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Chapter 14

VIGS: A Tool to Study Fruit Development in *Solanum lycopersicum*

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Abstract

A visually traceable system for fast analysis of gene functions based on Fruit-VIGS methodology is described. In our system, the anthocyanin accumulation from purple transgenic tomato lines provides the appropriate background for fruit-specific gene silencing. The tomato Del/Ros1 background ectopically express *Delila (Del)* and *Rosea1 (Ros1)* transgenes under the control of fruit ripening E8 promoter, activating specifically anthocyanin biosynthesis during tomato fruit ripening. The Virus-Induced Gene Silencing (VIGS) of *Delila* and *Rosea1* produces a color change in the silenced area easily identifiable. *Del/Ros1* VIGS is achieved by agroinjection of an infective clone of *Tobacco Rattle Virus* (pTRV1 and pTRV2 binary plasmids) directly into the tomato fruit. The infective clone contains a small fragment of *Del* and *Ros1* coding regions (named DR module). The co-silencing of reporter *Del/Ros1* genes and a gene of interest (GOI) in the same region enables us to identify the precise region where silencing is occurring. The function of the GOI is established by comparing silenced sectors of fruits where both GOI and reporter DR genes have been silenced with fruits in which only the reporter DR genes have been silenced. The Gateway vector pTRV2_DR_GW was developed to facilitate the cloning of different GOIs together with DR genes. Our tool is particularly useful to study genes involved in metabolic processes during fruit ripening, which by themselves would not produce a visual phenotype.

Key words: Virus-Induced Gene Silencing, Tomato fruit, Agroinjection, Tobacco Rattle Virus, Gateway, pTRV2_Del/Ros1_GW, Co-silencing, Anthocyanin, Gene function.

1. Introduction

Virus-Induced Gene Silencing (VIGS) is a technique based on RNA-mediated antiviral plant defense that has been used to analyze gene function in plants (1–4). A fragment of a plant gene of interest (GOI) is inserted into the recombinant viral genome used for infection. The specific degradation of endogenous GOI's mRNA is the result of plant antiviral defense and produces the silencing of the endogenous GOI (5). VIGS presents multiple advantages when compared to other loss-of-function techniques

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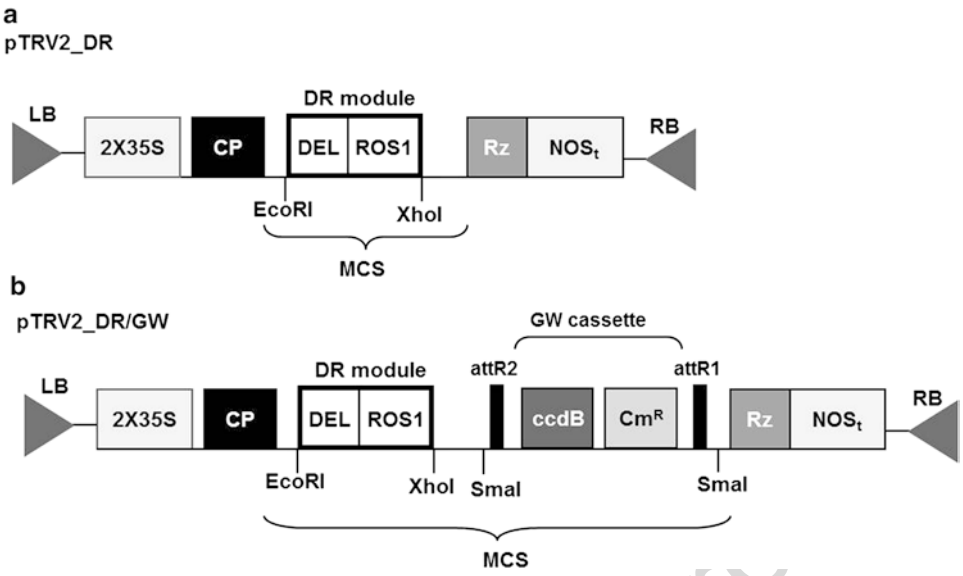
(3, 5–7) and therefore qualifies as an advantageous technique for reverse genetic studies.

Genomics project are generating an overwhelming amount of information, thanks to a more powerful RNA sequencing and array technologies. This is also happening with tomato, which is a particularly important crop, not only because its fruit contributes importantly to the human diet, but also because it is becoming a model crop species. To improve both the nutritional value and organoleptic features of this crop, first it is necessary to understand the genetic basis of the metabolic pathways that operate during fruit ripening processes (6). The easy way in which we can obtain genomic information contrasts with our current lack of understanding about the function of many genes in the genome. In many fruit crops, one way to investigate gene function is altering its expression by stable transformation. Different techniques can be used for that objective, but are often cumbersome and lengthy taking several months or years. A rapid and high-throughput method is required, which allows both to analyze the enormous amount of data from genomic projects and to link gene functions to phenotypes (6). VIGS technique can be successfully applied in tomato fruit for that purpose (3, 6, 8–10). The use of *Agrobacterium tumefaciens* as a vehicle for transfection is the common way to introduce effectively viral-modified vectors for VIGS approaches. We developed a new VIGS methodology in fruits named "Agroinjection" (Fig. 2) which introduces *Agrobacterium* suspension into tomato fruit tissues by stylar apex (8). This method speeds up the experimental procedures and confines the VIGS signal into the fruit, allowing to increase the throughput of VIGS by "one organ-one biological replicate" approaches (8).

Different viruses have been used as suitable VIGS vectors. *Tobacco Rattle Virus* (TRV) was described as a VIGS vector one decade ago (1), and since then it has been one of the most widely used (4). TRV-based vectors for VIGS approaches consist in pTRV1 and pTRV2 binary plasmids. GOIs are cloned into pTRV2 plasmid by digestion/ligation cloning or into pTRV2_GW vector by Gateway recombination (11). In tomato fruits, TRV-based vectors normally produce partial VIGS penetration and patchy tissue distribution as a result from partial and highly variable silencing from fruit to fruit (6). This causes serious limitation for its use in the investigation of gene loss-of-function that yields nonvisual phenotypes (6). An internal reference that monitors the levels of silencing was developed to overcome these limitations and increase the sensitivity of downstream analysis, allowing the dissection of silenced from non-silenced tissues (6). In our system, the anthocyanin accumulation in purple transgenic tomato lines provides the appropriate background for fruit-specific gene silencing. These lines were obtained in Dr. C. Martin's group (12) by ectopically expression of Delila (Del) and Rosea1 (Ros1) genes (two transcription factors



[AU1] Fig. 1. ■ ■ ■.

that activate the anthocyanin branch of flavonoid biosynthesis pathway in *Antirrhinum majus* flowers (13)) under the control of tomato E8 promoter. This resulted in the activation of anthocyanin biosynthesis specifically during tomato fruit ripening (6, 12). The silencing of both *Del* and *Ros1* genes, using small fragments of their coding regions (named reporter DR genes) by pTRV2_DR expression vector (developed in our laboratory, Fig.1a; (6, 9)), results in the lack of anthocyanin production. As reporter DR genes silencing involves the blockage of a pathway not normally active in tomato fruit, the lack of anthocyanin accumulation produces red silenced sectors that present similar characteristics in metabolism and development as "wild type" tomato fruit (6). To facilitate the dissection of silenced tissues and to increase the yield of silenced areas for downstream analysis, we transferred the *Del* and *Ros1* transgenes from cherry-type MicroTom (12) to a large globe-type Money-Maker tomato background (Fig. 3a) by standard crossing and selection (see Subheading 2.1, item 1; (6)). The integration of DR-reporter module and GOI in the same viral genome (pTRV2_DR_GOI VIGS vector) is required for an efficient co-silencing of both the reporter module and target gene in the same tissue area (co-silencing in *tandem*; example in Fig. 3b, c) (6). To facilitate high-throughput tandem cloning of subsequent GOIs, we modified pTRV2_DR vector into pTRV2_DR_GW vector (Fig. 1b) by the introduction of a Gateway recombination cassette (6). This system has proved to be particularly useful for the analysis of genes of unknown function involved in different stages of fruit ripening, especially of genes associated with different branches of metabolism in fruit (6, 10).

In the present chapter we describe the methodology of Fruit-VIGS based on anthocyanin accumulation in tomato fruit, as a tool to study the gene function during ripening stages related with quantitative characters, as secondary metabolites in tomato fruit. Details on plant cultivation and maintenance are explained. Some recommendations on silenced areas harvesting and their analysis are provided, too. At the end of the chapter, we present some future perspectives to use this tool with different reporter genes and different promoters aimed to study genes involved in different developmental stages of tomato fruit.

2. Materials

2.1. Plant Material

1. Globe-type purple tomatoes (Solanum lycopersicum) were obtained by crossing *Del/Ros1* MicroTom N line (T2 homozygous generation from Micro Tom plants transformed with *Delila* and *Rosea1* cDNAs under the control of the E8 ripening-specific promoter; (12)) with wild-type MoneyMaker plants (6). Segregating sibling lines were selfed and selected through to the F7 generation. Selection was based on globe-type fruit, smooth leaves, indeterminate growth, and best fruit VIGS response (6).
2. Plants were grown in a greenhouse supplemented with artificial light from mercury vapor lamps (OSRAM) of 400ω (PHILILPS HDK) 400HPI®N (96 μmol m^{-2} s^{-2}(14)).
3. Plants were irrigated four times per day with a HOAGLAND N°1 nutritive solution supplemented with oligo elements by automatic dripping irrigation system (14).

2.2. Cloning Procedures and Vectors Construction

Gateway technology (www.invitrogen.com) has been used to generate the different VIGS vectors following the manufacturer's instructions.

1. For amplification: Advantage® 2 DNA Polymerase Mix (Clontech, Mountain View, CA, USA; see Note 1), specific forward and reverse primers (10 mM each one), dNTPs mixture (10 mM each dNTP), and sterilized water.
2. For DNA cleanup: QUIAquick® PCR Purification kit (Qiagen, Valencia, CA, USA) and sterilized water; or appropriate percentage of agarose in TAE 1× (40 mM Tris–Acetate and 1 mM Na_2EDTA) and QUIAEXII® Gel Extraction kit (Qiagen, Valencia, CA, USA).
3. NanoDrop spectrophotometer (NanoDrop ND-100 Spectrophotometer; Thermo Fisher Scientific Inc., USA). Follow the manufacturer's instructions in each case.

4. For cloning in pDONOR to generate a pENTR clone: pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA) and freshly purified PCR product. Follow the manufacturer's instructions.
5. For final generation of a VIGS Vector (Expression clones): TRV-based silencing vectors pTRV1 and pTRV2 were provided by Prof. Dinesh Kumar (11); pTRV2_DR and pTRV2_DR_GW VIGS vectors were generated in our group (Fig. 1, (6)).
6. Gateway® LR Clonase™ II Enzyme Mix (Invitrogen, Carlsbad, CA, USA).
7. For *E. coli* transformation, use One Shot®TOP10 or One Shot®Mach1™T1R chemically competent *E. coli* kit (Invitrogen, Carlsbad, CA, USA).
8. Sterile LB liquid medium and solid LB agar plates containing 50 µg/mL Spectinomycin in case of Entry clones (TA-Cloning) or 50 µg/mL Kanamycin for Expression clones (LR reaction). 37 °C growing chamber.
9. For *E. coli* DNA plasmid extraction use Plasmid Mini Kit I E.Z.N.A. (Omega Biotek, Doraville, GA, USA).
10. For *E. coli* colony glycerol stocks: in a sterile Eppendorf tube mix 700 µL of fresh liquid *E. coli* culture and 300 µL of 50 % sterile glycerol. Freeze it quickly in liquid N2 and store it at –80 °C.

2.3. Agrobacterium Transformation and Agroinjection Suspension Preparation

1. For *Agrobacterium* transformation: Eppendorf tubes containing 40 µL of electrocompetent *Agrobacterium* C58 cells stored at –80 °C (see Note 2).
2. Electroporator (Bio-Rad, gene-pulser 165-2077) + 1 mm electroporation cuvettes (Bio-Rad Laboratories, CA, USA).
3. 15 mL plastic tubes containing 250 µL of S.O.C. medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose).
4. Sterile LB liquid medium and solid LB agar plates containing 50 µg/mL kanamycin and 50 µg/mL rifampicin antibiotics
5. Growing chamber set at 28 °C.
6. For *Agrobacterium* DNA plasmid extraction use QUIAprep® Miniprep Kit (Qiagen, Valencia, CA, USA).
7. For *Agrobacterium* glycerol stocks: in a sterile Eppendorf tube mix 700 µL of fresh liquid *Agrobacterium* culture and 300 µL of 50 % sterile glycerol. Freeze it quickly in liquid N2 and store it at –80 °C.
8. For *Agrobacterium* C58 cultures and subcultures for agroinjection: 15 mL plastic tubes.
9. MES infiltration buffer: 10 mM MES (Sigma-Aldrich, MO, USA; see Note 3), 10 mM MgCl (see Note 3), 200 µM

acetosyringone (Sigma-Aldrich, MO, USA; see Notes 3 and 4).
Rotating and swaying mixer (CAT RM-5).
10. Spectrophotometer (UV/VIS Spectrophotometer SP8001, DINKO) set at a wavelength of 600 nm and transparent plastic cuvettes.

2.4. Fruit Agroinjection

1. Tomato *Del/Ros1* fruits at Mature Green (MG) stage, 30–35 days post-anthesis (dpa).
2. Sterile 1 mL Plastipak needle syringes (25 GA 5/8 IN, needle: 0.5 × 16 mm, BD Plastipak™).

2.5. Dissection and Collection of Silenced Sectors

1. For silenced sectors dissection: Glass board to dissect the fruit with sharp knife (see Note 5).
2. For silenced sectors collection: Plastic screwed cap tubes (25 mL) to store the samples at –80 °C. Liquid Nitrogen (N2) in a suitable container.
3. For silenced sectors crushing: Thermal-cover mortar, metallic little spoon, thermal gloves, and protective glasses.

2.6. Evaluation of GOI Silencing

1. A suitable RNA extraction method for tomato fruits (15).
2. SuperScript TMFirst-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Follow the manufacturer's instructions.
3. Power SYBR® Green PCR Master Mix and RT-PCR (Applied Biosystems, Madrid, CA, USA) and 7500 Fast Real-Time PCR system (Applied Biosystems, Madrid, CA, USA).

3. Methods

3.1. Plant Cultivation and Maintenance

1. Estimation of the plant numbers required for the experiment. For that, determine the following: (a) how many genes will be silenced; (b) how many individual silencing constructions will be generated; and (c) how much material will be necessary for downstream analysis (see Note 6). For each construction we use three plants with 20 fruits each, ten of them for control DR-silencing and the other ten for GOI-DR silencing (see Note 7).
2. Sowing and seedlings. Sow more seeds than plants will be required and keep some extra seedlings to allow selection of best-performing plants (see Note 8).
3. Pruning and labeling. Remove secondary buds to give structure to the plant (a main axis with first lateral branches only) and to increase reproductive vigor (see Note 9). During flowering

period, flower unloading and labeling are required. Keep six flowers per truss and label them with the anthesis date. When small fruits appear, keep six or seven floral trusses and remove the rest. Later, the excess of fruit per truss should be removed. But before that, make sure that at least four of them have reached Inmature Green (IMG) stage (see Note 10). When the fruits get to Mature Green (MG) stage, select which ones will be used for DR control silencing and which others will be used for GOI-DR co-silencing. Then label them appropriately (see Note 11) before agroinjection.

3.2. Cloning Procedures and Vector Construction

1. Design the primers for a specific GOI region. Optimal GOI regions are between 100 and 500 bp in length (see Note 12).
2. GOI sequence amplification by PCR reaction using Advantage® 2 DNA Polymerase Mix. PCR conditions will depend on fragment length and primers Tm.
3. Amplified DNA fragment purification. If the PCR yields a single product, purify the PCR reaction using QUIAquick®PCR Purification kit, and elute it in 50 µL of volume. If the PCR yields several products, put the complete PCR reaction on an electrophoresis gel with appropriate percentage of agarose. Separate the product of interest and excise it from the gel. Purify it using QUIAEXII® Gel Extraction Kit, and elute in 30 µL of volume. In both cases, quantify the DNA by NanoDrop spectrophotometry.
4. Ligation to obtain the pCR8_GOI entry clone. Prepare as many 1.5 mL Eppendorf tubes as amplified GOIs. Prepare the TA-Cloning reaction following the pCR®8/GW/TOPO® TA Cloning® Kit manufacturer's instructions. Incubate the ligation 1 h at room temperature.
5. Transformation of entry clone in *E. coli*. Take from −80 °C a tube of chemically competent *E. coli* cells per each Entry clone to be generated (One Shot®TOP10 or One Shot®Mach1TMT1R (see Subheading 2.2, item 5)). Thaw competent cells on ice. Add 2 µL of pCR8-GOI ligation reaction to each tube, incubate without shaking and perform transformation following manufacturer's instruction (see Note 13). Collect 50 µL from the bacterial culture and spread in a solid LB plate containing 50 µg/mL spectinomycin (see Note 14). Incubate the plates overnight at 37 °C.
6. Validation of entry clone. Pick 4–6 colonies into 3 mL of liquid LB media with 50 µL/mL spectinomycin using toothpicks in sterile conditions. Allow them to grow overnight at 37 °C. Isolate the plasmid DNA using the E.Z.N.A.® Plasmid Mini Kit I, and elute in 50 µL of volume. Validate the pCR8_GOI entry clone by restriction analysis (see Note 15) and by sequencing using M13 forward and reverse primers. Generate

a glycerol stock (see Subheading 2.2, item 7) with a positive colony previously validated.

7. Generation of final expression vector. For each LR reaction, mix in a 1.5 mL sterile Eppendorf: 50–150 ng Entry clone (pCR8-GOI) (1–7 µL), 150 ng/µL (1 µL) of pTRV2_DR_GW destination vector, and TE buffer pH 8.0 up to 8 µL. Follow the instructions from Gateway® LR ClonaseTM II Enzyme Mix manufacturer's instructions.
8. Transformation of final expression vector. Proceed as Subheading 3.2, step 5 but using 1 µL of LR reaction and 50 µg/mL kanamycin in selective LB plates.
9. Validation of final expression vector. After overnight incubation, pick 4–6 colonies and proceed as Subheading 3.2, step 6. Validate the expression vector by restriction analysis and sequencing (see Note 16). Finally, generate a glycerol stock (see Subheading 3.2, step 6).

3.3. Agrobacterium Transformation and Agroinjection Suspension Preparation

1. *Agrobacterium* transformation. Take a sterile Eppendorf containing 40 µL of *Agrobacterium* C58 electrocompetent cells per GOI and thaw them on ice. Add to each tube 1 µL of a positive *E. coli* plasmid miniprep from Subheading 3.2, step 9. Electroporate the samples at 1.5 V. Add 250 mL of SOC medium and incubate them in 15 mL plastic cap tube for 2 h shaking (150–200 rpm) at 28 °C. Then, collect them by spin at 13,000 rpm in a micro-centrifuge. Remove the supernatant, leaving approximately 100 µL. Resuspend the cells and spread them on selective LB plates containing 50 µg/mL of both kanamycin and rifampicin. Incubate for at least 48 h at 28 °C.
2. Validation of *Agrobacterium* clones. Pick 4 colonies from selective plates in sterile conditions (as Subheading 3.2, step 6) and allow them grow in 5 mL of liquid LB media containing kanamycin and rifampicin (50 µg/mL) at 28 °C for 48 h. Isolate the plasmid DNA by miniprep and validate by digestion with the suitable restriction enzyme (see Note 17). Generate a glycerol stock with a positive colony previously validated.
3. Culturing and subculturing of *Agrobacterium* clones. Grow pTRV1, pTRV2_DR, and each pTRV2_DR_GOI construct from frozen stocks individually in kanamycin and rifampicin (50 µg/mL) selective LB plates. Pick a colony of each LB plate and put them into a 50 mL plastic tube containing 5 mL of LB medium with kanamycin and rifampicin (50 µg/mL). Grow them shaking 48 h at 28 °C. Based on the number of labeled MG fruit (Subheading 3.1, step 3), make an estimation of *Agrobacterium* suspension volume required for each final expression vector. The volume of agroinjection suspension mix (pTRV1:each pTRV2) varies depending on the fruit size, but

1 mL should be enough to infiltrate one MG Del/Ros1 MM fruit. For small fruits, use 0.5 mL of agroinjection suspension. Prepare a fresh pre-culture of *Agrobacterium*. Take 100 µL of each pre-cultures and inoculate a 50 mL plastic tube containing 5 mL LB medium with kanamycin and rifampicin (50 µg/mL) (Fig. 2a). Grow by shaking overnight at 28 °C.

4. Agroinjection suspension preparation. Collect the *Agrobacterium* cells by centrifugation at 3,000 rpm for 15 min. Discard the supernatant by inversion. Prepare acetosyringone solution and add it to MES infiltration buffer. Protect it from light wrapping the bottle with aluminum foil. Resuspend the pellet with the cells in 15 mL MES infiltration buffer and vortex it (see Note 18) to produce the agroinjection suspension. Wrap the plastic tubes in aluminum foil, too. Incubate them at room temperature with gently agitation (20 rpm) in a rotating and swaying mixer for at least 2 h. Check the optical density (OD) at 600 nm wavelength of each suspension and dilute them adding more MES infiltration buffer to reach 0.05 OD (see Note 19). Prepare the agroinjection suspension by mixing 1:1 (volume–volume) the pTRV1 suspension with each pTRV2 suspensions, including pTRV2_DR control (Fig. 2a, b, see Note 20).
5. Fruit agroinjection. Use different sterile 1mL needled syringes for each agroinjection suspensions (containing pTRV1 and pTRV2_DR or PTRV1 and each pTRV2_DR/GOI vectors) and agroinject them into MG fruits (30–35 dpa) (2c, e). Agroinjection proceeds by inserting the needle about 3–4 mm into the fruit through the calyx region, between sepals and peduncle junction (carpopodium). This was found to be more efficient than injection through the stylar end as initially described (9). Inject the suspension carefully (see Note 21). The successful spread of agroinjection suspension into the fruit can be monitored by the color change observed in the fruit tissues from light to dark green. Agroinjection is finished when the fruit is fully infiltrated, and a few drops appear in the sepal hydatodes (Fig. 2f). Dry the drops on the fruit and keep the fruit surface clean. DR and GOI gene silencing can be observed 10 days after agroinjection, when the fruit reaches the breaker stage.

3.4. Dissection and Collection of Silenced Sectors

1. Harvesting. Fruits are harvested at different ripening stages depending on the particular interest of each study. Harvest DR- and DR/GOI-silenced fruits separately and keep their labels.
2. Dissection and collection. Rinse fruits with tap water and dry them. Separate silenced from non-silenced areas by cutting them with a sharp knife (Fig. 2d). For pericarp tissue studies, slice the fruit and discard seeds and gel. Sort out silenced from non-silenced areas (see Note 21), transfer them quickly to conveniently labeled screw cap tubes (25 mL) and hold in liquid N_2.

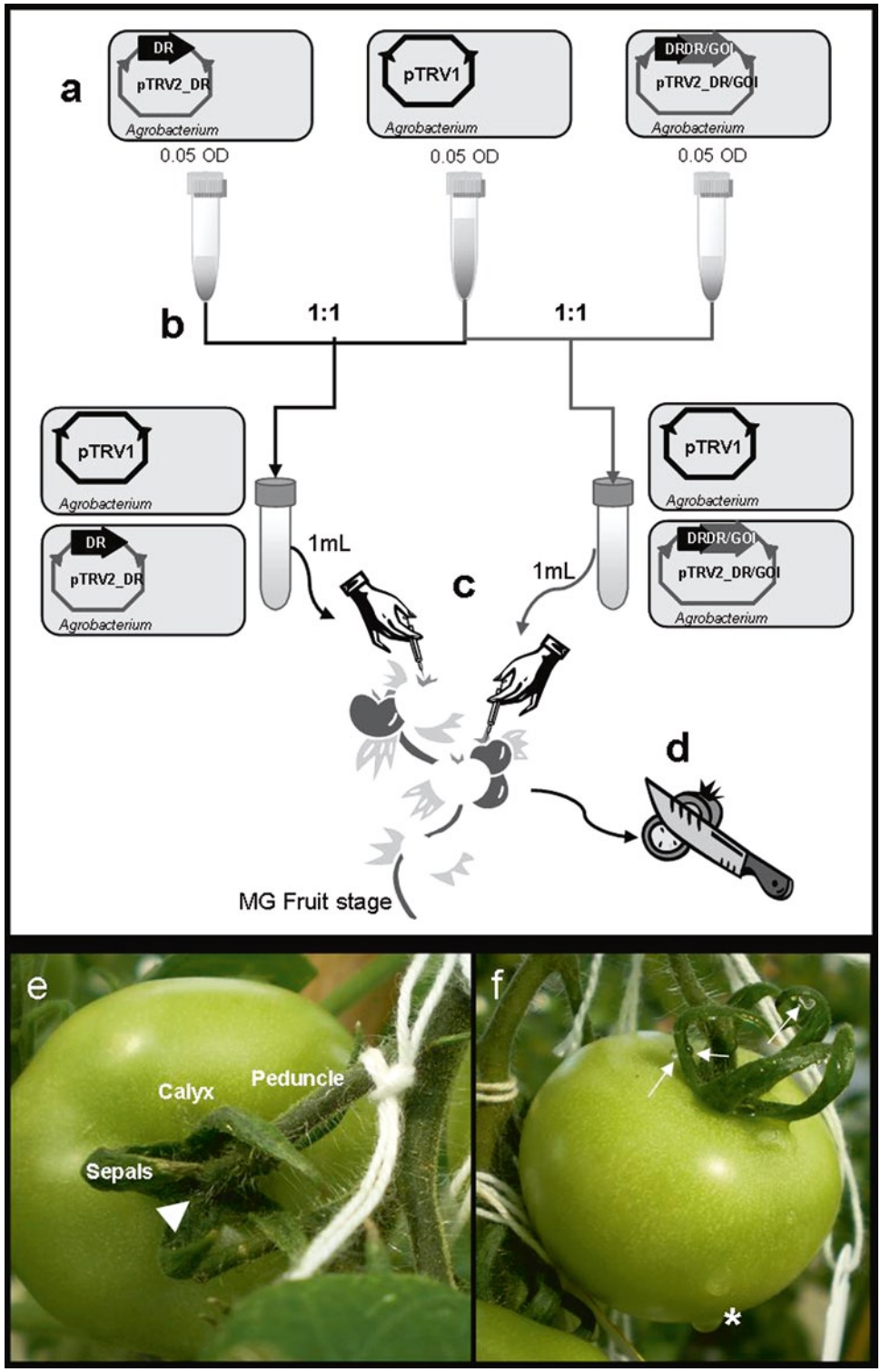


Fig. 2. ■ ■ ■.

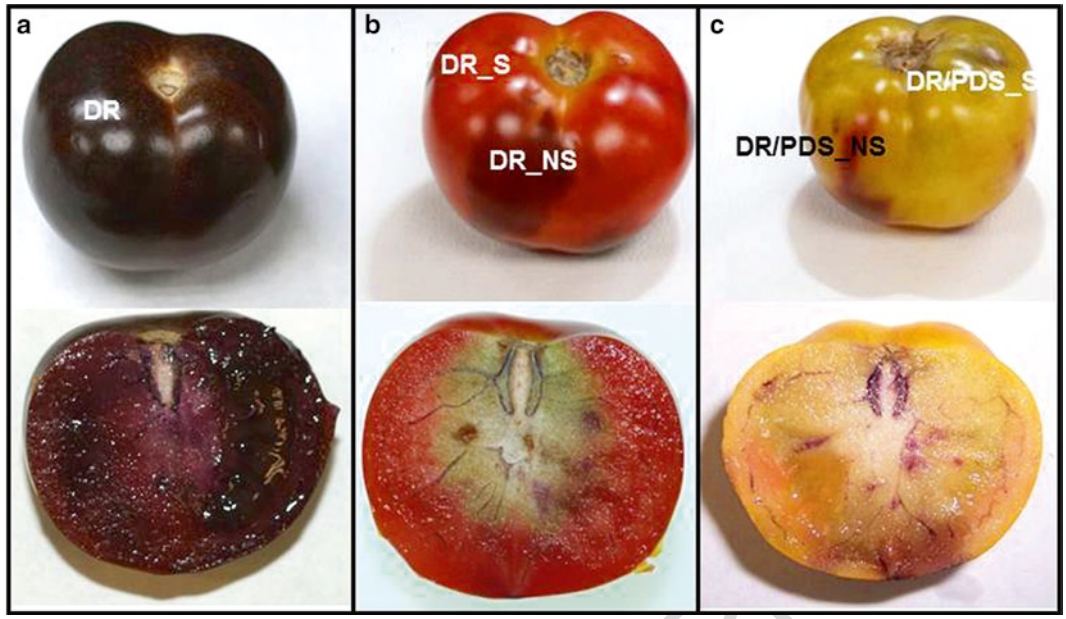


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Fig. 3. ■ ■ ■.

Store at −80 °C. Grind the samples up in a mortar with liquid N_2 to obtain a fine frozen powder. In Fig. 3 we show an example of DR- and DR/GOI-silenced fruits in comparison with DR-non-silenced fruit. We used the *Phytoene desaturase (PDS)* gene as GOI because it provides a visual phenotype by itself, which gives us the opportunity to evaluate the co-silencing of the DR module and the PDS gene (6).

3.5. Evaluation of GOI Silencing

1. Extract the RNA from the silenced region using a suitable RNA isolation protocol (15).
2. Synthesize cDNA from isolated RNA with SuperScriptTMFirst-Strand Synthesis System for RT-PCR following the manufacturer's instructions.
3. Perform a relative quantification of transcript abundance e.g., in 7,500 Fast Real-Time PCR system (Applied Biosystems) using Power SYBR® Green PCR Master Mix and an established RT-PCR protocol.

3.6. Future Perspectives

The strategy used in purple *Del/Ros1* transgenic plants as background that provides a monitoring system for VIGS can be adapted to other developmental stages of tomato fruit by engineering. Different approaches are possible to achieve this objective: (a) to change the stage-specific promoter. The pENFRUIT collection developed in our laboratory can be used as promoter source for specific developmental stages (16); (b) to change the reporter gene. Other visual reporters easily traceable can be used, e.g., fluorescent

DsRed protein. The *35S-DsRed* MM tomato transgenic line developed in our laboratory, works as a new and attractive monitoring VIGS strategy for early stage of fruit development (9); and (c) to combine both different specific-stage promoters with different reporter genes. In this way, complete developmental and ripening processes could be studied in tomato fruit.

4. Notes

1. We have used Advantage™ as a high fidelity polymerase which adds a single adenine overhang at 3 ends of each amplicon. It is necessary for TA cloning reaction.
2. In tomato fruit (*Solanum lycopersicum*) we observed that *Agrobacterium* C58 strain is more infective and efficient in transient silencing assays than LBA4404.
3. Stocks for MES infiltration buffer: (a) MES 100 mM (10×) pH 5,6. Dissolve in sterile water and adjust the pH with KOH 1M. Require sterilization by autoclaving or filtration; (b) Magnesium Chloride (MgCl2) 1M (100×). Dissolve in sterile water. Require sterilization by filtration; and (c) 200 mM acetosyringone solution. Dissolve 78.48 mg in 2 mL of dimethyl sulfoxide and filter-sterilize it. Split in 200 µL aliquots and store at −20 °C. IMPORTANT: It is better to prepare the 200 mM stock acetosyringone on the same day you plan to use it.
4. Acetosyringone is photosensitive and it needs to be under darkness conditions. Tubes are wrapped with aluminum foil.
5. A sharp knife is important to avoid crushing the tissue.
6. Depending on many variables, such as the final expression vector size or environmental conditions, the yield of silenced sectors per fruit will be different. For example, in our experience we observed that expression vectors with larger sizes produce less silenced areas per fruit than vectors with shorter sizes. In these cases, we advise to use more plants per experiment in order to increase the amount of silenced samples.
7. Based on our experience, good results were obtained with 20 fruits per plant. We try to keep five trusses per plant, containing four fruits each. In each truss, two fruits are used to control silencing and other two fruits are used for DR/GOI co-silencing. With three plants per construction, enough material is obtained for most downstream analysis (e.g., metabolomics).
8. Even though our *Del/Ros1* MM background is a F7 generation, sometimes one or two plants with MT trait appear. They should be removed.

9. If pruning can affect the expression of your trait, do not prune the plants. If this is not the case, prune them every week until the last fruit is collected.
10. Put special attention at plant pruning after first fruit reach Mature Green (MG) stage: Remove every floral truss and secondary bud.
11. Label the MG fruits indicating date, silencing vectors agroinjected and write a code that contains fruit, truss, and plant number. IMPORTANT: keep this code for downstream analysis because it represents a unique biological replicate.
12. Selection of a specific GOI region is particularly important when working with gene families. Overlapping primer designs can be used to clone several gene fragments in *tandem* inside the same vector.
13. We routinely use *E. coli* (One Shot®TOP10 or One Shot®Mach1 TMT1R chemically competent *E. coli* kit) following the transformation conditions from pCR®8/GW manual.
14. When the transformation efficiency has not been optimal, we spread the rest of culture (150 µL) in a different plate for recover some colonies.
15. We use Vector NTI 10.3.0 (Jul 31, 2006© Invitrogen, Carlsbad, CA, USA) program to choose the suitable restriction enzymes and predict the expected sizes after plasmid DNA digestion. Proper enzymatic reaction conditions can be found at the enzyme manufacturer website. The pCR®8/GW/TOPO® TA Cloning® Kit, recommend *EcoRI* restriction enzyme because it releases the cloned GOI from the plasmid. Be careful with additional *EcoRI* digestion products which can be obtained as a result of internal *EcoRI* sites in your fragment.
16. We use Vector NTI 10.3.0 program, too, to select the best restriction enzyme. We commonly use *EcoRV* (Takara Bio Europe, France; Takara Bio Inc., Shiga, Japan).
17. To increase the yield of *Agrobacterium* plasmid DNA minipreps, collect the cells from 5 mL liquid LB medium and follow QUIAprep® Miniprep Kit procedures. Elute with 20 µL to obtain a more concentrated plasmid DNA preparation.
18. It is IMPORTANT to resuspend completely the cells in the MES infiltration buffer. Vortex them for around 1–2 min.
19. Usually, we make a 0.5 OD intermediate dilution for each agroinjection suspension in order to equalize them before reaching the final 0.05 OD.
20. In some fruits you may find a higher initial resistance to agroinjection than normal. In those cases try to find a more suitable position by changing the depth of needle insertion in the fruit.

21. Sometimes you may find it difficult to distinguish between silenced and non-silenced areas. Small patchy silencing or a gradual silencing aggravates the dissection of the sectors. If there are enough samples, discard those fruits. If not, try to select areas in patchy silenced fruit with high similarity to well-silenced fruit.

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