Chapter 13

Virus-Induced Gene Silencing in Olive Tree (Oleaceae)

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Abstract

Research on gene functions in non-model tree species is hampered by a number of difficulties such as time-consuming genetic transformation protocols and extended period for the production of healthy transformed offspring, among others. Virus-induced gene silencing (VIGS) is an alternative approach to transiently knock out an endogenous gene of interest (GOI) by the introduction of viral sequences encompassing a fragment of the GOI and to exploit the posttranscriptional gene silencing (PTGS) mechanism of the plant, thus triggering silencing of the GOI. Here we describe the successful application of *Tobacco rattle virus* (TRV)-mediated VIGS through agroinoculation of olive plantlets. This methodology is expected to serve as a fast tracking and powerful tool enabling researchers from diversified fields to perform functional genomic analyses in the olive tree.

Key words Virus-induced gene silencing (VIGS), Posttranscriptional gene silencing (PTGS), RNA interference (RNAi), Functional genomics, Olive, *Olea europaea*, *Agrobacterium tumefaciens*, *Tobacco rattle virus* (TRV)

1 Introduction

Olive tree (*Olea europaea* L., Oleaceae) is an emblematic crop and its cultivation in the Mediterranean basin goes back millennia. Recent advances in sequencing technologies have led to the availability of an enormous amount of genomic and transcriptomic datasets [1, 2] giving researchers the opportunity to focus on various aspects of olive tree biology. Functional characterization of a gene should ideally be initiated with a knockout mutation and complemented with observation of the phenotype. However, this approach is rather complicated when somebody is working with a non-model plant such as olive, member of a plant family that comprises perennial woody species [3]. Thus, precise characterization of biological/physiological mechanisms governing economically important traits (e.g., juvenile period [4], alternate bearing [5]) or Oleaceae-specific traits (e.g., biosynthesis of

Vincent Courdavault and Sébastien Besseau (eds.), *Virus-Induced Gene Silencing in Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 2172, https://doi.org/10.1007/978-1-0716-0751-0_13,

oleosidic secoiridoids [6–9]) or even olive-specific traits (e.g., genes involved in olive oil quality [10], responses to (a)biotic stresses [11, 12], and morphogenesis of uncommon type of trichomes [13, 14]) is hampered. Few examples of successful genetic transformation of olives and development up to the stage of plantlet have been reported [15–17], even though these approaches are heavily time-consuming—a common problem for all the perennial trees recalcitrant to transformation.

A very powerful tool to study the functional attitudes of a gene/protein as well as the signaling process is the approach of reverse genetics using virus-induced gene silencing (VIGS). This approach is an alternative fast-track route in model plants and in plant species where stable genetic transformation is difficult or even impossible. This strategy has been successfully recruited in numerous species from several plant families [18, 19]. VIGS takes advantage of the posttranscriptional gene silencing (PTGS) mechanism that plants enable as a defense mechanism against viruses. In order to silence an endogenous plant gene of interest (GOI), a small fragment of this gene is cloned in a plasmid which encompasses viral sequences, and this sequence is delivered in the plant either through bombardment or via *Agrobacterium*. During viral replication, dsRNA is produced triggering PTGS and therefore systemic silencing of the mRNAs of the gene of interest. The major advantages of this approach are: there is no need for stable transformation; the transgene is not incorporated into the plant genome—except in agroinfiltrated tissues; and the silencing of the gene of interest is transient, and in many cases the virus is not transmitted to the offsprings [18, 20].

Due to the aforementioned advantages of this approach, VIGS has already gained attention as a tool to functionally characterize gene/protein function and signaling processes in numerous plant species. Although few examples were initially reported in which VIGS was recruited as a tool in woody tree species, recent advances provide additional approaches and strategies to study economically important crops. Up to date, VIGS has been successfully applied in different species of the *Prunus*, *Pyrus*, and *Malus* genera (Rosaceae) [21, 22], species of the *Jatropha* and *Vernicia* genera (Euphorbiaceae) [23, 24], species of the *Xanthoceras* and *Litchi* genera (Sapindaceae) [25, 26], and species of the *Populus* (Salicaceae) [27], *Citrus* (Rutaceae) [28], *Actinidia* (Actinidiaceae) [29], *Camptotheca* (Nyssaceae) [30], and *Morus* (Moraceae) [31] genera. This number is expected to drastically increase in the following years bridging the research in woody tree species and the field of functional genomics.

As a proof of concept, and in order to estimate when and where VIGS is taking place in a plant, silencing of a gene that results in distinguishable phenotype is performed in parallel with silencing of a GOI. Typically, constructs designed to trigger silencing of either

the phytoene desaturase (*PDS*) or the H subunit of Mg-protoporphyrin chelatase (*ChlH*) gene serve as a positive control of successful VIGS process [32]. PDS is involved in the biosynthesis of carotenoids, and silencing of this gene reduces the photo-protective carotenoids, thus causing a photo-bleaching phenotype due to chlorophyll photooxidation [33]. ChlH is involved in the biosynthesis of chlorophyll, and successful silencing causes a yellowish leaf phenotype due to chlorophyll reduction [34]. Although silencing of *PDS* is often used as a visual marker in VIGS studies, it is accepted that silencing of *ChlH* serves as a higher sensitivity visual marker when compared to *PDS* [35–37].

We have successfully applied VIGS in olive plantlets with *Agrobacterium* harboring plasmids encoding the bipartite RNA genome of *Tobacco rattle virus* (TRV). Although it is unknown whether olive is naturally susceptible to the nematode-transmitted TRV, we chose to perform VIGS studies with TRV-based constructs for two reasons. First, TRV is known to have a wide host range and can infect about 400 species in more than 50 plant families [38]. Second, reports of TRV-infected plants from other genera of the Oleaceae family can be found in the literature [39–41]. From a taxonomic point of view, it is worth noting that olive is the fifth plant example that VIGS has been successfully applied in Lamiales, an order with almost 24,000 species distributed in more than 1000 genera and 24 families [42]. The other four reported cases are the sweet basil (*Ocimum basilicum*; Lamiaceae) [43], the snapdragon (*Antirrhinum majus*; Plantaginaceae) [44], the yellow monkeyflower (*Mimulus guttatus*; Phrymaceae) [45], and the purple witchweed (*Striga hermonthica*; Orobanchaceae) [19, 46].

This detailed protocol describes the pipeline to successfully silence endogenous genes in olives by cloning a small fragment of the GOI in a binary vector encompassing the TRV2 genome. Co-inoculation of *Agrobacterium* cells harboring the TRV1 genome with cells harboring the TRV2:GOI genome results in reconstitution of the bipartite genome of TRV in plant cells eliciting the viral infection. This triggers the PTGS mechanism of the plant and thus systemic silencing of the GOI. This approach provides researchers an additional tool to precisely and easily study gene/protein function, contributes to the understanding of signaling processes, implements strategies to tailor-change biochemical pathways, and is expected to boost functional genomic analysis in olive, a perennial woody tree species recalcitrant to genetic transformation. Taking into consideration that VIGS is known to persist *in planta* for several years [47] and can be induced after grafting [48], this approach could potentially be used as a tool to manipulate agronomic traits or even fortify olives against emerging (a) biotic challenges in already established orchards, thus giving new dimensions in sustainable agriculture of tomorrow.

2 Materials

2.1 Vector Construction

1. Sterilized tips, 0.2 and 1.5 mL tubes.
2. Agarose gel apparatus (gel tank, gel tray, well combs, and casting tray) and relative equipment (power supply, microwave oven, UV transilluminator, and gel documentation system).
3. 6× gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% sucrose.
4. 50× Tris-acetate buffer (TAE): mix 242 g Tris-base, 57.1 mL glacial acetic acid, and 100 mL of 0.5 M EDTA (pH 8.0) in 1 L of ddH_2O.
5. Powdered agarose.
6. Ethidium bromide (10 mg/mL, stored in room temperature and protected from light). Working solution is 0.5 μg/mL.
7. Restriction enzymes (*Kpn*I, *Xba*I, *Sma*I, and any other enzyme needed for cloning of the GOI) and the corresponding buffers.
8. Heat blocks or water baths.
9. pTRV1 and pTRV2-MCS vectors: VIGS plasmids encoding the bipartite RNA genome of *Tobacco rattle virus* (TRV) can be obtained from the Arabidopsis Biological Resource Center (ABRC, www.arabidopsis.org) under the stock numbers CD3-1039 (pTRV1) and CD3-1040 (pTRV2-MCS).
10. Liquid nitrogen, mortar, and pestle.
11. RNA extraction buffer: 100 mM Tris–HCl pH 9.5, 0.5% SDS.
12. Phenol, equilibrated to pH 8.
13. 24:1 (v/v) chloroform/isoamyl alcohol.
14. 3 M sodium acetate, pH 4.8.
15. Ethanol.
16. Spectrophotometer.
17. DNase I treatment kit and RNase inhibitor.
18. cDNA synthesis kit including oligo$(dT)_{17}$.
19. Proofreading DNA polymerase and buffer.
20. 10 mM dNTPs set.
21. GOI-specific primers including desired restriction site(s) (*see* **Note 1**):

    OePDS (GenBank Acc. no.: GABQ01079853.1) (232 bp fragment) primers OePDS-F 5′-GATGGCAATCCACCA GAAAGAC-3′ and OePDS-R 5′-ACTGTATCTCCTT CCAGTCCTC-3′.

    OeChlH (GenBank Acc. no.: GABQ01080755.1) (297 bp fragment) primers OeChlH-F 5′-GCAGCTTATTA TTCCTATGTGG-3′ and OeChlH-R 5′-GGACGAGA TCAACTCACTCAAC-3′.

22. PCR machine.
23. T4 DNA ligase.
24. PCR purification and miniprep kits.
25. Chemically competent *Escherichia coli* (DH5α) cells.
26. Luria-Bertani broth medium: 1% peptone, 0.5% yeast extract, and 1% NaCl. In case of solid medium, agar is added in a final concentration of 1.4% before autoclaving.
27. Kanamycin, 1000× stock solution: 50 mg/mL prepared in ddH_2O and stored at −20 °C.
28. Orbital shakers.
29. Centrifuges.
30. Incubators at 37 °C.

2.2 Handling of Agrobacterium *Cells*

1. 10% (v/v) sterile glycerol.
2. *Agrobacterium tumefaciens* strain C58C1 Rif^R (GV3101) containing the T-DNA-deficient Ti plasmid pMP90.
3. LB medium.
4. Antibiotic stock solutions: 5 g/L rifampicin resuspended in methanol (100×), 50 g/L gentamicin resuspended in ddH_2O (1000×), 50 g/L kanamycin resuspended in ddH_2O (1000×). Store all antibiotic stocks at −20 °C. Working solution for each antibiotic is 50 mg/L.
5. Electroporator and electroporation cuvettes of a 0.1 cm gap.
6. Refrigerated centrifuge, centrifugation bottles and tubes.
7. Erlenmeyer flasks, shakers, and incubators at 28 °C.
8. Dilution buffer: prepare stock solutions of 100 mM 2-N-morpholino-ethanesulfonic acid (MES) pH 5.6 and 100 mM $MgCl_2$. Prepare fresh dilution buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$) prior agroinoculations.
9. 100 mM acetosyringone stock solution in DMSO.
10. Syringes of 1 mL with needle.
11. Sterile glycerol (100%).

2.3 Preparation of Olive Plantlets

1. Growth chamber maintained at 22 °C with a 16/8 h light/dark cycle equipped with artificial lighting.
2. Olive seeds collected from *O. europaea* L. cv. 'Koroneiki' trees.
3. Solution of 10% NaOH.
4. A bench vice or a pipe cutter.
5. Solution of 20% NaClO containing 0.01% Triton-X.
6. Soil, pots, and trays.

2.4 Real-Time PCR

1. Reagents for RNA extraction (*see* Subheading 2.1, **items 10–15**).
2. DNAse I treatment kit.
3. Single-strand cDNA synthesis kit including oligo$(dT)_{17}$.
4. GOI-specific primers for qPCR analysis (*see* **Note 2**):
   OePDS: OePDSrt-F 5′-AAACTCCAAGGTCCGTCTATAA-3′ and OePDSrt-R 5′-GCTTTGTGTAATCACCAGCTAAA-3′ primers.
   OeChlH: OeChlHrt-F 5′-GTACACTTTCGGAGACGGTAAG-3′ and OeChlHrt-R 5′-CTTGTCCTGAAGTTGCACTCCA-3′ primers.
   OeActin (GenBank Acc. no.: GABQ01079399.1): OeActin-F 5′-GTATGTTGCTATCCAGGCTGTT-3′ and OeActin-R 5′-AAATGGGTACTGTGTGACTCAC-3′ primers.
5. 2× SYBR Select Master Mix.
6. 96-Well reaction plates with optically transparent lids.
7. Real-time PCR cycler.

3 Methods

3.1 Cloning Procedures and Vector Construction

1. Grind young leaves (and/or other desired plant material) from *Olea europaea* L. cv. 'Koroneiki' plants into powder using a mortar and pestle in the presence of liquid nitrogen.
2. Transfer 200 mg of powdered tissue in a sterile and pre-chilled tube, and add 200 μL of RNA extraction buffer and 200 μL of phenol (pH 8.0). Mix thoroughly by vortexing and centrifuge the sample for 5 min at 13,000 × *g*.
3. Transfer the aqueous (upper) phase in a new tube and add an equal volume of phenol. Mix thoroughly and centrifuge the sample for 5 min at 13,000 × *g*.
4. Transfer the aqueous phase in a new tube, and add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Mix thoroughly and centrifuge the sample for 5 min at 13,000 × *g*.
5. Transfer the aqueous phase in a new tube, and add an equal volume of chloroform/isoamyl alcohol (24:1). Mix thoroughly and centrifuge the sample for 5 min at 13,000 × *g*.
6. Transfer the aqueous phase in a new tube, and add 1/10 volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of ice-cold ethanol (100%). Mix gently and precipitate the nucleic acids by incubating the sample at −20 °C for 16 h.
7. Centrifuge the sample at 13,000 × *g* for 20 min at 4 °C. Discard the supernatant, air-dry the pellet, and resuspend the nucleic acids at 30 μL of RNase-free ddH_2O.

8. Determine the quantity and the quality of the extracted nucleic acids using a spectrophotometer and agarose gel electrophoresis, respectively. The sample can be stored at −80 °C.
9. Proceed with RNase-free DNase I treatment to remove the DNA using 5–15 μg of the extracted nucleic acids and following the manufacturer's instructions. The reaction is incubated at 37 °C for 1 h. When the reaction is finished, add ddH_2O in a total volume of 400 μL, and proceed with phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol/sodium acetate precipitation (**steps 4–7**) to recover the DNA-free RNA sample(s).
10. Determine the quantity and the quality of the extracted DNA-free RNA(s) using a spectrophotometer and agarose gel electrophoresis, respectively.
11. Reverse transcribe 1.5 μg of RNA sample(s) in the presence of the oligo$(dT)_{17}$ primer using a standard reverse transcription enzyme or commercial kits following the manufacturer's instructions. The synthesized cDNA can be stored at −20 °C until use.
12. Dilute a fraction of the synthesized single-strand cDNA(s) five times, and use this as a template for PCR amplifications with a proofreading polymerase following the manufacturer's instructions. Typically a PCR reaction is set up in a final volume of 25 μL containing 5 μL of 5× polymerase buffer, 500 nM of each gene-specific primer, 200 μM of dNTPs (each), 0.5 U of proofreading polymerase, and 10–50 ng of cDNA.
13. PCR amplify a 200–400 bp fragment of the gene of interest (GOI) with sequence-specific primers that introduce the desired restriction site(s) at the ends of the fragment. The fragment can be a partial part of either the coding sequence (CDS) or the untranslated regions (UTRs) of the transcribed GOI. Select the fragment carefully to avoid triggering any off-target silencing (*see* **Note 3**). Amplify also the *OeChlH* and/or the *OePDS* fragments using the respective primers OeChlH-F/OeChlH-R and OePDS-F/OePDS-R. Set up the PCR machine to perform up to 35 cycles for the amplification of the fragments to avoid introducing point mutations.
14. Digest and ligate the fragments in the corresponding restriction site(s) of the multiple cloning site (MCS) of pTRV2 in the antisense orientation relative to the TRV coat protein using common molecular cloning protocols, and verify the constructs by sequencing. The restriction sites available in the multiple cloning site region of pTRV2 are *Eco*RI, *Xba*I, *Stu*I, *Nco*I, *Bam*HI, *Kpn*I, *Sac*I, *Mlu*I, *Xho*I, *Srf*I, and *Sma*I.

3.2 Handling of Agrobacterium Cells

3.2.1 Preparation of Electro-competent Agrobacterium tumefaciens

1. Streak the *Agrobacterium tumefaciens* strain C58C1 Rif^R (GV3101) containing the T-DNA-deficient Ti plasmid pMP90 Gen^R on LB plate supplemented with 50 mg/L rifampicin and 50 mg/L gentamicin, and grow for 36–48 h at 28 °C.
2. Inoculate a single colony in 5 mL liquid LB medium supplemented with 50 mg/L rifampicin and 50 mg/L gentamicin, and grow at 28 °C under continuous shaking (200–250 rpm) until the stationary phase (at least 16 h).
3. Use 1 mL of the culture to inoculate a 1 L flask containing 100 mL of LB medium supplemented with 50 mg/L rifampicin and 50 mg/L gentamicin, and grow at 28 °C under continuous shaking (200–250 rpm) until the $OD_{600} = 0.5$ (typically 5–8 h).
4. Place the flask containing the culture on ice for 15 min, transfer the culture in ice-cold centrifuge bottles, and centrifuge at 1500 × *g* for 5 min at 4 °C.
5. Remove the supernatant carefully, and gently resuspend the pellet in 100 mL of ice-cold 10% glycerol solution. Centrifuge at 1500 × *g* for 5 min at 4 °C.
6. Remove the supernatant carefully, and gently resuspend the pellet in 50 mL of ice-cold 10% glycerol solution. Centrifuge at 1500 × *g* for 5 min at 4 °C.
7. Remove the supernatant carefully, and gently resuspend the pellet in 2 mL of ice-cold 10% glycerol solution. Divide the cell suspension in aliquots of 40 μL in ice-cold Eppendorf tubes, freeze immediately the cells in liquid nitrogen, and store at −80 °C.

3.2.2 Transformation of Agrobacterium tumefaciens Through Electroporation

1. Defreeze the appropriate number of *Agrobacterium* electro-competent cells to be transformed by letting the Eppendorfs on ice for 5–10 min (i.e., do not warm the cells above 4 °C).
2. Add 10–50 ng of plasmid DNA in 40 μL of cells, incubate on ice for 1 min, and transfer the mix in an ice-cold electroporation cuvette.
3. Adjust the settings of the electroporation apparatus to deliver a pulse of 1.8 kV, 400 Ω resistance, and 25 μF capacitance for electroporation cuvettes of 0.1 cm gap size of electrodes.
4. Dry well the outer part of the cuvette with a paper, place it in the cuvette holder of the electroporation apparatus, and deliver the electric pulse. A time constant of approximately 5 ms with an electric field strength of 18 kV/cm should register on the apparatus (in case of arcing, *see* **Note 4**).
5. Immediately add 1 mL of LB (without antibiotics) to the cuvette, transfer the cell suspension to an Eppendorf, and incubate for 3 h at 28 °C under slow agitation.

6. Centrifuge the cell suspension at 4000 × *g* for 5 min; resuspend gently the pellet in 200 µL of LB; streak the cell suspension on LB plates supplemented with 50 mg/L rifampicin, 50 mg/L gentamicin and 50 mg/L kanamycin; and incubate at 28 °C. Transformed colonies are visible after 2–3 days.
7. Inoculate at least one colony per transformation in 5 mL liquid LB supplemented with 50 mg/L rifampicin, 50 mg/L gentamicin, and 50 mg/L kanamycin. Grow at 28 °C under continuous shaking (200–250 rpm) for 36–48 h, and prepare glycerol stocks in sterilized Eppendorfs by mixing 650 µL of each culture with 350 µL of glycerol (100%) for archiving the strains at −80 °C.

3.3 Agroinoculation of Olive Plantlets

3.3.1 Preparation of Olive Plantlets

1. Harvest ripe fruits from *Olea europaea* L. cv. 'Koroneiki' naturally growing trees (*see* **Note 5**).
2. Remove the mesocarps, and subject the woody endocarps in 10% NaOH for 10 min.
3. Wash thoroughly the woody endocarps with water to remove any fleshy remnants.
4. Dry naturally the woody endocarps (Fig. 1a), and store them in cool and ventilated area until use. Stones can be stored for several months without loss of germination rate.
5. Carefully crack the woody endocarps with a bench vice (Fig. 1b) or a pipe cutter to remove seeds (Fig. 1c). Apply pressure gently to avoid damaging seeds.
6. Surface-sterilize the seeds with 20% NaClO containing 0.01% Triton-X for 5 min, and wash three to four times with sterile ddH_2O (Fig. 1d, e).
7. After 7 days of stratification at 4 °C in sterile ddH_2O, transfer the seeds in soil (Fig. 1f), and grow at 22 °C in a growth chamber with a 16/8 h light/dark cycle and 100 µmol/m^2/s light intensity. Typically, olive plantlets develop the first pair of true leaves after 1 month.

3.3.2 Preparation of *Agrobacterium* Strains Harboring the VIGS Vectors for Agroinoculation

1. Streak each of the *Agrobacterium* strains in LB plates supplemented with 50 mg/L rifampicin, 50 mg/L gentamicin, and 50 mg/L kanamycin, and grow for 2 days at 28 °C. The strains to be used are transformed with the following plasmids:
   pTRV1.
   pTRV2-EV.
   pTRV2-OePDS or *pTRV2-OeChlH*.
   pTRV2-GOI.
2. Inoculate single colonies (*see* **Note 6**) from each strain in 5 mL of LB supplemented with 50 mg/L rifampicin, 50 mg/L

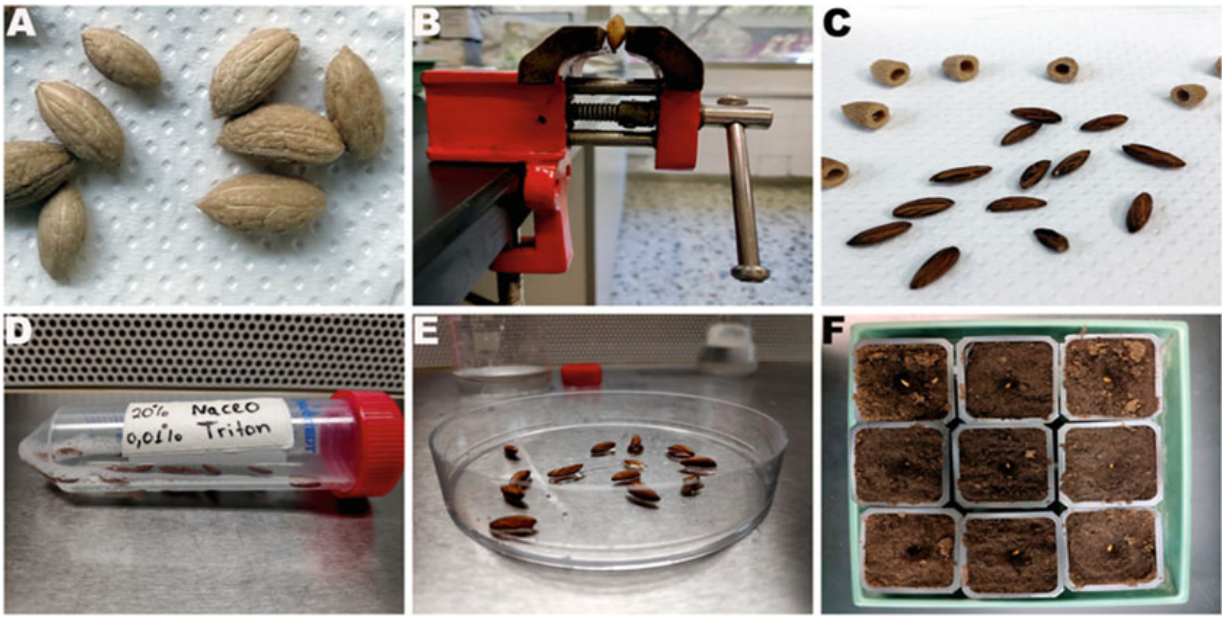

Fig. 1 Preparation of olive plantlets. Dried olive stones (**a**) were carefully cracked with a bench vice (**b**) in order to remove seeds (**c**). After surface sterilization (**d**, **e**), the seeds were potted in soil (**f**) for germination

gentamicin, and 50 mg/L kanamycin, and grow at 28 °C for ~36–48 h under continuous shaking (200–250 rpm).

3. Transfer the *Agrobacterium* cultures in Falcon tubes, and centrifuge for 10 min at 1000 × *g* in room temperature.
4. Discard the supernatant and carefully wash the cells with 2.5 mL dilution buffer (1/2 of the initial volume of the culture) without disrupting the pellet in order to remove any remaining LB medium.
5. Gently resuspend the pellet in 1 mL of dilution buffer (1/5 of the initial culture), and measure the OD_{600} of a diluted fraction for each cell suspension.
6. Mix each of the cell suspensions harboring a variant of pTRV2 in a 1:1 ratio with the cell suspension harboring the pTRV1 plasmid so that each culture has a final OD_{600} of 3 in dilution buffer supplemented with acetosyringone to a final concentration of 150 μM.
7. Incubate the cell suspensions at 28 °C for 3 h under slow agitation.

3.3.3 Agroinoculation

1. Use sterile 1 mL syringes (Fig. 2a) to inoculate the *Agrobacterium* cell suspensions prepared. Use a different syringe for each combination of strains to be used.
2. Olive plantlets developing the first true leaf pair should be used for agroinoculation (Fig. 2b).

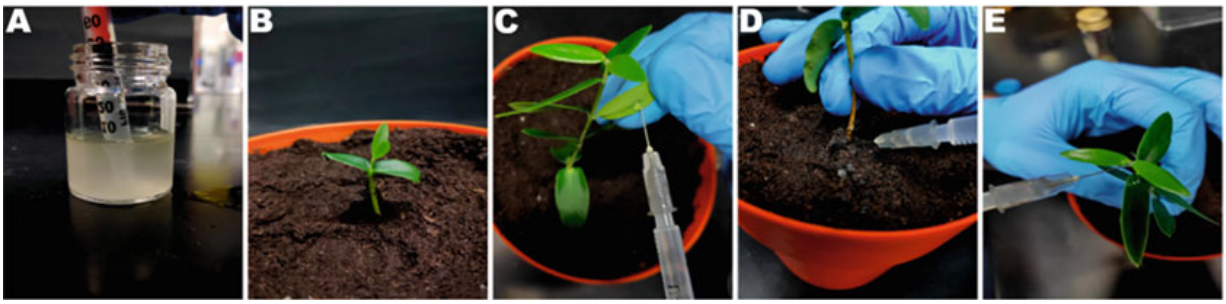

Fig. 2 Agroinoculation of TRV constructs in olive plantlets. *Agrobacterium* cell suspensions (**a**) were inoculated on olive plantlets that have developed the first true leaf pair (**b**) by gently traumatizing the abaxial side of a leaf (**c**). Agrobacteria were also inoculated at the crown region (**d**) and drenched onto the shoot apical meristem of the plant (**e**). Panels (**c**) and (**d**) show older plantlets on the fifth round of booster inoculations

3. Attach the needle and inoculate the abaxial side of a leaf by gently traumatizing the leaf (Fig. 2c) or by pricking. Avoid harming the veins or puncturing across the leaf.
4. Agroinoculate also the crown region (Fig. 2d), and drench the remaining cell suspension (i.e., without traumatizing the plantlets) onto the shoot apical meristem of the plant (Fig. 2e).
5. In order to boost the TRV infection, repeat the agroinoculation process on the same plants every 2 weeks until the desired phenotype is observed.

3.4 Evaluation of GOI Silencing by Real-Time PCR

1. Typically, after ~3–4 months the plants will exhibit the expected phenotype (Fig. 3). Agroinoculating plants with the silencing markers (either *pTRV2-OePDS* or *pTRV2-OeChlH*) in parallel with *pTRV2-GOI* helps to roughly estimate when and in which leaf nodes the GOI is expected to be silenced (*see* **Note 7**).
2. Collect leaves from the two pairs of leaves that emerged above the agroinoculated region, freeze immediately in liquid nitrogen, and store at −80 °C for RNA extraction.
3. Grind each leaf into powder using a mortar and pestle in the presence of liquid nitrogen, and extract RNA using either a phenol/chloroform protocol (Subheading 3.1) or a commercial kit following the manufacturer's instructions. Proceed with RNase-free DNase I treatment to remove the DNA and determine the quantity and the quality of the extracted DNA-free RNAs using a spectrophotometer and agarose gel electrophoresis, respectively.
4. Reverse transcribe equal quantities of each RNA sample in the presence of the oligo$(dT)_{17}$ primer using a standard reverse transcription kit following the manufacturer's instructions.
5. Set up the real-time PCR reactions according to the manufacturer's instructions. Typically, each reaction is set up in a final

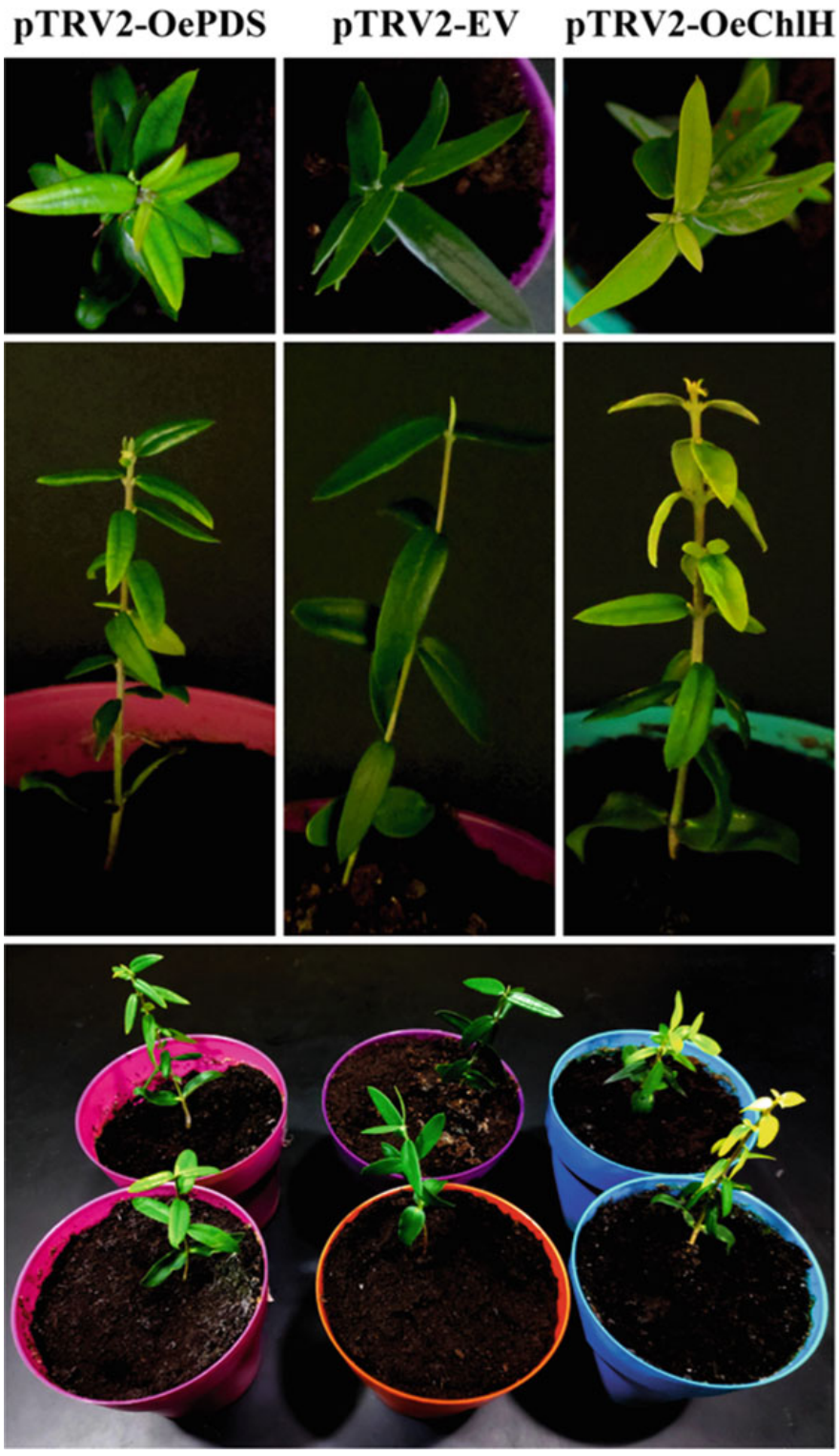

Fig. 3 Phenotypes of representative olive plants agroinoculated with a combination of pTRV1 and either pTRV2-OePDS or pTRV2-EV or pTRV2-OeChlH constructs

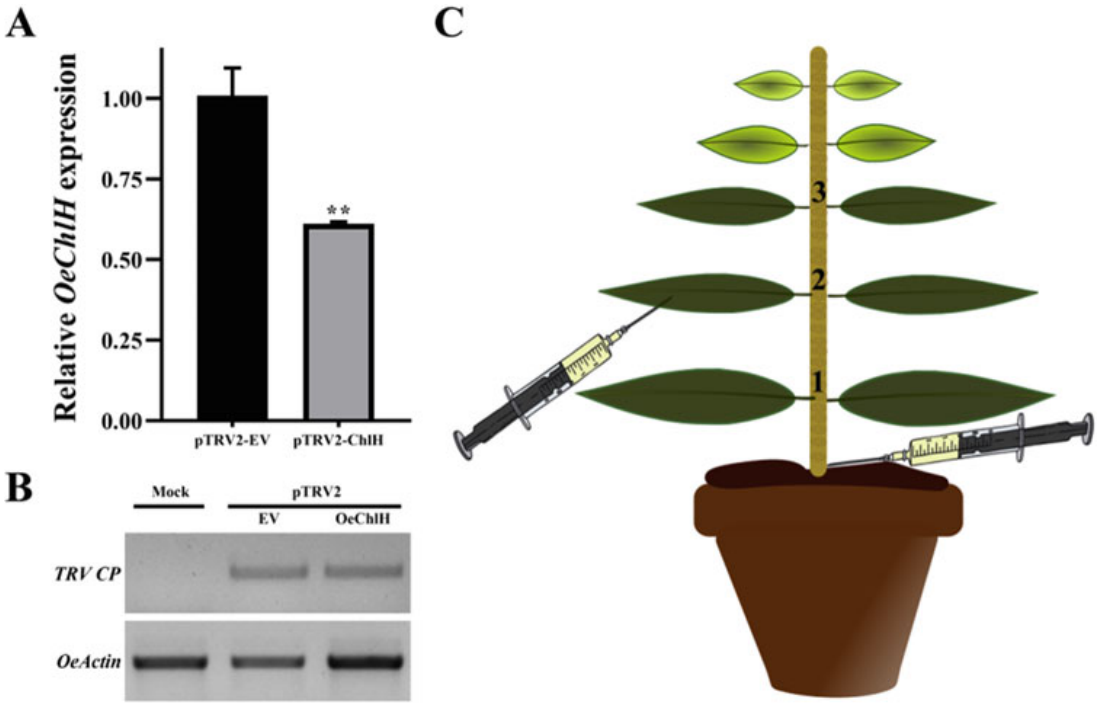

Fig. 4 Evaluation of GOI silencing. (**a**) Real-time PCR analysis of OeChlH expression level between plants agroinoculated with pTRV2-EV or pTRV2-OeChlH constructs. Three technical replicates of three biological replicates per treatment were analyzed. Mean ± SE and asterisks denote statistical significance ($P \leq 0.01$, Student's *t*-test). (**b**) RT-PCR of the TRV coat protein (CP) in the newly emerged (e.g., non-treated) leaves per treatment, as indicated. (**c**) Graphical representation of agroinoculation of an olive plantlet. Agrobacteria harboring either the pTRV1 or pTRV2-ChlH plasmid were co-inoculated, by using a syringe, onto the abaxial side of the leaf and on the crown region. Agroinoculation starts when olive plantlets have developed the first true leaf pair at one leaf at a time. The procedure is repeated after 2 weeks onto the other leaf of each pair and on the crown region. Typically silencing is observed after 3 months when all six leaves have been inoculated. Dark-green leaves represent inoculated non-silenced leaves, and light-green leaves represent non-agroinoculated (non-treated) gene-silenced leaves. Numbers 1, 2, and 3 represent the pairs of leaves used to perform initial and booster inoculations

volume of 20 μL containing 10 μL of 2× SYBR Select Master Mix, 300 nM for each gene-specific primer, and 5 ng of cDNA. The transcript of *OeActin* [13] can be used as an endogenous control for all the samples using the respective primers OeActin-F and OeActin-R (*see* **Note 2**).

6. The cycling conditions typically used in the real-time PCR machine are initial denaturation at 95 °C for 7 min followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s.
7. Perform the quantitative gene expression analysis (Fig. 4a) in an appropriate real-time PCR cycler, and analyze the data by the ΔΔCt method [49] (Fig. 4a).
8. If needed, perform semiquantitative RT-PCR to screen for the presence of the TRV coat protein (Fig. 4b) (*see* **Note 8**) [32].

9. The same plants can be used for further boosting the TRV infection by agroinoculating again with the corresponding strains until the desired silencing is observed (Fig. 4c). Use booster inoculations (i.e., starting from the pair of leaves of the first node, then the second and then the third) every 2 weeks (*see* **Note 9**).

4 Notes

1. Both sets of primers do not introduce any restriction sites; thus the PCR products need to be first blunt-end ligated in a *Sma*I digested pUC19 plasmid, and then the fragments can be directly subcloned in pTRV2-MCS (EV) utilizing the *Kpn*I and *Xba*I restriction sites of pUC19. The cloning strategy for any GOI should be adjusted accordingly.
2. The primers used for amplification of the fragment of the GOI to be silenced (i.e., for the pTRV2 constructs) should not be used for qPCR analysis. At least one primer should hybridize upstream or downstream the region used to trigger silencing of the GOI.
3. Several bioinformatic approaches have been developed in order to assist in the prediction of specific regions of a GOI that is expected to produce efficient and specific siRNAs [50]. An easy-to-use software that is very helpful when working with non-model plants is si-Fi (http://labtools.ipk-gatersleben.de/) since researchers can create local databases with in-house and/or publicly available datasets of unigenes.
4. Arcing is usually caused due to the presence of ions resulting in increased conductivity of the solution. The transformation efficiency is dramatically reduced due to uneven transfer of the electrical charge through the cuvette. Use a commercial kit to purify the plasmids that will be electroporated, and resuspend plasmids in salt-free water. If needed, increase the washing steps during the preparation of the electro-competent cells, and prepare the buffers in ddH_20 of low electrical conductance. Another reason for arcing could emerge by the presence of moisture in the outer (side) of the electroporation cuvette. Ensure that the cuvette is well dried before the delivery of the pulse.
5. In order to ensure that the seeds are mature and able to germinate, collect a number of fruits at their full ripening stage when the pulp and the peel have a similar purple-black color.
6. The number of colonies and/or the volume of LB to be inoculated is adjustable and depends on the number of plants

to be agroinoculated and the desired optical density of the cultures to be mixed in the final solution. Note that the strain harboring the pTRV1 plasmid will be mixed in 1:1 ratio with each one of the strains harboring the pTRV2 constructs, so ensure that sufficient number of cultures are initiated.

7. In our experience, silencing of *OeChlH* results in a more intense and easily to identify phenotype than silencing of *OePDS*.
8. A convenient way to narrow down the number of the samples expected to exhibit the higher degree of silencing of the GOI is to screen the plants for the presence of transcripts of the TRV coat protein. Detection of the TRV coat protein can be performed by semiquantitative RT-PCR using the TRV2cp-F 5′-CTGGGTTACTAGCGGCACTGAATA-3′ and TRV2cp-R 5′-TCCACCAAACTTAATCCCGAATAC-3′ set of primers (GenBank Acc. no.: AF406991.1).
9. When the experiment is finished, ensure that the plants are disposed only after autoclaving.

Acknowledgments

P.H. acknowledges funding from General Secretariat of Research and Technology, Greece, National Emblematic Action "Olive Routes." M.T. acknowledges funding from Greece and the European Union (European Social Fund, ESF) through the Operational Programme "Human Resources Development, Education and Lifelong Learning" in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" (MIS-5000432), implemented by the State Scholarships Foundation (IKY). We also acknowledge the editors of this volume for the helpful suggestions.

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