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Generation and Selection of Transgenic Olive Plants

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[Abstract] Olive (*Olea europaea* L.) is one of the most important oil crops in the Mediterranean basin. Biotechnological improvement of this species is hampered by the recalcitrant nature of olive tissue to regenerate *in vitro*. In previous investigations, our group has developed a reliable *Agrobacterium*-mediated transformation protocol using olive somatic embryos as explants (Torreblanca *et al*., 2010). Embryogenic cultures derived from radicles of matured zygotic embryos are infected with *Agrobacterium tumefaciens*, AGL1 strain, containing a binary plasmid with the gene of interest and the *nptII* selection gene. After a meticulous selection procedure, carried out using solid and liquid media supplemented with paromomycin, the putative transformed lines are established. A preliminary confirmation of their transgenic nature is carried out through PCR amplification. Afterwards, plants can be obtained through an efficient regeneration protocol, whose main characteristics are the use of a low-ionic-strength mineral formulation, a phase in liquid medium for synchronization of cultures and the use of semi-permeable cellulose acetate membranes for embryo maturation (Cerezo *et al.*, 2011). Final confirmation of transgene insertion is carried out through Southern or Northern analysis using leaf samples of regenerated plants.

Keywords: *Olea europaea*, Genetic transformation, *Agrobacterium tumefaciens*, Somatic embryogenesis

[Background] The protocol developed by Torreblanca *et al*. (2010) differs from the previous olive transformation protocol, developed by Rugini *et al*. (2000), in several aspects; mainly, kind of explant, *Agrobacterium* strain and the selection method used. Rugini *et al*. (2000) used embryogenic masses as explants, which were incubated in a bacterial suspension of LBA4404 *Agrobacterium* strain for 48 h. After the infection, the explants were rinsed in water and cultured in embryogenic medium supplemented with 250 mg/L cefotaxime; however, the selection of transgenic embryos was not started until 30 days after the infection, with the addition of 100 mg/L kanamycin. To speed up the process, the explants were transferred to liquid medium in light, and the embryos which turned green were selected and cultured in isolation on solid multiplication medium with 150 mg/L kanamycin. Later on, the plant regeneration process was carried out without kanamycin. In contrast, Torreblanca *et al*. (2010) used globular somatic embryos as explants and the AGL1 *Agrobacterium* strain, with an incubation period of only 2 h and 2 days co-culture. Afterwards, the explants were transferred to selection medium with 200 mg/L paromomycin, and re-cultured onto fresh selection medium weekly during the first month and

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bi-weekly thereafter. In addition, a 3 weeks selection period in liquid medium supplemented with 50 mg/L paromomycin was included. The selection process and the use of somatic embryos as explants solved the problems of chimaeric transgenic embryos appearance, and higher transformation efficiencies were obtained. The protocol for olive plant regeneration published by Cerezo *et al*. (2011) improved whole plant recovery (shoots and roots) from 1.5% up to 50%. Both protocols together, have allowed the development of a reliable regeneration and transformation procedure in olive, recently used in flower induction studies (Haberman *et al*., 2017). Indeed, these protocols have been employed to analyze the effect of overexpression of *MtFT1* gene in olive.

Materials and Reagents

A. Biological material
   1. Embryogenic olive cultures, formed by callus and globular embryo structures of yellow-creamed colour
   2. *Agrobacterium tumefaciens* AGL1 strain harbouring a binary vector, containing the gene of interest and the *nptII* selection gene

B. Chemicals and materials
   1. Sterile filter paper cut 10 x 10 cm (Filtros Anois, FILTER-LAB®, catalog number: RM13054252)
   2. Petri plates (90 cm) (J. D. CATALAN, S. L.)
   3. Mesh, 3 x 3 (ALBUS Suministros de Laboratorio)
   4. Active charcoal (Sigma-Aldrich, catalog number: C9157)
   5. Assay tubes (25 x 150 mm) (Kimble Chase Life Science and Research Products, catalog number: 73500-20150)
   6. Dialysis tubing cellulose membrane (Sigma-Aldrich, catalog number: D9777-100FT)
   7. Jiffystrips 5-50 peat pots square, 4.5 x 4.5 cm (Jiffy, catalog number: 110007)
   8. Plant pots (12.5 and 20 cm)
   9. 1:1 peat moss:perlite substrate (Projar professional)
   10. *Agrobacterium* liquid growth medium (LB medium) (AppliChem, catalog number: 414753)
   11. 10 mM magnesium sulphate ($MgSO_4$) (AppliChem, catalog number: 131404)
   12. Antibiotics:
      a. Paromomycin (Duchefa Biochemie, catalog number: P0141)
      b. Cefotaxime (PhytoTechnology Laboratories, catalog number: C380)
      c. Timentin (Duchefa Biochemie, catalog number: 011258)
   13. ¼ OM (Cañas and Benbadis, 1988) macroelements
   14. ¼ MS (Murashige and Skoog, 1962) microelements
   15. ½ OM Vitamins
   16. *Myo*-inositol (Sigma-Aldrich, catalog number: I5125)
   17. Sucrose (D(+)-Saccharose) (VWR, catalog number: 27478.467)

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18. L-Glutamine (Biowest, catalog number: P1012)
19. Casein hydrolysate (N-Z-Amine® A) (Sigma-Aldrich, catalog number: C0626)
20. Mannitol (Sigma-Aldrich, catalog number: M9647)
21. Plant hormones:
    a. N6-2-Isopentenyladenine (2iP) (Duchefa Biochemie, catalog number: D0934)
    b. N6-benzyladenine (BA) (Duchefa Biochemie, catalog number: B0904)
    c. Indole-3-butyric acid (IBA) (Sigma-Aldrich, catalog number: I5386)
    d. Zeatin riboside (ZR) (Duchefa Biochemie, catalog number: Z0917)
22. Olive cyclic embryogenesis medium (ECO) (see Recipes)
23. Germination medium (see Recipes)
24. Shoot proliferation medium (see Recipes)
25. Plant rooting medium (see Recipes)

Equipment

1. Culture flasks (125 ml) (Nalgene)
2. Autoclave (JP SELECTA, model: Autester MOD 437-G)
3. Constant temperature/orbital shaker incubator (Optic Ivymen System)
4. Laminar flow hood (Telstar, model: BH-100)
5. Laboratory centrifuge (Sigma Laborzentrifugen, model: 3K30)
6. Spectrophotometer (JP SELECTA, model: UV-2005)
7. Walk in plant growth cabinet with controlled light and temperature conditions

Procedure

Note: All this protocol must be conducted under strictly sterile conditions in a laminar flow hood; except B7 step and molecular analysis.

A. *Agrobacterium*-mediated transformation of olive embryogenic callus
   1. Obtain an embryogenic olive culture, derived from radicle of mature seed (Orinos and Mitrakos, 1991). Embryogenic friable callus (Figure 1A), containing globular embryos, is maintained and multiplied on ECO medium (see Recipes), transferring to fresh medium at 4-week intervals in darkness at 25 ± 2 °C (Pérez-Barranco *et al.*, 2009).
   2. Grow *Agrobacterium tumefaciens* AGL1 strain, containing a binary plasmid harbouring the gene of interest and the selection gene *nptII* (pBINUbiGUSInt as example; containing *uidA* and *nptII* genes), in LB medium at 28 °C and 250 rpm for 24 h (with suitable antibiotics for the plasmid used), to obtain a culture of 40 ml at 0.5 $OD_{600\ nm}$. Then, centrifuge the culture at 4,000 *x g*, wash the pellet with 10 mM $MgSO_4$ without shaking and dilute it in liquid ECO medium, keeping a final 0.5 $OD_{600\ nm}$.

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3. Inoculate globular somatic embryos 1-2 mm diameter, isolated from embryogenic callus, in the diluted *Agrobacterium* suspension for 20 min under mild agitation; about 20 embryos into 10 ml of *Agrobacterium* suspension with a total of 80 embryos, approximately (Figure 1B). Then, dry the embryos out on sterile filter paper (Figure 1C).
4. Co-culture the infected somatic embryos on ECO solid medium for 48 h in darkness, 15-20 embryos per plate (Figure 1D).
5. Afterwards, wash the somatic embryos with ECO liquid medium supplemented with 250 mg/L cefotaxime and 250 mg/L timentin (Figure 1E). Blot the embryos dry on filter paper (Figure 1F) and transfer them onto selection medium; *i.e.,* ECO solid medium supplemented with 250 mg/L cefotaxime, 250 mg/L timentin and 200 mg/L paromomycin (Figure 1G). Re-culture the explants onto fresh selection medium weekly during the first month and bi-weekly thereafter.
6. When the explants show proliferation of paromomycin resistant embryogenic callus on selection medium (Figure 1H), after 3 months of culture approximately, transfer those calli individually to 250 ml culture flasks with 40 ml of ECO liquid medium supplemented with 25-50 mg/L paromomycin (Figure 1I). Incubate the suspensions in an orbital shaker at 120 rpm for 3 weeks in darkness.
7. Afterwards, sieve the suspensions through a 3 x 3 mm screen (Figure 1J) and culture the small globular embryos separately in a plate on ECO selection medium with 200 mg/L paromomycin. After two months, proliferating callus is transferred to test tubes (Figure 1K).
8. Calli growing on selection medium can be analysed through PCR to obtain a preliminary verification of transformation (Figure 1L).

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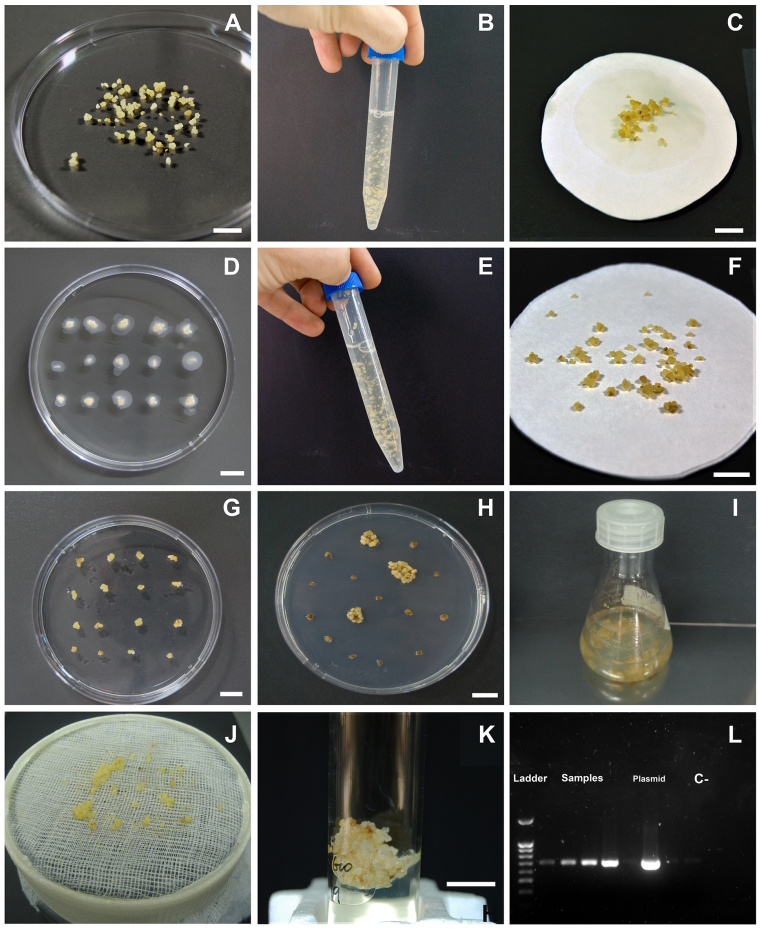

Figure 1. Sequence of olive somatic embryos transformation via *Agrobacterium tumefaciens*. A. Isolated somatic embryos; B. Inoculation of somatic embryos in a diluted *Agrobacterium tumefaciens* suspension; C. Somatic embryos drying onto sterile paper after inoculation; D. Co-culture of somatic embryos with *Agrobacterium* on solid medium; E. Somatic embryos washing in liquid medium supplemented with antibiotics; F. Somatic embryos drying onto filter paper after washing; G. Culture of somatic embryos onto selection medium, supplemented with antibiotics; H. Growth of resistant embryogenic callus on selection medium; I. Incubation of the resistant embryogenic callus in liquid selection medium; J. Obtainment of the fine fraction of the embryogenic callus (1-3 mm); K. Growth of putative transgenic callus on solid selection medium for multiplication; L. PCR verification in an electrophoresis gel. Scale bars = 10 mm.

B. Regeneration of transformed olive plants

1. Once the transgenic nature of the paromomycin resistant embryogenic calli has been confirmed by PCR, the process of transgenic plants regeneration from these calli can be started, following the protocol described by Cerezo *et al.* (2011). Firstly, grow each independent embryogenic calli in ECO liquid medium to obtain globular embryos of small size, 1-3 mm, *e.g.*, culture 0.5 g of callus into 50 ml of medium at 120 rpm for 4 weeks in darkness and later filter through a 3 x 3 mm screen (Figures 2A-2C).

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2. Culture the isolated small globular embryos onto ECO maturation medium for a 4 weeks period (without hormones or cefotaxime and supplemented with 1 g/L active charcoal) in Petri dishes. Incubate the embryos in darkness, 20 globular embryos per plate (Figure 2D).
3. Transfer the embryos directly on top of 4 x 4 cm dialysis tubing cellulose membrane sections lied onto fresh ECO maturation medium and incubate them for another 4 weeks period in darkness (Figure 2E). These membranes are prepared according to the manufacturer's instructions and sterilized by autoclave.
4. Afterwards, culture mature embryos at cotyledonary stage in germination medium (see Recipes) under light conditions (16 h photoperiod, 40 µmol m^{-2} sec^{-1} irradiance level). (Figure 2F)
5. Transfer the shoots obtained to medium supplemented with 5.6 µM ZR (Figure 2H) for further proliferation.
6. To induce rooting, transfer shoots with two or three nodes (2-3 cm) to DKW rooting medium (see Recipes) supplemented with 0.5 µM IBA (Figures 2G-2I).

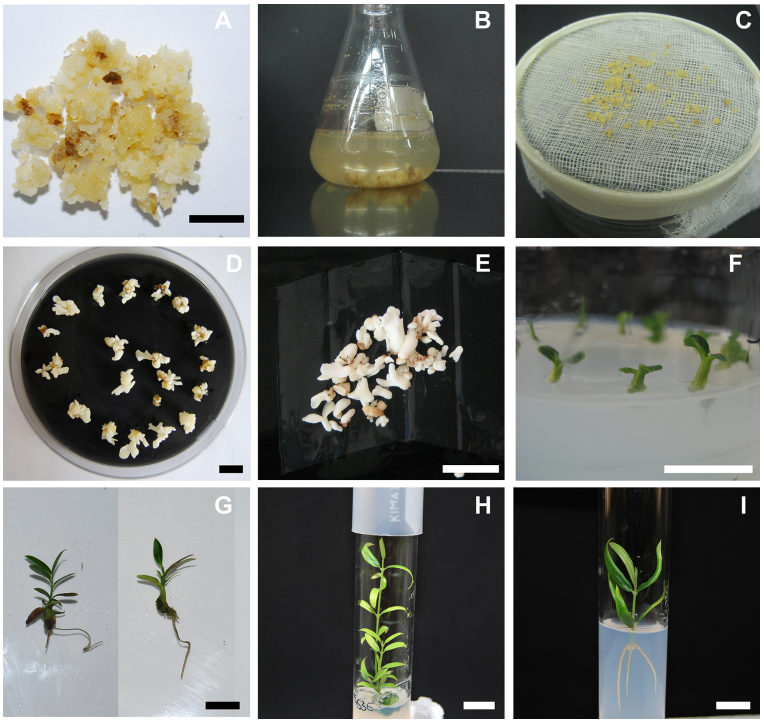

Figure 2. Sequence of regeneration of olive transformed plants. A. Growth of transformed embryogenic callus on selection medium; B. Incubation of the transgenic callus in liquid medium; C. Obtaining a fine fraction embryogenic culture (1-3 mm); D. Culture of small transformed isolated embryos onto ECO maturation medium; E. Culture of transformed pre-maturated embryos onto cellulose acetate membrane lied on ECO maturation medium; F. Culture of transformed maturated embryos in germination medium; G. Transformed olive plantlets; H. Transformed plant growing in multiplication medium; I. Transformed olive plant in rooting medium. Scale bars = 10 mm.

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7. Acclimate rooted plants to *ex vitro* conditions in a greenhouse. Wash roots with distilled water and transfer the plants to a jiffy tray with 1:1 peat moss:perlite substrate and cover the tray with a translucent plastic top. Gradually, adapt the plants to *ex vitro* conditions by increasing exposure to ambient humidity opening the cover. Transfer the plants to larger pots as they grow (Figures 3A-3D).
8. Carry out a Southern or Northern analysis to confirm the transgenic nature of regenerated plants.

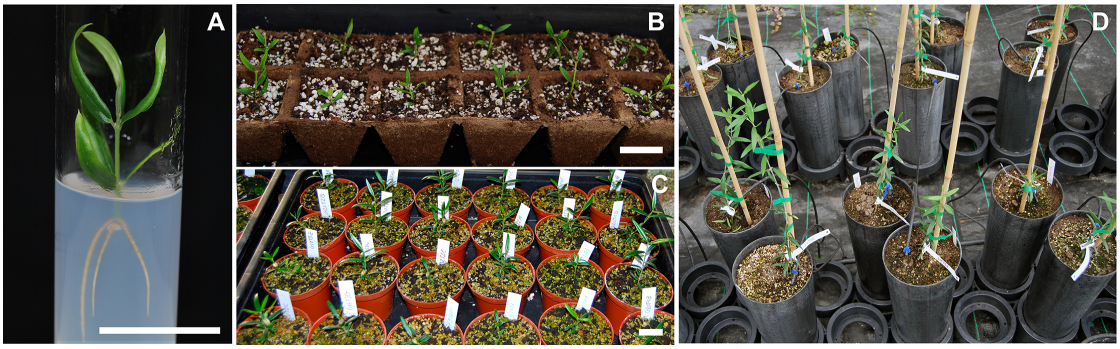

Figure 3. Sequence of recovery of transformed olive plants in a greenhouse. A. A well-developed transformed olive plant; B. Transformed olive plants acclimated into a jiffy tray with 1:1 peat moss:perlite substrate; C. Transformed olive plants in 12.5 cm pots; D. Transformed olive plants in 15 cm pots. Scale bars = 25 mm.

Data analysis

At least 50 explants were used per each transformation experiment, 3 replicates of each one. Transformation efficiency was estimated as the percentage of explants growing on selection medium, in the presence of paromomycin, after selection in liquid medium.

Notes

The selection pressure used after the infection is genotype dependent; *e.g.*, the concentration of paromomycin may vary between 150-200 mg/L in solid medium and 25-50 mg/L in liquid medium. In addition, for some genotypes, an increasing selection pressure is advisable, starting with 50 mg/L paromomycin in solid medium and increasing up to 100, 150 and finally, 200 mg/L.

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Recipes

Note: The pH of all media has been adjusted to 5.7 using NaOH or HCl.

1. Olive cyclic embryogenesis medium (ECO) (Pérez-Barranco *et al.*, 2009)
   ¼ OM (Cañas and Benbadis, 1988) macroelements
   ¼ MS (Murashige and Skoog, 1962) microelements
   ½ OM Vitamins
   0.05 g/L *myo*-inositol
   20 g/L sucrose
   0.550 g/L glutamine
   1 g/L casein hydrolysate
   0.5 µM 2iP
   0.44 µM BA
   0.25 µM IBA
   0.42 µM cefotaxime
2. Germination medium (Cerezo *et al.*, 2011)
   Modified MS medium
   ⅓ macroelements
   0.1 g/L *myo*-inositol
   10 g/L sucrose
3. Shoot proliferation medium (Vidoy *et al.*, 2012)
   Modified RP medium (Roussos and Pontikis, 2002)
   1 g/L *myo*-inositol
   20 g/L mannitol
   1.2 g/L glutamine
   5.6 µM ZR
4. Plant rooting medium (Revilla *et al.*, 1996)
   Half-strength DKW medium (Driver and Kuniyuki, 1984) salts with no vitamins or amino acids
   2% sucrose
   0.5 µM IBA

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