169)

c. Gelrite (Duchefa Biochemie, catalog number: G1101)

4. Growth regulators

a. 6-Benzylaminopurine (6-BAP) (Duchefa Biochemie, catalog number: B0904)

b. 2,4-Dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie, catalog number: D0911)

c. α-Naphthalene acetic acid (NAA) (Duchefa Biochemie, catalog number: N0903)

d. β-Naphthoxyacetic acid (NOA) (Duchefa Biochemie, catalog number: N0912)

5. Kit

a. Qubit™ RNA BR Assay kit, 500 assays (Invitrogen, Thermo Fisher Scientific, catalog number: Q10211)

b. GeneArt Precision gRNA Synthesis kit (Invitrogen, Thermo Fisher Scientific, catalog number: A29377)

6. Proteins

a. TrueCut ™ Cas9 protein v2 (Invitrogen, Thermo Fisher Scientific, catalog number: A36499)

7. Salts and biochemicals

a. $CaCl_2 \cdot 2H_2O$ (Duchefa Biochemie, catalog number: C0504)

b. $Ca(NO_3)_2 \cdot 4H_2O$ (Duchefa Biochemie, catalog number: C0505)

c. KCl (Sigma-Aldrich, catalog number: 31219)

d. KH_2PO_4 (Duchefa Biochemie, catalog number: P0574)

e. KNO_3 (Duchefa Biochemie, catalog number: P0519)

f. K_2SO_4 (Duchefa Biochemie, catalog number: P0535)

g. $MgCl_2 \cdot 6H_2O$ (Duchefa Biochemie, catalog number: M0533)

h. $MgSO_4 \cdot 7H_2O$ (Duchefa Biochemie, catalog number: M0513)

i. NaCl (Duchefa Biochemie, catalog number: S0520)

j. NH_4NO_3 (Duchefa Biochemie, catalog number: A0501)

k. NH_4Cl (Duchefa Biochemie, catalog number: A0528)

l. $CuSO_4 \cdot 5H_2O$ (Duchefa Biochemie, catalog number: C0508)

m. FeNaEDTA (Duchefa Biochemie, catalog number: E0509)

n. $FeSO_4 \cdot 7H_2O$ (Duchefa Biochemie, catalog number: F0512)

o. H_3BO_3 (Duchefa Biochemie, catalog number: B0503)

p. $MnSO{\cdot}H_2O$ (Duchefa Biochemie, catalog number: M0514)
q. $Na_2MO_4{\cdot}2H_2O$ (Duchefa Biochemie, catalog number: S0525)
r. $Na_2EDTA{\cdot}2H_2O$ (Duchefa Biochemie, catalog number: E0511)
s. $ZnSO_4{\cdot}7H_2O$ (Duchefa Biochemie, catalog number: Z0526)
t. KI (Duchefa Biochemie, catalog number: P0518)
u. $CoCl_2{\cdot}6H_2O$ (Duchefa Biochemie, catalog number: C0507)
v. Sucrose (Duchefa Biochemie, catalog number: S0809)
w. D-Mannitol (Duchefa Biochemie, catalog number: M0803)
x. D-Glucose monohydrate (Duchefa Biochemie, catalog number: G0802)
y. Polyethylene glycol 4000 (PEG 4000) (Duchefa Biochemie, catalog number: P0804)
z. 2-(N-morpholino) ethane sulfonic acid (Duchefa Biochemie, catalog number: M1503)
aa. Biotin (Duchefa Biochemie, catalog number: B0603**)**
bb. Folic acid (Duchefa Biochemie, catalog number: F0608)
cc. Myo-inositol (Duchefa Biochemie, catalog number: I0609)
dd. Glycine (Duchefa Biochemie, catalog number: G0709)
ee. Nicotinic acid (Duchefa Biochemie, catalog number: N0611)
ff. Pyridoxine HCl (Duchefa Biochemie, catalog number: P0612)
gg. Thiamine HCl (Duchefa Biochemie, catalog number: T0614)
hh. D-pantothenate calcium (vitamin B5 calcium) (Duchefa Biochemie, catalog number: C0604)
ii. L-glutamic acid (Duchefa Biochemie, catalog number: G0707)
jj. L-phenylalanine (Duchefa Biochemie, catalog number: P0716)
kk. TopVision agarose (Thermo Scientific, Thermo Fisher Scientific, catalog number: R0491)
ll. 100 bp DNA ladder (Life Technologies, Thermo Fisher Scientific, catalog number: SM0241)
mm. Syber® safe DNA gel stain (Invitrogen, Thermo Fisher Scientific, catalog number: S33102)
nn. TriTrack DNA loading dye 6× (Life Technologies, Thermo Fisher Scientific, catalog number: R1161)
oo. Acetocarmine staining (Sigma-Aldrich, catalog number: 280370)
pp. NaClO (Sigma-Aldrich, catalog number: 1056142500)
qq. Tween-20 (Sigma-Aldrich, catalog number: P1379)

8. Supplements
a. Casein hydrolysate (Duchefa Biochemie, catalog number: C1301)
b. Activated charcoal (Sigma-Aldrich, catalog number: 31616)

Solutions

1. Macronutrients
a. NN macronutrients 10× (see Recipes)
b. MS macronutrients 10× (see Recipes)
c. C2D macronutrients 10× (see Recipes)

2. Micronutrients
a. NN micronutrients 100× (see Recipes)
b. MS micronutrients 1,000× (without FeEDTA) (see Recipes)
c. MS micronutrients 100× (see Recipes)
d. C2D micronutrients 1,000× (see Recipes)

3. Hormones

a. 2,4-Dichlorophenoxyacetic acid 1,000 μM (see Recipes)
b. 6-Benzylaminopurine 1,000 μM (see Recipes)
c. 1-Naphthaleneacetic acid 1,000 μM (see Recipes)
d. β-Naphthoxyacetic acid 1,000 μM (see Recipes)

4. Vitamins

a. NN vitamins 500× (see Recipes)
b. MS vitamins 500× (see Recipes)
c. C2D vitamins 1,000× (see Recipes)
d. B5 vitamins 500× (see Recipes)
e. Vitamins mix C1 500× (see Recipes)
f. Vitamins T 1,000×: (see Recipes)

5. Amino acids

a. Amino acids mix 1,000× (see Recipes)

6. Chemicals

a. FeEDTA 200× (see Recipes)
b. KCl 500 mM (see Recipes)
c. 2-(N-morpholino) ethanesulfonic acid 100 mM, pH 5.7 (see Recipes)
d. $CaCl_2 \cdot 2H_2O$ 1 M (see Recipes)
e. $MgCl_2 \cdot 6H_2O$ 500 mM (see Recipes)
f. NaCl 1 M (see Recipes)

7. Organics

a. Mannitol 1 M (see Recipes)
b. Glucose 3 M (see Recipes)

8. Culture media for induction of embryogenic calli (see Recipes)

a. NB2 (culture medium for induction of embryogenic calli from in vitro leaves)
b. PIV (culture medium for induction of embryogenic calli from stamens and pistils)
c. MSII (culture medium for induction of embryogenic calli from stamens and pistils)

9. Culture medium for long-term maintenance of embryogenic calli ($C1^P$) (see Recipes)

10. Culture medium for somatic embryos full germination (see Recipes)

11. Culture media for somatic embryos shooting (see Recipes)

a. C2D
b. C2D4B
c. MG1
d. MG1–10B

12. Culture media for full plant development and rooting (see Recipes)

a. RIM

b. MSN

13. Culture media for protoplasts cultivation (see Recipes)

a. Solid culture medium

b. Liquid culture medium

14. Solution for protoplast isolation, purification, and transfection (see Recipes)

a. Digestion solution

b. Digestion solution without enzymes

c. Wash solution

d. W5 solution

e. MMG solution

f. PEG solution

Recipes

1. Macronutrients

a. NN macronutrients 10×: 2.2 g/L $CaCl_2·2H_2O$, 9.5 g/L KNO_3, 7.2 g/L NH_4NO_3, 1.85 g/L $MgSO_4·7H_2O$, 0.68 g/L KH_2PO_4

b. MS macronutrients 10×: 16.5 g/L of NH_4NO_3, 4.4 g/L of $CaCl_2·2H_2O$, 3.7 g/L of $MgSO_4·7H_2O$, 19.7 g/L of KNO_3 and 1.7 g/L of KH_2PO_4

c. C2D macronutrients 10×: 16.5 g/L NH_4NO_3, 19 g/L KNO_3, 3.7 g/L $MgSO_4·7H_2O$, 1.7 g/L KH_2PO_4, 7.1 g/L $Ca(NO_3)_2·4H_2O$, 0.28 g/L $FeSO_4·7H_2O$, 0.37 g/L Na_2EDTA

2. Micronutrients

a. NN micronutrients 100×: 2.5 mg/L $CuSO_4·5H_2O$, 3.67 g/L FeNaEDTA, 1 g/L H_3BO_3, 1.9 g/L $MnSO_4·H_2O$, 25 mg/L $Na_2MoO_4·2H_2O$, 1g/L $ZnSO_4·7H_2O$

b. MS micronutrients 1,000× (without FeEDTA): 6.2 g/L H_3BO_3, 16.9 g/L $MnSO_4·H2O$, 8.6 g/L $ZnSO_4·7H_2O$, 0.83 g/L KI, 0.25 g/L $Na_2MoO_4·2H_2O$, 25 mg/L $CuSO_4·5H_2O$, 25 mg/L $CoCl_2·6H_2O$

c. MS micronutrients 100×: 0.62 g/L H_3B0_3, 3.67 g/L FeNaEDTA, 1.69 g/L of $MnSO_4·H_2O$, 0.86 g/L $ZnSO_4·7H_2O$, 0.083 g/L of KI, 0.025 g/L $Na_2MoO_4·2H_2O$, 0.0025 g/L $CuSO_4·5H_2O$, 0.0025 g/L $CoCl_2·6H_2O$

d. C2D micronutrients 1,000×: 0.64 g/L $MnSO_4·H_2O$, 6.2 g/L H_3BO_3, 8.6 g/L $ZnSO_4·7H_2O$, 0.25 g/L $Na_2MoO_4·2H_2O$, 25 mg/L $CuSO_4·5H_2O$, 25 mg/L $CoCl_2·6H_2O$

3. Hormones

Note: All the following hormone powders are soluble in NaOH 1 M. It is important to add NaOH 1 M to the weighed powder before the addition of water.

a. 2,4-Dichlorophenoxyacetic acid 1,000 µM: 0,221 g/L of 2.4-dichlorophenoxyacetic acid

b. 6-Benzylaminopurine 1,000 µM: 0.2252 g/L of 6-benzylaminopurine

c. 1-Naphthaleneacetic acid 1,000 µM: 0.1862 g/L of 1-naphthaleneacetic acid

d. β-Naphthoxyacetic acid 1,000 µM: 0.2022 g/L of β-naphthoxyacetic acid

4.Vitamins

a. NN vitamins 500×: 25 mg/L biotin, 0.25 g/L folic acid, 1 g/L glycine, 50 g/L myo-Inositol, 2.5 g/L nicotinic acid, 0.25 g/L Pyridoxine HCl, 0.25 g/L thiamine HCl

b. MS vitamins 500×: 1 g/L glycine, 50 g/L myo-inositol, 0.25 g/L nicotinic acid, 0.25 g/L pyridoxine HCl, 0.05 g/L thiamine HCl

c. C2D vitamins 1,000×: 1 g/L thiamine HCl, 10 g/L myo-inositol, 1 g/L nicotinic acid, 1 g/L pyridoxine HCl

d. B5 vitamins 500×: 50 g/L myo-inositol, 5 g/L thiamine HCl, 0.5 g/L nicotinic acid, 0.5 g/L pyridoxine HCl

e. Vitamins mix C1 500×: 50 g/L of myo-inositol, 5 g/L of nicotinic acid, 5 g/L of thiamine-HCI, 0.5 g/L of pyridoxine-HCl, 0.5 g/L of calcium pantothenate, 0.005 g/L of biotin

f. Vitamins T 1,000×: 50 g/L myo-inositol, 1 g/L nicotinic acid, 1 g/L thiamine HCl, 1 g/L pyridoxine HC, 1 g/L calcium pantothenate, 0.01 g/L biotin.

5. Amino acids

a. Amino acids mix 1,000×: 100 g/L L-glutamic acid (monosodium salt), 10 g/L L-phenylalanine, 2 g/L glycine

6. Chemicals

a. FeEDTA 200×: 7.44 g/L of $Na_2EDTA{\cdot}2H_2O$, 1.86 g/L of $FeSO_4{\cdot}7H_2O$

b. KCl 500 mM: 37.775 g/L of KCL

c. 2-(N-morpholino) ethanesulfonic acid 100 mM, pH 5.7: 19.52 g/L of 2-(N-morpholino) ethanesulfonic acid.

Note: Adjust the pH of this solution to 5.7 with KOH 1 M

d. $CaCl_2{\cdot}2H_2O$ 1 M: 146.9 g/L of $CaCl_2{\cdot}2H_2O$

e. $MgCl_2{\cdot}6H_2O$ 500 mM: 101.655 g/L of $MgCl_2{\cdot}6H_2O$

f. NaCl 1 M: 58.44 g/L of NaCl

7. Organics

a. Mannitol 1 M: 182.17 g/L of D-mannitol

b. Glucose 3 M: 594.6 g/L of glucose

8. Culture media for induction of embryogenic calli

a. NB2 (culture medium for induction of embryogenic calli from in vitro leaves)

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
NN macronutrients	10×	1×	100 mL
NN micronutrients	100×	1×	10 mL
MS vitamins	500×	1×	2 mL
6-benzylaminopurine	1,000 µM	1.0 µM	1 mL
2,4-dichlorophenoxyacetic acid	1,000 µM	5.0 µM	5 mL
Myo-inositol	/	0.1 g/L	0.1 g
Sucrose	/	20 g/L	20 g
Adjust to final pH 6.0 with KOH 1 M			
Agar TC	/	7 g/L	7 g

b. PIV (culture medium for induction of embryogenic calli from stamens and pistils)

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
NN macronutrients	10×	1×	100 mL
MS micronutrients	1,000×	1×	1 mL
FeEDTA	200×	1×	5 mL
B5 vitamins	500×	1×	2 mL
6-benzylaminopurine	1,000 µM	8.9 µM	8.9 mL
2,4-dichlorophenoxyacetic acid	1,000 µM	4.5 µM	4.5 mL
Sucrose		60 g/L	60 g
Adjust to final pH 5.7 with KOH 1 M			
Phytagel	/	3 g/L	3 g

c. MSII (culture medium for induction of embryogenic calli from stamens and pistils)

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
MS macronutrients	10×	1×	100 mL
MS micronutrients	100×	1×	10 mL
MS vitamins	500×	1×	2 mL
β-naphthoxyacetic acid	1,000 µM	2.5 µM	2.5 mL
6-benzylaminopurine	1,000 µM	5 µM	5 mL
2,4-dichlorophenoxyacetic acid	1,000 µM	2.5 µM	2.5 mL
Myo-inositol	/	0.1 g/L	0.1 g
Sucrose	/	20 g/L	20 g
Adjust to final pH 6.0 with KOH 1 M			
Agar TC	/	7 g/L	7 g

9. Culture medium for long-term maintenance of embryogenic calli ($C1^P$)

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
MS macronutrients	10×	1×	100 mL
MS micronutrients	1,000×	1×	1 mL
Vitamins C1	500×	1×	2 mL
AA mix	1,000×	1×	1 mL
Fe-EDTA	200×	1×	5 mL
2,4-dichlorophenoxyacetic acid	1,000 µM	5 µM	5 mL
6-benzylaminopurine	1,000 µM	1 µM	1 mL
Casein hydrolysate	/	1 g/L	1 g
Sucrose	/	30 g/L	30 g
Adjust to final pH 5.8 with KOH 1 M			
Phytagel	/	5g/L	5 g

10. Culture medium for somatic embryos full germination

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
NN macronutrients	10×	1×	100 mL
NN micronutrients	100×	1×	10 mL
NN vitamins	500×	1×	2 mL

Sucrose	/	30 g/L	30 g
Adjust to final pH 5.8 with KOH 1 M			
Gelrite	/	2 g/L	2 g

11. Culture media for somatic embryo shooting

a. C2D

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
C2D macronutrients	10×	1×	100 mL
C2D micronutrients	1,000×	1×	1 mL
C2D vitamins	1,000×	1×	1 mL
Sucrose	/	30 g/L	30 g
Adjust to final pH 5.8 with KOH 1 M			
Agar TC	/	7 g/L	7 g

b. C2D4B

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
C2D macronutrients	10×	1×	100 mL
C2D micronutrients	1,000×	1×	1 mL
C2D vitamins	1,000×	1×	1 mL
6-benzylaminopurine	1,000 µM	4 µM	4 mL
Sucrose	/	30 g/L	30 g
Adjust to final pH 5.8 with KOH 1 M			
Agar TC	/	7 g/L	7 g

c. MG1

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
NN macronutrients	10×	1×	100 mL
MS micronutrients	1,000×	1×	1 mL
Fe-EDTA	200×	1×	5 mL
B5 vitamins	500×	1×	2 mL
Sucrose	/	30 g/L	30 g
Adjust to final pH 5.8 with KOH 1 M			
Agar TC	/	7 g/L	7 g
Activated charcoal	/	2.5 g/L	2.5 g

d. MG1-10B

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
NN macronutrients	10×	1×	100 mL
MS micronutrients	1,000×	1×	1 mL
Fe-EDTA	200×	1×	5 mL
B5 vitamins	500×	1×	2 mL
6-benzylaminopurine	1,000 µM	10 µM	10 mL
Sucrose	/	30 g/L	30 g
Adjust to final pH = 5.8 with KOH 1 M			

Agar TC	/	7 g/L	7 g
Activated charcoal	/	2.5 g/L	2.5 g

12. Culture media for full plant development and rooting

a. RIM

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
MS macronutrients	10×	1×	100 mL
MS micronutrients	1,000×	1×	1 mL
Fe-EDTA	200×	1×	5 mL
Vitamins T	1,000×	1×	1 mL
1-naphthaleneacetic acid	1,000 µM	0.5 µM	500 µL
Sucrose	/	30 g/L	30 g
Adjust to final pH 6.0 with KOH 1 M			
Agar TC	/	7 g/L	7 g

b. MSN

Component	Stock concentration	Final concentration	Quantity or Volume (1 L)
MS macronutrients	10×	1×	100 mL
MS micronutrients	100×	1×	10 mL
MS vitamins	500×	1×	2 mL
1-naphthaleneacetic acid	1,000 µM	0.5 µM	500 µL
Sucrose	/	30 g/L	30 g
Adjust to final pH 5.8 with KOH 1 M			
Agar TC	/	7 g/L	7 g

13. Culture media for protoplast cultivation

a. Solid culture medium

Component	Stock concentration	Final concentration	Quantity or Volume (50 mL)
NN macronutrients	10×	1×	5 mL
NN micronutrients	100×	1×	500 µL
NN vitamins	500×	1×	100 µL
1-naphthaleneacetic acid	1,000 µM	10 µM	500 µL
6-benzylaminopurine	1,000 µM	2 µM	100 µL
Glucose	3 M	0.3 M	5 mL
Sucrose	/	30 g/L	1.50 g
Adjust to final pH 5.7 with KOH 1 M			
Gelrite	/	2 g/L	0.1 g

b. Liquid culture medium

Component	Stock concentration	Final concentration	Quantity or Volume (50 mL)
NN macronutrients	10×	1×	5 mL
NN micronutrients	100×	1×	500 µL
NN vitamins	500×	1×	100 µL
1-Naphthaleneacetic acid	1,000 µM	10 µM	500 µL

6-benzylaminopurine	1,000 µM	2 µM	100 µL
Glucose	3 M	0.3 M	5 mL
Sucrose	/	30 g/L	1.50 g
Adjust to final pH 5.7 with KOH 1 M			
Activated charcoal	/	3 g/L	0.15 g

14. Solution for protoplast isolation, purification, and transfection

a. Digestion solution

Component	Stock concentration	Final concentration	Quantity or Volume (20 mL)
Cellulase R10	/	2% w/v	0.4g
Macerozyme R10	/	1 % w/v	0.2 g
Pectolyase Y-23	/	0.05 % w/v	0.01 g
$CaCl_2 \cdot 2H_2O$	1 M	10 mM	200 µL
2-(N-morpholino) ethanesulfonic acid, pH 5.7	100 mM	5 mM	1 mL
Mannitol	1 M	0.5 M	10 mL
Adjust to final pH 5.7 with KOH 1 M			

b. Digestion solution without enzymes

Component	Stock concentration	Final concentration	Quantity or Volume (20 mL)
$CaCl_2 \cdot 2H_2O$	1 M	10 mM	200 µL
2-(N-morpholino) ethanesulfonic acid, pH 5.7	100 mM	5 mM	1 mL
Mannitol	1 M	0.5 M	10 mL
Adjust to final pH 5.7 with KOH 1 M			

c. Wash solution

Component	Stock concentration	Final concentration	Quantity or Volume (50 mL)
$CaCl_2 \cdot 2H_2O$	1 M	10 mM	500 µL
Mannitol	1 M	0.5 M	25 mL
Adjust to final pH 5.7 with KOH 1 M			

d. W5 solution

Component	Stock concentration	Final concentration	Quantity or Volume (40 mL)
$CaCl_2 \cdot 2H_2O$	1 M	125 mM	5 mL
2-(N-morpholino) ethanesulfonic acid, pH 5.7	100 mM	2 mM	800 µL
NaCl	1 M	154 mM	6.16 mL
KCl	500 mM	5 mM	400 µL

e. MMG solution

Component	Stock concentration	Final concentration	Quantity or Volume (10 mL)
Mannitol	1 M	0.4 M	4 mL
$MgCl_2 \cdot 6H_2O$	500 mM	15 mM	300 µL

2-(N-morpholino) ethanesulfonic acid, pH 5.7	100 mM	4 mM	400 µL

f. PEG solution

Component	Stock concentration	Final concentration	Quantity or Volume (10 mL)
Mannitol	1 M	0.2 M	2 mL
$CaCl_2 \cdot 2H_2O$	500 mM	100 mM	1 mL
PEG 4000	/	40%	4 g

Laboratory supplies

1. Steri vent container 107 × 94 × 96 mm (Duchefa Biochemie, catalog number: S1682)
2. Nylon filter 60 µM (AGRINOVA)
3. Petri dishes 92 × 16 (VWR, catalog number: 391-0493)
4. Petri dishes 60 × 15 (Greiner, catalog number: 628161)
5. Petri dishes 35 × 15 (Thermo Scientific, catalog number: 153066)
6. Sterile needles 0.7 × 50 mm (Henke Sass Wolf, catalog number: 471,0007050)
7. Sterile syringes 1 mL (Terumo, catalog number: MDSS01SE)
8. Sterile syringes 50 mL (NIPRO, catalog number: BSS131)
9. Sterile syringes 20 mL (BD Plastipak, catalog number: 300613)
10. Sterile centrifuge tubes, Falcon 15 mL (VWR, catalog number: VWRI525-0607)
11. Sterile centrifuge tubes, Falcon 50 mL (VWR, catalog number: VWRI525-0612)
12. Pipettes (Eppendorf)
13. Fast-Read102® (Kova International, catalog number: BVS100H)
14. Filter 0.2 µm (Sarstedt, catalog number: 83.1826.001)
15. Pasteur 3 mL (Sarstedt, catalog number: D-51588)
16. Filter tips (2,5 µL, 20 µL, 200 µL, 1,000 µL, 5 mL)
17. Scalpel blades 21 and 11 (Duchefa)
18. Microscope slides
19. Coverslips
20. PCR tubes
21. 1.5 mL Eppendorf tubes
22. Sterile scalpels
23. Sterile forceps
24. Parafilm

Equipment

1. Heating magnetic stirrer (VWR®, model: 442-0664)
2. pH meter (CRISON, model: BASIC 20^+)
3. Weighing balance (OHAUS, model: AX422/E)
4. Analytical balance (OHAUS, model: PA114C)
5. Glass microsphere sterilizer (AgnTho's AB, model: steri 250, Art-Nr 31'101)

6. ChemiDoc imaging system (Bio-Rad, model: 12003153)
7. Thermal cycler (Bio-Rad, model: S1,000)
8. Horizontal laminar flow cabinet (BIOAIR, model: aura HZ72)
9. PowerPac™ basic power supply (Bio-Rad, model: 1645050)
10. Horizontal electrophoresis cells (Bio-Rad, model: Sub-Cell GT)
11. Horizontal electrophoresis cells (ELETTROFOR, models: OA-50; OA-78)
12. INCU-Line 150R (VWR®, model: 390-1338)
13. Benchtop microcentrifuge (Eppendorf, catalog number: 5420)
14. Centrifuge (Eppendorf, model: 5804 R)
15. Stereomicroscopes (Leica, models: MZ16 F; EZ4)
16. Optical microscope (Leica, model: DM2500)

Procedure

All the following steps must be done under a laminar flow hood in order to maintain sterile conditions unless otherwise specified. Be sure to use sterile laboratory supplies and to have an available bead sterilizer in order to be able to periodically sterilize all the tools used to handle the plant material.

Embryogenic calli induction and long-term maintenance

A. Embryogenic calli induction from unopened leaves

1. Open the container in which the in vitro grapevine plantlets are present (Figure 1A).
2. Cut the unopened leaves near the apex of the shoots with a scalpel.
3. Collect all the leaves inside a Petri dish using forceps.
4. Prepare some Petri dishes with NB2 solid medium inside.
5. Still using forceps, place up to five unopened leaves inside each NB2 Petri dish (Figure 1B), with one side fully adjacent to the induction medium (abaxial side).
6. Incubate the NB2 petri dishes prepared with the plant material in full darkness at 27 °C for 6–7 weeks.
7. Check weekly for the evolution of the plant material in NB2 medium and the induction of embryogenic calli (Figure 1L).

B. Embryogenic calli induction from stamens and pistils

1. Examination of the microsporogenesis development stage in anthers

Steps B1a–B1j can be performed in non-sterile conditions.

a. Collect a single flower from an inflorescence (Figure 1C).
b. Using a stereomicroscope, open the flower (Figure 1D) by cutting just below the calyptra with the sharp tip of a needle connected with a syringe in order to eliminate the calyx from the flower and maintain just the upper part with the calyptra, stamens, and pistil.
c. Still using a stereomicroscope, with two syringes, open the calyptra and reveal the stamens (Figure 1E) and the pistil (Figure 2F) inside.
d. Pick up all the stamens and place them on a microscope slide.
e. Crush the anthers in order to reveal the reproductive cells hidden inside (Figure 1G, H, I).

f. Soak the crushed material with 2–3 droplets of acetocarmine.

g. Cover the sample with a coverslip.

h. Observe the structures revealed in the crushed material under an optical microscope.

i. Repeat steps *B1a–B1i* by sampling different flowers from different parts of the inflorescence.

j. If all the observations show the microsporogenesis stage (Figure 1G, H, I) required, it is possible to proceed with the inflorescence sampling.

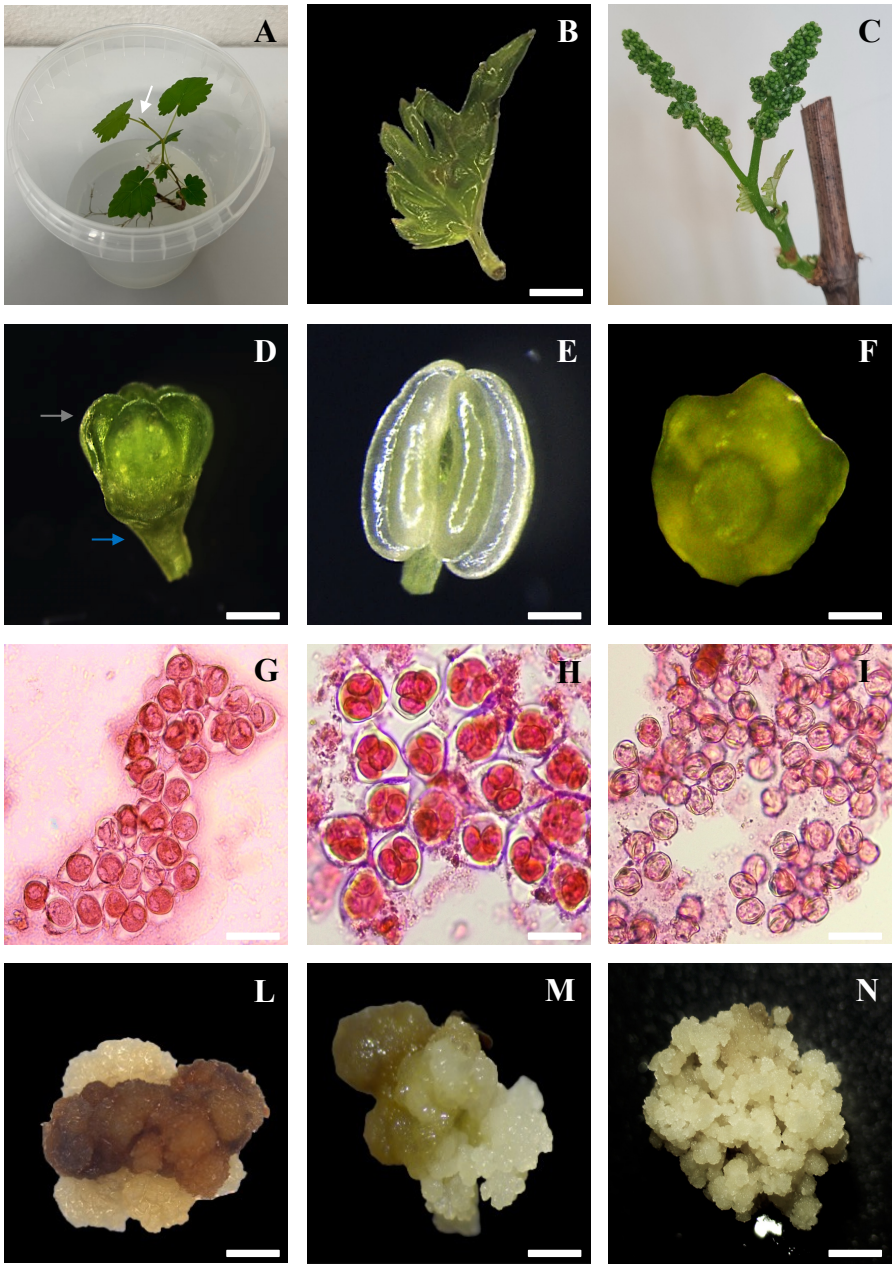


Figure 1. Embryogenic calli induction and proliferation. A. In vitro grapevine plantlet. The arrow indicates the apex. B. In vitro leaf in NB2 medium (scale bar: 6 mm). C. Inflorescence from greenhouse-grown cutting. D. Grapevine flower (scale bar: 3 mm). Grey arrow indicates the calyptra, and blue arrow indicates the calyx. E. Stamen (scale bar: 0.5 mm). F: Pistil (scale bar: 0.5 mm). Pollen microsporogenesis stages: mother cells (G), tetrads (H), uninucleate pollen cells (I) (scale bars: 50 μm). L. In vitro leaf-induced embryogenic callus in NB2

medium (scale bar: 2 mm). M. Stamen/pistil-induced embryogenic callus in MSII/PIV media (scale bar: 2 mm). N. Embryogenic calli proliferated in $C1^P$ medium (scale bar: 3 mm).

2. Inflorescence sampling and sterilization
a. Prepare the solution for the inflorescence sterilization (NaClO 3% and Tween-20 0.1%) in a Falcon tube.
b. Cut the entire inflorescence and immerse it in the sterilization solution.
c. Mix continuously by inversion for 10 min.
d. Discard the sterilization solution inside a collection container.
e. Fill the empty Falcon, which contains the inflorescence, with sterile double-distilled H_2O.
f. Mix continuously by inversion for 5 min.
g. Discard the water inside the collection container.
h. Repeat steps B2e, B2f and B2g two more times.
i. Using forceps, gently pick the inflorescence from the Falcon and place it in a new Petri dish.
j. Store the sterilized inflorescence at 4 °C up to 72 h.

3. Flower explants cultivation
a. Prepare a Petri dish with single drops of sterile double-distilled H_2O on different sides of the Petri dish.
b. Cut some flowers from the inflorescence with forceps and a scalpel and transfer them to the Petri dish prepared in the previous step.
c. Be sure to place the flowers inside sterile double-distilled H_2O droplets.
d. Using a stereomicroscope, with the sharp tip of a couple of sterile needles connected to two syringes, apply a cut in the flower just below the calyptra and discard the lower part of the organ (calyx).
e. Using needles, eliminate the calyptra and reveal the stamens and the pistil.
f. Gently pick the stamens and the pistil with the smooth part of the tip of a sterile needle, and one by one place them in a Petri dish with solid induction media (MSII or PIV) inside.
g. Place 52 explants (16 pistils and 36 stamens) in each induction media Petri dish.
h. Prepare up to 10 plates for each embryogenic calli induction medium.
i. Incubate for 2–4 months in darkness at 27 °C.
j. Using the stereomicroscope, check weekly the formation of embryogenic calli (Figure 1M).

C. Embryogenic calli proliferation

1. Selection of embryogenic calli and transfer in $C1^P$ medium
a. Using a stereomicroscope, select white and granular shaped embryogenic calli (Figure 1M).
b. Mark the top of the Petri dish in correspondence with the explant selected for the propagation of the embryogenic calli.
c. Using a stereomicroscope, for each embryogenic callus selected (step C1b), collect all the embryogenic material emitted by the explant selected and transfer it to a new $C1^P$ Petri dish.
d. Prepare one or more $C1^P$ Petri dishes, each containing new embryogenic calli.
e. Using a scalpel, put embryogenic calli close to each other.
f. Repeat from step C1c to step C1e for each embryogenic callus (step C1b) selected.
g. Incubate the $C1^P$ petri dishes in darkness at 27 °C for 4 weeks.

2. First subculture in $C1^P$ medium and subsequent subcultures

a. Using a stereomicroscope, collect embryogenic material using a scalpel from 4-week-old $C1^P$ Petri dishes (Figure 2N).
b. Transfer selected embryogenic material to a new $C1^P$ Petri dish.
c. Prepare up to six new $C1^P$ Petri dishes.
d. Adjust the position of the embryogenic callus as described in step C1e.
e. Repeat from step C2a to step C2d for all embryogenic callus selected (step C1b).
f. Incubate in darkness at 27 °C for 4 weeks.
g. Repeat the subculture process every 4 weeks and prepare in each cycle six $C1^P$ Petri dishes with fresh embryogenic material for each embryogenic callus selected.

Protoplast isolation and cultivation

A. Preparation of embryogenic calli

1. Using a stereomicroscope, select compact and white cream-colored embryogenic callus from 4-week-old embryogenic culture masses grown in $C1^P$ medium.
2. Transfer them to fresh $C1^P$ medium.
3. Prepare up to seven $C1^P$ plates.
4. Incubate them at 27 °C in darkness for 7–10 days.

B. Protoplasts isolation

1. Using forceps, transfer embryogenic calli (Figure 2A) to the 60 × 10 mm Petri dish. Additionally, transfer a small amount of embryogenic calli as control to the 35 × 10 mm Petri dish.
2. Weigh embryogenic calli collected in the 60 × 10 mm Petri dish and add 10 g/mL of filtered-sterilized digestion solution.
3. Add 1 mL of filtered-sterilized digestion solution without enzymes in the 35 × 10 mm Petri dish.
4. Incubate both Petri dishes in darkness for 5–6 h on a rotating shaker.

C. Monitoring of progression of cell wall digestion

1. After 90 min, take an aliquot from the Petri dish containing calli in digestion solution (step B4) and observe the progress of isolation under an optical microscope.
2. Repeat step C1 after 3 h of incubation.
3. After about 5–6 h of incubation (Figure 2B), evaluate the correct digestion of the cell wall by staining both protoplasts and control with 2 μM fluorescent brightener 28.
4. Mix by pipetting.
5. Prepare a microscope slide and observe both protoplasts (Figure 2C) and control (Figure 2E) under UV light (Figure 2D, F).

D. Protoplast filtration and washing

1. Filter protoplast in a new Petri dish using a 60 μm nylon sieve.
2. Gently transfer filtered protoplast to a 15 mL Falcon.

3. Centrifuge the mixture at 100× *g* for 6–8 min.
4. Remove the supernatant.
5. Completely resuspend the protoplast pellet in 2–3 mL of wash solution.
6. Add wash solution until a final volume of 5–10 mL.
7. Centrifuge at 100× *g* for 3 min at 20 °C.
8. Remove the supernatant.
9. Repeat steps D5 to D8.
10. Resuspend protoplast pellet in 3–5 mL of wash solution.

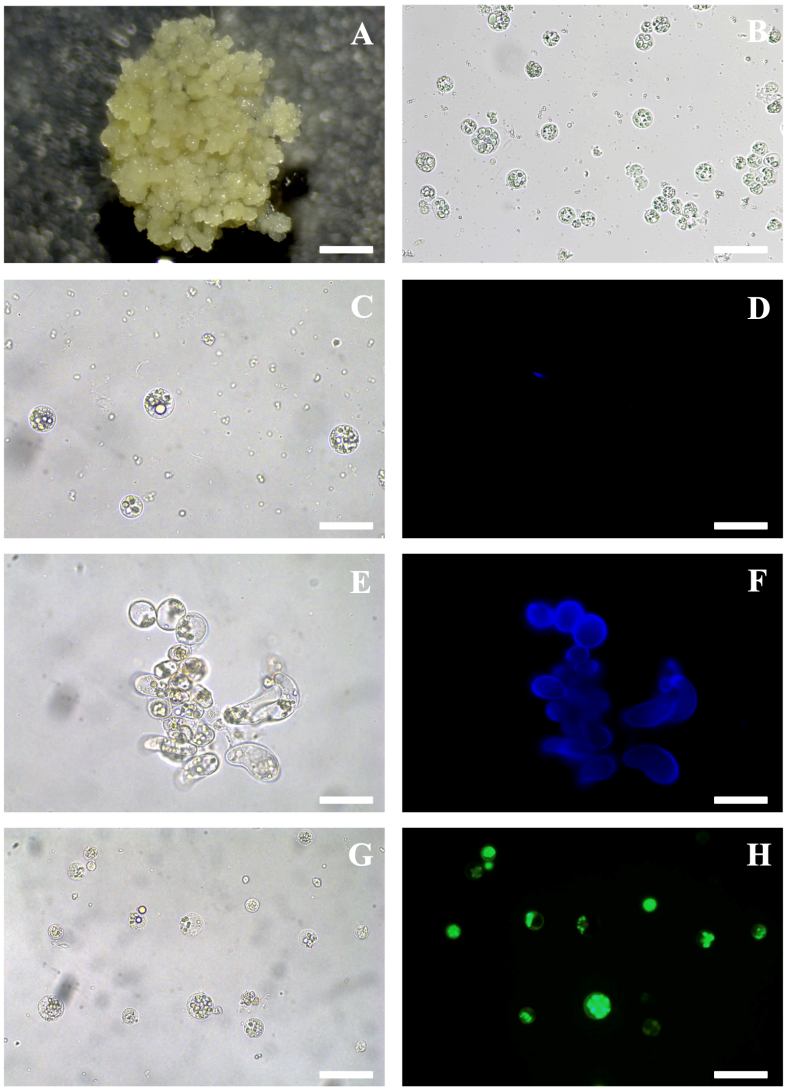


Figure 2. Protoplast isolation from embryogenic calli, analysis of cell wall digestion, and protoplast viability. A: Embryogenic callus (7–10 days) in C1 medium (scale bar: 2 mm). B: Protoplasts isolated from embryogenic calli following 5 h of incubation with digestion solution. C, D: Protoplasts stained with fluorescent brightener 28 under white (C) and UV (D) light. The absence of blue signal indicates the correct cell wall digestion. E, F: Embryogenic calli (control) stained with fluorescent brightener 28 under white (E) and UV (F) light. The blue signal indicates the presence of the cell wall. G, H: Protoplasts stained with FDA under white (G) and UV (H) light. The green fluorescent signal indicates that protoplasts are viable and therefore can be used for transfection and cultivation (Scale bars: 20 μm).

E. Evaluation of protoplasts viability by fluorescein diacetate (FDA) staining

1. Add 0.5 mg/mL FDA solution to a previously obtained aliquot of protoplasts mixture.
2. Mix by pipetting.
3. Prepare a microscope slide and observe protoplasts (Figure 2G) under UV light (Figure 2H).

F. Protoplasts count, cultivation, and analysis of regenerative structures

1. Prepare a 1:10 protoplast dilution in wash solution and place an aliquot in a counting chamber.
2. Determine the number of protoplasts obtained in 1 mL (protoplast/mL) using an optical microscope.
3. Proceed to protoplast cultivation, using a final concentration of 1×10^5 ppt/mL.
4. Based on the yield obtained per milliliter, calculate the volume of suspension to obtain a quantity of 1×10^5 protoplasts and transfer it into 15 mL Falcons.
5. Centrifuge at $100 \times g$ for 3 min.
6. Remove the supernatant.
7. Resuspend each pellet in 1 mL of solid culture medium for protoplast cultivation and prepare four drops for each 60 mm Petri dish.
8. Once the drops have solidified, add 4 mL of liquid culture medium for protoplast cultivation.
9. Incubate plates at 27 °C.
10. Monitor cell divisions and development of regenerative structures every week using an inverted microscope (Figure 3A–F).

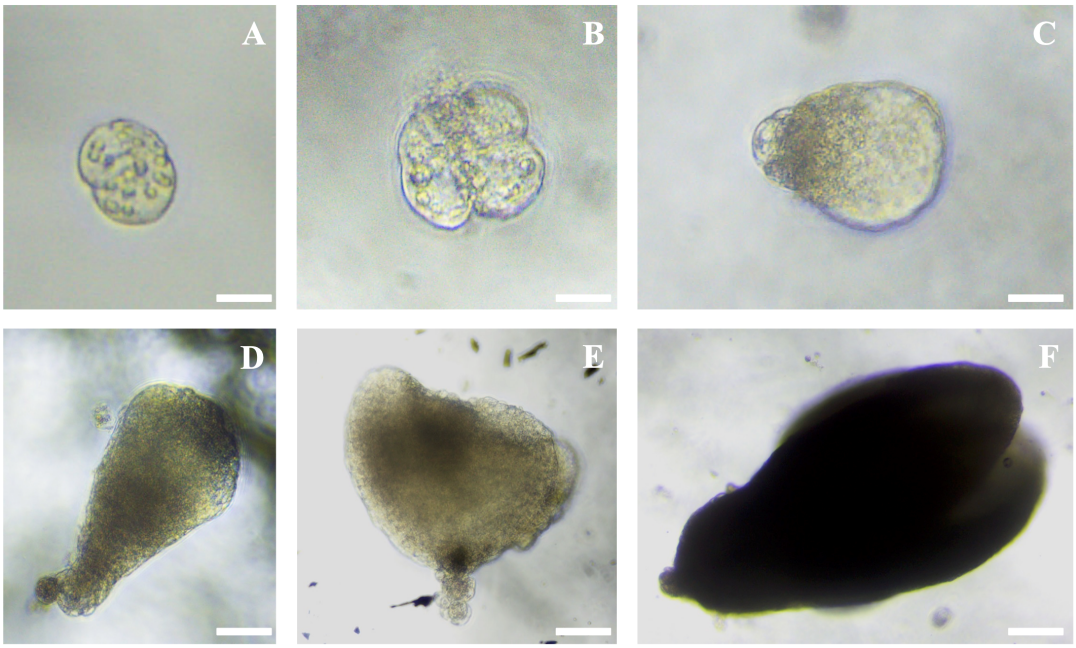


Figure 3. Stages of regeneration from protoplasts. A: First cell division (scale bars: 30 μm). B: Further cell divisions (scale bars: 40 μm). C: Somatic embryo at early globular stage of development (scale bars: 50 μm). D: Somatic embryo at the early globular stage of embryo development (scale bars: 70 μm). E: Somatic embryo at the heart stage of embryo development (scale bars: 70 μm). F: Somatic embryo at the torpedo stage of embryo development (scale bars: 100 μm).

Protoplasts PEG-mediated transfection

A. RNA guide design

RNA guide design was performed using CRISPOR online software (http://crispor.tefor.net) and CRISPR-RGENE (http://www.rgenome.net).

B. RNA guide synthesis

RNA guide synthesis was conducted using the GeneArt Precision gRNA Synthesis kit following the instructions provided by the manufacturer. The main steps are as follows:
1. Design forward and reverse oligonucleotides for PCR assembly.
2. PCR-assemble the gRNA DNA template using the Phusion™ High-Fidelity PCR master mix.
3. Generate the gRNA by in vitro transcription using the TranscriptAid™ enzyme mix.
4. Remove the DNA template by DNase I degradation.
5. Purify the in vitro–transcribed gRNA using the GeneJET™ purification columns.
6. Measure the purified gRNA concentration using Qubit RNA BR kit following the instructions provided by the manufacturer.

C. Cas9-sgRNA ribonucleoprotein complex assembly

To assemble the Cas9-sgRNA ribonucleoprotein complex, proceed as follows:
1. Add the Cas9 protein and sgRNA at a 1:1 weight ratio in a tube and mix gently.
2. Incubate for 10 min in darkness at room temperature.

D. PEG-mediated protoplasts transfection, cultivation, and analysis of regenerative structures

1. Add 2×10^5 protoplasts in a sterile 15 mL falcon tube.
2. Centrifuge the protoplasts at $100 \times g$ for 3 min.
3. Remove the supernatant.
4. Gently resuspend the protoplasts in 200 µL of MMG solution.
5. Add the Cas9-sgRNA ribonucleoprotein complex preassembled as described above in section “Cas9-sgRNA ribonucleoprotein complex assembly”.
6. Add 200 µL of PEG solution.
7. Mix gently until the solution is completely homogenized.
8. Incubate the solution for 20 min in the dark at room temperature.
9. Wash the protoplasts by adding 2 mL of sterile W5 solution and mix gently.
10. Centrifuge the protoplasts at $100 \times g$ for 3 min.
11. Remove the supernatant.
12. Repeat steps D9 to D11.
13. Remove the supernatant and resuspend the protoplasts in 1 mL of solid culture medium for protoplast cultivation. Proceed with the protoplasts cultivation as indicated in section above “Protoplasts count, cultivation, and analysis of regenerative structures” (steps F7 to F9).

14. Monitor cell divisions and the development of regenerative structures every week using an inverted microscope (Figure 3A–3F).

Somatic embryogenesis and plant regeneration

A. Identification of somatic cotyledonary embryos

1. After 2–3 months, verify the correct development of mature somatic cotyledonary embryos (Figure 4A) regenerated from protoplasts using a stereomicroscope.
Note: This step can be performed in non-sterile conditions.

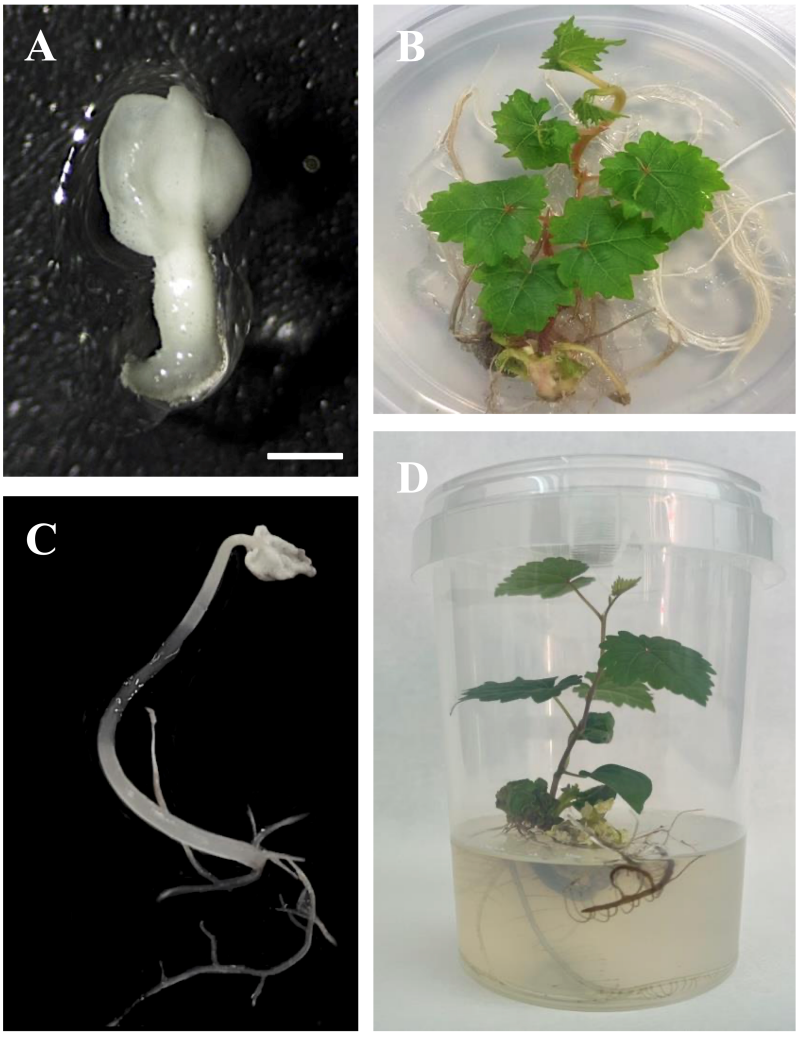


Figure 4. Plants regeneration from protoplasts. A. Mature cotyledonary somatic embryo (scale bar: 0.5 mm). B. Germinated somatic embryo. C. Young plantlet in shooting medium. D. Whole plant regenerated in vitro.

B. Germination of somatic cotyledonary embryos

1. Using a stereomicroscope, transfer mature somatic cotyledonary embryos from DC medium to full germination medium.

2. Incubate for 3–4 weeks in darkness at 27 °C.

C. Shoots and plantlets development

1. Transfer well-developed germinated somatic embryos (Figure 4B) with forceps to C2D, C2D-4B, MG1, and MG1-10B media.
2. Incubate for 5–6 weeks under light with a 16-h photoperiod.

D. Plantlets regeneration and in vitro whole plant development

1. Check the correct development of regenerated plantlets (Figure 4C).
2. Transfer plantlets with forceps to MSN and RIM plant development media.
Note: Regenerated plantlets from C2D and C2D-4B were transferred to MSN medium, while regenerated plantlets from MG1 and MG1-10B were transferred to RIM medium.
3. Maintain fully regenerated plantlets (Figure 4D) under light with a 16-h photoperiod.

Validation of protocol

As mentioned in Najafi et al. 2022, to confirm genome editing events, genomic DNA was extracted from leaves of each regenerated plantlet derived from RNP-transfected protoplasts. A PCR amplification was performed targeting the specific genomic region of interest. Finally, Sanger sequencing verified mutations of the target site, confirming the success and precision of the editing process.

General notes and troubleshooting

General notes

1. Collect young unopened leaves from the apex of in vitro grapevine plantlets (5–6 weeks old) using sterile forceps. "Unopened" refers to leaves where the two sides of the leaf flap are still closed. Place droplets of sterile double-distilled H_2O in a sterile Petri dish to maintain humidity.
2. Submerge inflorescences in water during processing and use two syringes to remove the calyptra, ensuring stamens remain attached to the pistil.
3. Embryogenic callus formation efficiency is dependent on the cultivar. Examine explants under a stereomicroscope for embryogenic material, which should appear white and granular. High-quality calli crumble apart when touched with a sterile scalpel.
4. After being placed in $C1^P$ medium, not all selected calli will proliferate effectively; use only high-quality material for subsequent steps.
5. Use cut tips to minimize damage to protoplasts and evaluate viability with fluorescein diacetate (FDA) staining. Monitor cell wall digestion and handle protoplasts carefully to maintain integrity.

6. The tissue of origin for protoplast isolation, details in the purification steps, and the composition of the regeneration media may vary across plant species, whereas the type and concentration of enzymes in the digestion solution are quite standard regardless of the starting material.

Acknowledgments

E.B., E.D. and S.Ze. designed the research; E.B., S.Za., S.L., L.C. and C.C. performed the research and analyzed the data; A.A., M.F. and S.Ze. contributed to data analysis; E.B., E.D., S.Za., S.L., L.C. and C.C. wrote the paper. This work was supported by EdiVite s.r.l., Grant BAYER (University of Verona awarded to Sara Zenoni) and PRIN 2022 (University of Verona awarded to Sara Zenoni). This protocol is based on our recent publication [13].

Competing interests

The patent application N. 102021000023468 is based on this protocol.

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