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An Efficient Clearing Protocol for the Study of Seed Development in Tomato (*Solanum lycopersicum* L.)

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Abstract

Tomato (*Solanum lycopersicum* L.) is one of the major cash crops worldwide. The tomato seed is an important model for studying genetics and developmental biology during plant reproduction. Visualization of finer embryonic structure within a tomato seed is often hampered by seed coat mucilage, multi-cell-layered integument, and a thick-walled endosperm, which needs to be resolved by laborious embedding-sectioning. A simpler alternative is to employ tissue clearing techniques that turn the seed almost transparent using chemical agents. Although conventional clearing procedures allow deep insight into smaller seeds with a thinner seed coat, clearing tomato seeds continues to be technically challenging, especially in the late developmental stages.

Presented here is a rapid and labor-saving clearing protocol to observe tomato seed development from 3 to 23 days after flowering when embryonic morphology is nearly complete. This method combines chloral hydrate-based clearing solution widely used in *Arabidopsis* with other modifications, including the omission of Formalin-Aceto-Alcohol (FAA) fixation, the addition of sodium hypochlorite treatment of seeds, removal of the softened seed coat mucilage, and washing and vacuum treatment. This method can be applied for efficient clearing of tomato seeds at different developmental stages and is useful in full monitoring of the developmental process of mutant seeds with good spatial resolution. This clearing protocol may also be applied to deep imaging of other commercially important species in the Solanaceae.

Introduction

Tomato (*S. lycopersicum* L.) is one of the most important vegetable crops around the world, with an output of 186.8 million tons of fleshy fruits from 5.1 million hectares in 2020[1]. It belongs to the large Solanaceae family with about 2,716 species[2], including many commercially important crops such as eggplant, peppers, potato, and tobacco. The cultivated

tomato is a diploid species (2n = 2x = 24) with a genome size of approximately 900 Mb[3]. For a long time, great effort has been made toward tomato domestication and breeding by selecting desirable traits from wild *Solanum* spp. There are over 5,000 tomato accessions listed in the Tomato Genetics Resource Center and more than 80,000 germplasm of tomatoes are stored worldwide[4]. The tomato plant is perennial in the greenhouse and propagates by seeds. A mature tomato seed consists of three major compartments: a full-grown embryo, residual cellular-type endosperm, and a hard seed coat[5,6] (**Figure 1A**). After double fertilization, the development of cellular-type endosperm precedes the development of zygotes. At ~5-6 days after flowering (DAF), two-celled proembryo is first observed when the endosperm consists of six to eight nuclei[7]. In *Solanum pimpinellifolium*, the embryo approaches its final size after 20 DAF, and seeds are viable for germination after 32 DAF[8]. As the embryo develops, the endosperm is gradually absorbed and only a small amount of endosperm remains in the seed. The residual endosperm consists of micropylar endosperm surrounding the radicle tip, and lateral endosperm in the rest of the seed[9,10]. The outer seed coat is developed from thickened and lignified outer epidermis of the integument, and with the dead layers of integument remnants, they form a hard shell to protect the embryo and endosperm[5].

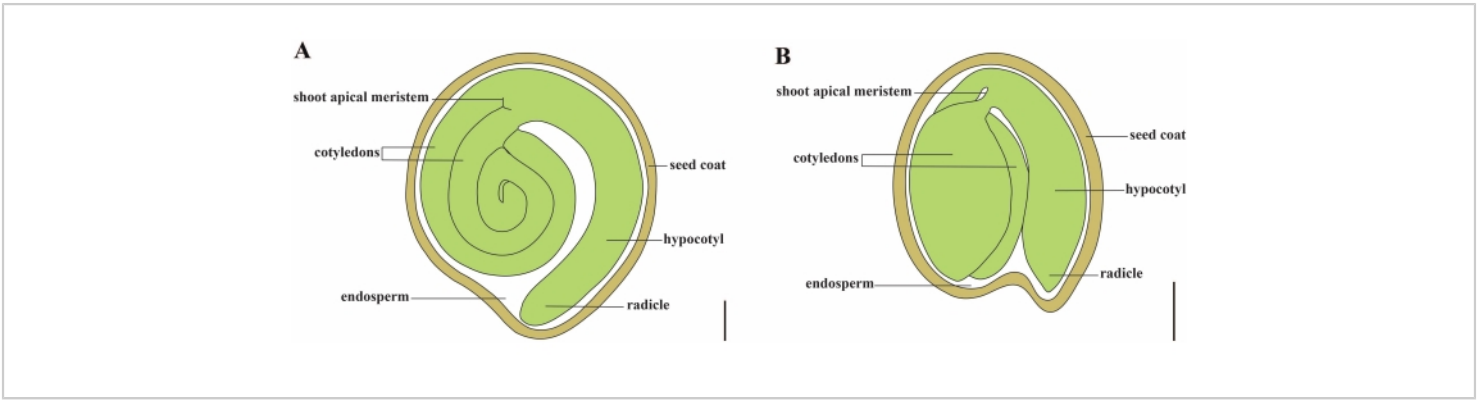

Figure 1: Schematic representation of a mature seed in *Solanum lycopersicum* and *Arabidopsis thaliana*. (**A**) Longitudinal anatomy of a mature tomato seed. (**B**) Longitudinal anatomy of a mature *Arabidopsis* seed. A mature tomato seed is approximately 70 times larger in size than an *Arabidopsis* seed. Scale bars = (**A**) 400 µm, (**B**) 100 µm. Please click here to view a larger version of this figure.

Production of high-quality tomato seeds depends on the coordination between the embryo, the endosperm, and the maternal seed components[11]. Dissecting key genes and networks in seed development requires a deep and full-track phenotypic recording of mutant seeds. Conventional embedding-sectioning techniques, such as the semi-thin section and paraffin section, are widely applied to tomato seeds to observe the local and finer structures of the embryo[12,13,14,15]. However, analyzing the seed development from thin sections is usually laborious and lacks z-axis spatial resolution. In comparison, tissue clearing is a fast and efficient method to pinpoint the developmental stage of embryo defects that are most likely to occur[16]. The clearing method reduces the opaqueness of internal

tissue by homogenizing the refractive index with one or more biochemical agents[16]. Whole tissue clearing allows observation of a plant tissue structure without destroying its integrity, and the combination of clearing technology and three-dimensional imaging has become an ideal solution to obtain information on the morphology and developmental state of a plant organ[17,18]. Over the years, seed clearing techniques have been used in various plant species, including *Arabidopsis thaliana*, *Hordeum vulgare*, and *Beta vulgaris*[19,20,21,22,23]. Among these, the whole-mount ovule clearing technology has been an efficient approach to studying seed development of *Arabidopsis*, due to its small size, 4-5 layers of the seed coat cell, and the nuclear-type endosperm[24,25]. With the continuous updating of different clearing mixtures, such as the emergence of Hoyer's solution[26], internal structures of the barley ovule were imaged with a high degree of clarity although its endosperm makes up the bulk of the seeds. Embryogenesis of sugar beet can be observed by clearing combined with vacuum treatment and softening with hydrochloric acid[19]. Nonetheless, unlike the species mentioned above, embryological observations by clearing protocols in tomato seeds have not been reported. This prevents detailed investigation into the embryonic and seed development of tomatoes.

Chloral hydrate is commonly used as a clearing solution that allows the immersed tissues and cells to be displayed on different optical planes, and substantially preserves the cells or tissue components[27,28,29]. Chloral hydrate-based clearing protocol has been successfully used for the whole-mount clearing of seeds to observe the embryo and endosperm of *Arabidopsis*[21,28]. However, this clearing solution is not efficient in clearing tomato seeds, which are more impermeable than *Arabidopsis* seeds. The physical barriers include: (1) the tomato integument has nearly 20 cell layers at 3 to 15 DAF[30,31], (2) the tomato endosperm is cellular-type, not nuclear-type[32], and (3) tomato seeds are about 70 times larger in size[33,34] and (4) produce large amounts of seed coat mucilage, which blocks the penetration of clearing reagents and affects the visualization of embryo cells.

Therefore, this report presents an optimized chloral hydrate-based clearing method for whole-mount clearing of tomato seeds at different stages, which allows deep imaging of the embryo development process (**Figure 2**).

Protocol

1. Preparation of solutions

1. Prepare FAA fixative by adding 2.5 mL of 37% formaldehyde, 2.5 mL of glacial acetic acid, and 45 mL of 70% ethanol in a 50 mL centrifuge tube. Vortex and store it at 4 °C. Freshly prepare FAA fixative just before use.
   CAUTION: 37% formaldehyde is corrosive and potentially carcinogenic if exposed or inhaled. The fixative must be performed in a fume hood while wearing appropriate personal protective equipment.
2. Prepare clearing solution by adding 5 mL of 100% glycerol, 40 g chloral hydrate, and 10 mL of distilled water in a 100 mL glass bottle wrapped with tin foil. Dissolve using a magnetic stirrer overnight at room temperature. The prepared solution can be stored at 4 °C for up to 6 months.
   CAUTION: Chloral hydrate is carcinogenic and has a pungent smell. Perform the experiment in a fume hood and wear appropriate personal protective equipment. Chloral hydrate is easy to deliquesce in the air and should not be stored in large quantities. The clearing solution

can decompose when exposed to light and should be kept away from light.

3. Prepare the disinfectant solution by adding 10 mL of 6% sodium hypochlorite, 40 mL distilled water, and 50 µL Tween-20 in a 50 mL centrifuge tube. Vortex and store it at room temperature. Freshly prepare the disinfectant solution just before use.

   NOTE: The effective chlorine content of sodium hypochlorite that has been placed for a long time may decrease, and the content of 6% sodium hypochlorite can be increased according to the actual situation.

2. Seed collection

1. Sow tomato seeds (*S. lycopersicum* L. cv. Micro-Tom, see **Table of Materials**) in 8 cm × 8 cm × 8 cm square basins full with a 1:1 mixture of flower nutrient soil and substrate (v/v) (see **Table of Materials**), and grow in a growth room with 16/8 h light/dark periods at 24 ± 2 °C (day) and 18 ± 2 °C (night), 60%-70% relative humidity, and a light intensity of 4,000 Lux. Approximately 6 weeks later, plants entered the flowering stage.
2. Tag the flowering date of independent flowers at anthesis (opening angle of the petals is 90°), and record the day after flowering (DAF).
3. Harvest the fruits from 3 to 23 DAF tomato plants and immediately put them on ice. Divide fruits (seeds or embryos) from 3 to 23 DAF into early (3-10 DAF), middle (11-16 DAF), and late (17-23 DAF) fruits (seeds or embryos).

   NOTE: Do not collect too many fruits at a time and ensure that the seeds in each fruit are stripped within 1 h for further treatment.
4. For early fruits, break the fruit and put it on a slide, and collect fresh seeds carefully with precision forceps under the stereomicroscope (see **Table of Materials**) at 1x to 4x magnification. For middle and late fruits, break the fruit and directly collect fresh seeds using precision forceps.

3. Chloral hydrate-based clearing of seeds

NOTE: Conventional[35] and optimized protocols were compared in this study for their seed clearing efficiency.

1. **Clearing using a conventional protocol**
   1. Place fresh seeds (obtained in step 2.4) immediately in a 2 mL centrifuge tube containing 1.5 mL FAA fixative and incubate on an orbital shaker (5 rpm, see **Table of Materials**) for 4 h at room temperature.
   2. Dehydrate these seeds in an ethanol series of 70%, 95%, and 100% ethanol (v/v) for 1 h each on an orbital shaker (5 rpm).
   3. Place the seeds in 3-5 drops of clearing solution (~100 µL) on the slide and gently cover the sample with a coverslip. Replace the slide with a single concave slide (see **Table of Materials**) for middle and late seeds.
   4. Keep these slides or single concave slides at room temperature for 30 min (3 DAF), 2 h (6 DAF), 1 day (9 DAF), 3 days (12 DAF), or 7 days (14 to 22 DAF) depending on the developmental stages of the seed material (the younger the materials, the faster the clearing speed).
   5. Observe the samples with a differential interference contrast (DIC) microscope equipped with a digital camera (see **Table of Materials**) at 10x, 20x, and 40x magnification. Adjust and optimize the

transmitted light brightness, DIC slider, and condenser aperture in real time for each sample and capture images.

2. **Clearing using the optimized protocol**

1. Place fresh seeds (obtained in step 2.4) directly into a 2 mL centrifuge tube containing 1.5 mL disinfectant solution (step 1.3).
NOTE: The number of seeds collected in the 2 mL centrifuge tube varies according to the developmental stage of the seeds. Details are listed in **Table 1**.

2. Incubate the samples on an orbital shaker (30 rpm) at room temperature for 3 to 50 min until the innermost seed coat outline is clearly visible. Discard the disinfectant solution.
NOTE: The incubation time required depends on the seed developmental stage (the later the seeds, the longer will be the incubation time). Details are listed in **Table 1**.

3. For middle and late seeds, transfer the seeds onto a slide and remove the seed mucilage with forceps and a dissecting needle under a stereomicroscope at 1x to 4x magnification. Transfer the seeds back into the original centrifuge tube using forceps.
NOTE: This step is not necessary for early seeds.

4. Wash the seeds 5x with 1.5 mL deionized water, 10 s each time. Discard the deionized water.

5. Add a clearing solution of 2 × the volume of the seeds, followed by vacuum treatment for 0 to 50 min (**Table 1**) with a vacuum pump (see **Table of Materials**). Intermittent vacuum treatment is performed with each vacuum treatment for 10 min spaced at 10 min intervals.

6. Replace with a fresh clearing solution of 2 × the volume of the seeds. Keep the 2 mL centrifuge tube containing the seeds at room temperature and protected from light for 30 min to 7 days to facilitate clearing (**Table 1**). During the incubation, for late embryos, replace the clearing solution daily with fresh solution and subject the seeds to vacuum treatment for 10 min.

7. Prepare the cleared seeds on slides or single-concave slides and observe with the DIC microscope equipped with a digital camera at 10x, 20x, and 40x magnification. Adjust and optimize the transmitted light brightness, DIC slider, and condenser aperture in real time for each sample and capture images.

Representative Results

When tomato seeds were cleared using a conventional method as in *Arabidopsis*, dense endosperm cells blocked the visualization of early tomato embryos at 3 DAF and 6 DAF (**Figure 3A**,**B**). As the total volume of the embryo increased, a globular embryo was barely distinguishable at 9 DAF (**Figure 3C**). However, as the seed size continued to increase, its permeability decreased, resulting in a fuzzy heart embryo at 12 DAF (**Figure 3D**). From 13 DAF onward, seed mucilage and seed coat gradually became denser, preventing the penetration of clearing agents. The outline of the embryo inside 14-19 DAF seeds was extremely blurred even when the treatment time was extended to 7 days (**Figure 3E-G**). In 22 DAF seeds, the internal structure of the seed was completely invisible (**Figure 3H**).

Therefore, the conventional chloral hydrate-based clearing procedure was optimized to enable the efficient clearing of

tomato seeds. Details on all steps are listed in **Table 1**. Firstly, the FAA fixation and ethanol dehydration steps, which are often required in conventional clearing procedures, were omitted from the protocol. While FAA fixation is generally used to preserve the morphological structure and cellular components from decay, it was found (in this study) that the internal structure of tomato seeds was not easily deformed and was well-preserved despite the absence of FAA fixation and ethanol dehydration.

Secondly, seed coat mucilage produced by the seed coat epidermal cells is very prominent from 13 DAF onward (**Figure 4**). The polysaccharide-rich seed mucilage showed a high viscosity and a significantly low permeability[35,36]. Initial trials to remove it without destroying the seed coat using fine forceps and needles under a dissecting microscope unfortunately failed. This is because the seed coat is brittle, and connected tightly to the mucilage. Moreover, the existence of pigment in the seed coat further leads to the decline in the quality of the bright-field image[37]. To overcome this problem, the seeds were treated with a disinfectant solution of 1.2% sodium hypochlorite and 0.1% Tween 20. The bleaching effect of sodium hypochlorite enables a clear identification of the inner seed coat and creates a relatively sterile environment that allowed samples to be stored for a long time. The use of detergent Tween 20 could lower the surface tension and increase seed permeability. More importantly, after this step, most of the adherent mucilage can be detached from the seed without damaging the seed coat (**Figure 4**). Henceforth, the treated seeds were pooled in a 2 mL centrifuge tube and were incubated at room temperature in an orbital shaker (30 rpm).

Thirdly, vacuum treatment was used to accelerate the penetration of the clearing solution to the embryos at the middle and late stages. Finally, because tomato seeds are especially larger after 12 DAF, conventional slides were replaced with single concave slides when DIC imaging.

After treatment according to the optimized clearing protocol, tomato seeds showed satisfactory transparency in all tested developmental stages (**Figure 5A-L**). In contrast to fuzzy cell contours obtained using conventional protocols, distinct cell layers of the seed coat at 3 DAF were visible (**Figure 5A**). Endosperm cells were more distinguishable at 5 DAF (**Figure 5B**). The stick-shaped embryo appeared at 7 DAF, and then the embryo reached the globular stage at 9 DAF and the heart stage at 11 DAF (**Figure 5C-E**). During these developmental stages, the outlines of the cells inside the embryo were most clearly visible. Compared with the conventional method, the optimized protocol produced significantly better quality images from the heart stage to the mature embryo stage. When embryonic development reached the early torpedo stage at 13 DAF, the middle torpedo stage at 14 DAF, the late torpedo stage at 15 DAF, the early-cotyledon stage at 16 DAF, the bent-cotyledon stage at 19 DAF and 21 DAF, and the mature embryo at 23 DAF, the degree of the curl of cotyledons and shoot apical meristem was very easily captured (**Figure 5F-L**). However, beyond 23 DAF, it was not possible to visualize the details in the embryo, even when the clearing treatment was prolonged to over 1 week.

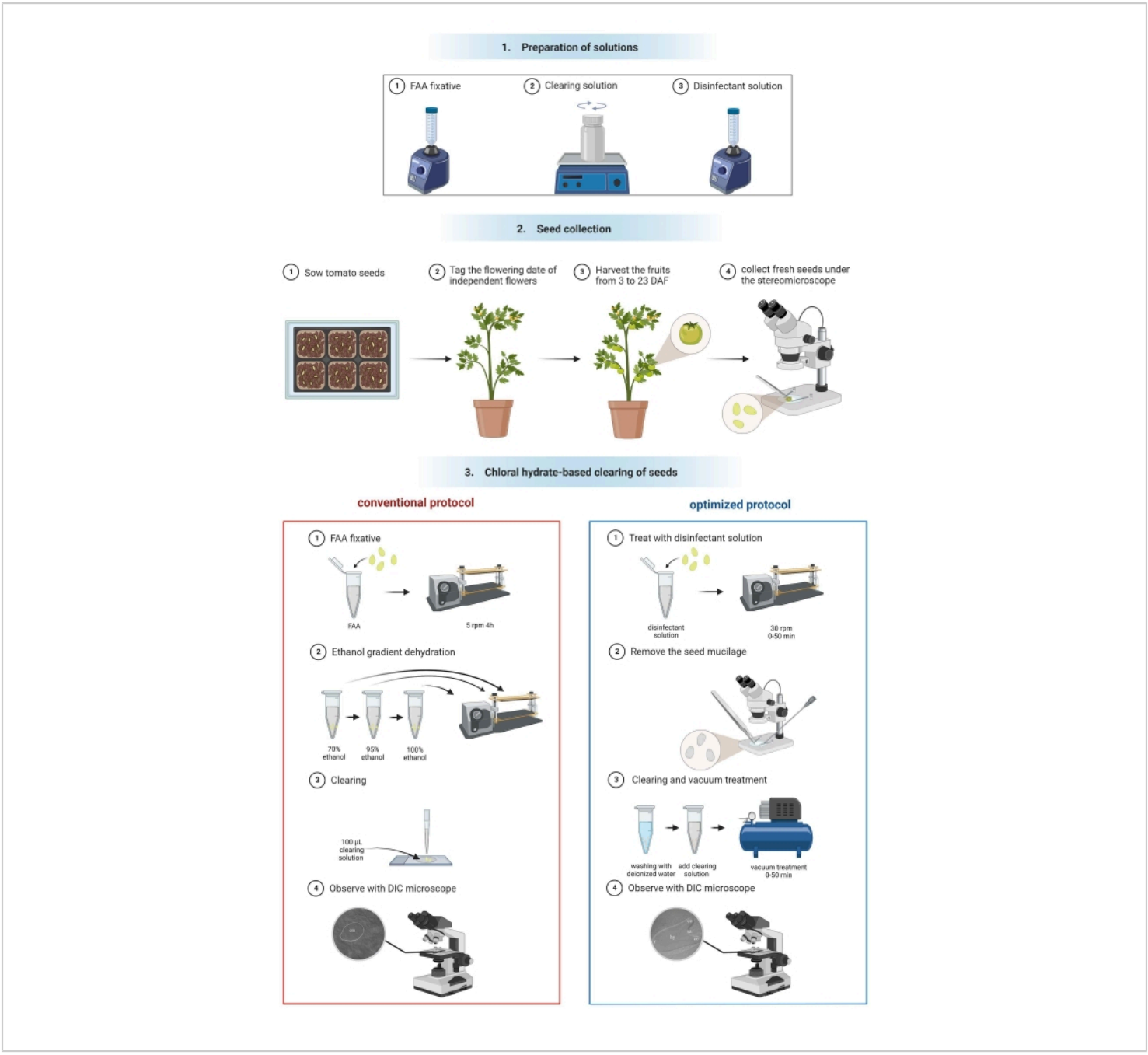

Figure 2: Flow chart of conventional protocol and optimized protocol. Please click here to view a larger version of this figure.

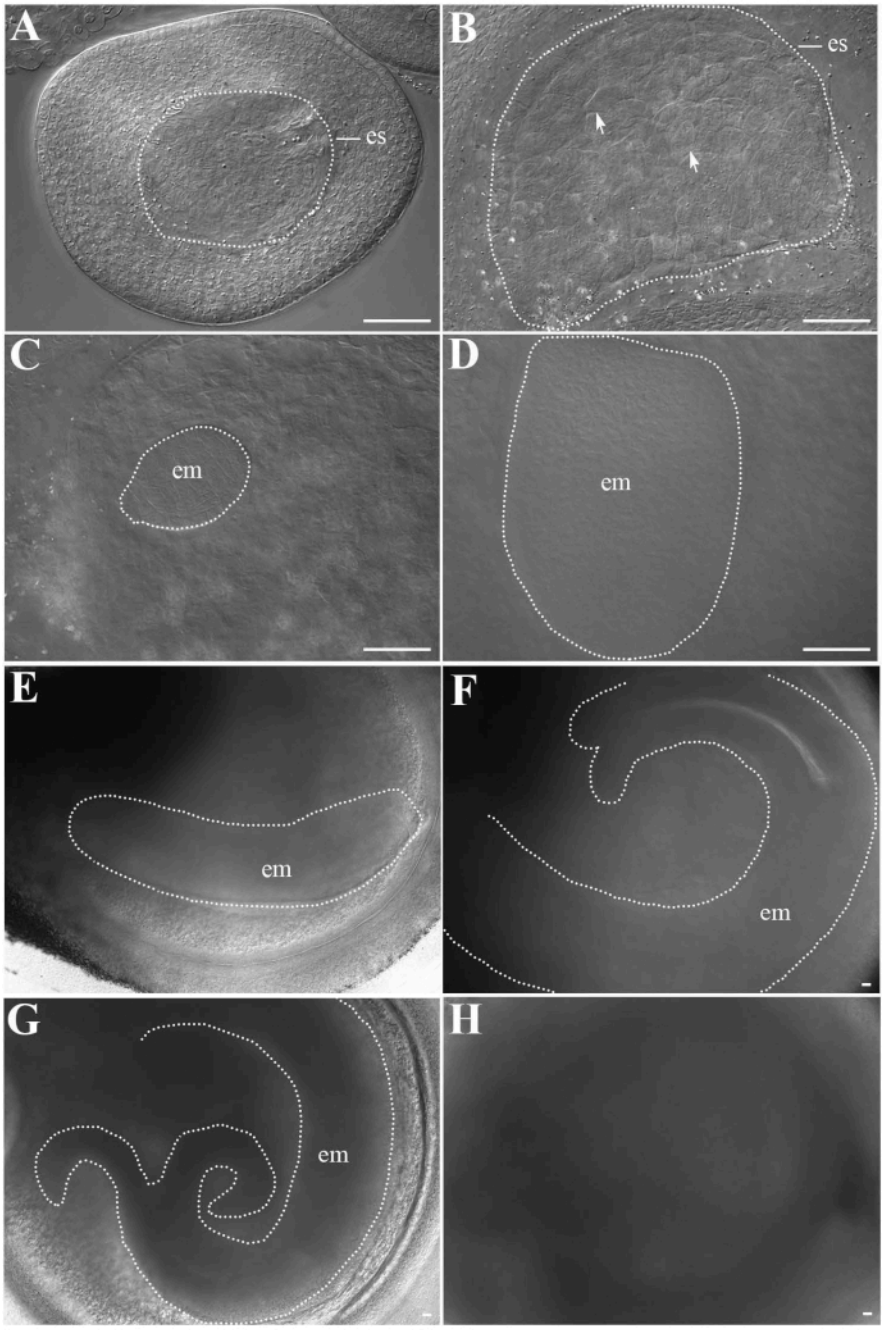

Figure 3: Differential interference contrast images of *S. lycopersicum* ovules treated using the conventional clearing method. (**A**) An ovule with fuzzy embryo sac at 3 DAF. (**B**) Embryo sac with visible endosperm cells (arrowheads) at 6 DAF. (**C**) A barely distinguishable globular embryo at 9 DAF. A fuzzy embryo at (**D**) 12, (**E**) 14, (**F**) 16, and (**G**) 19 DAF. (**H**) The internal structure of the seed is completely invisible at 22 DAF. Scale bars = 25 µm; em = embryo; es = embryo sac. Please click here to view a larger version of this figure.

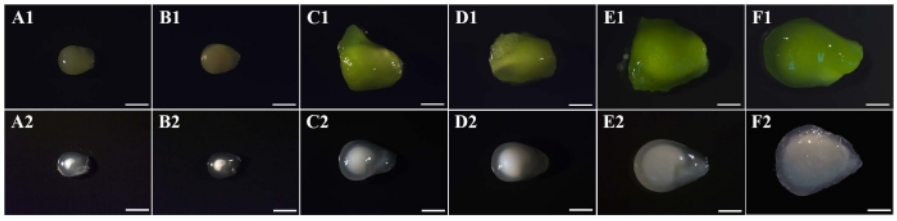

Figure 4: Disinfection of *S. lycopersicum* seeds at different developmental stages. The top images (**A1-F1**) are the striped seeds from the developing fruits at (**A1**) 11, (**B1**) 12, (**C1**) 14, (**D1**) 16, (**E1**) 18, and (**F1**) 21 DAF, while the bottom images (**A2-F2**) are seeds treated with disinfectant solution. In **A2-F2**, the inner seed coat contour can be identified clearly. Scale bars = 1 mm. Please click here to view a larger version of this figure.

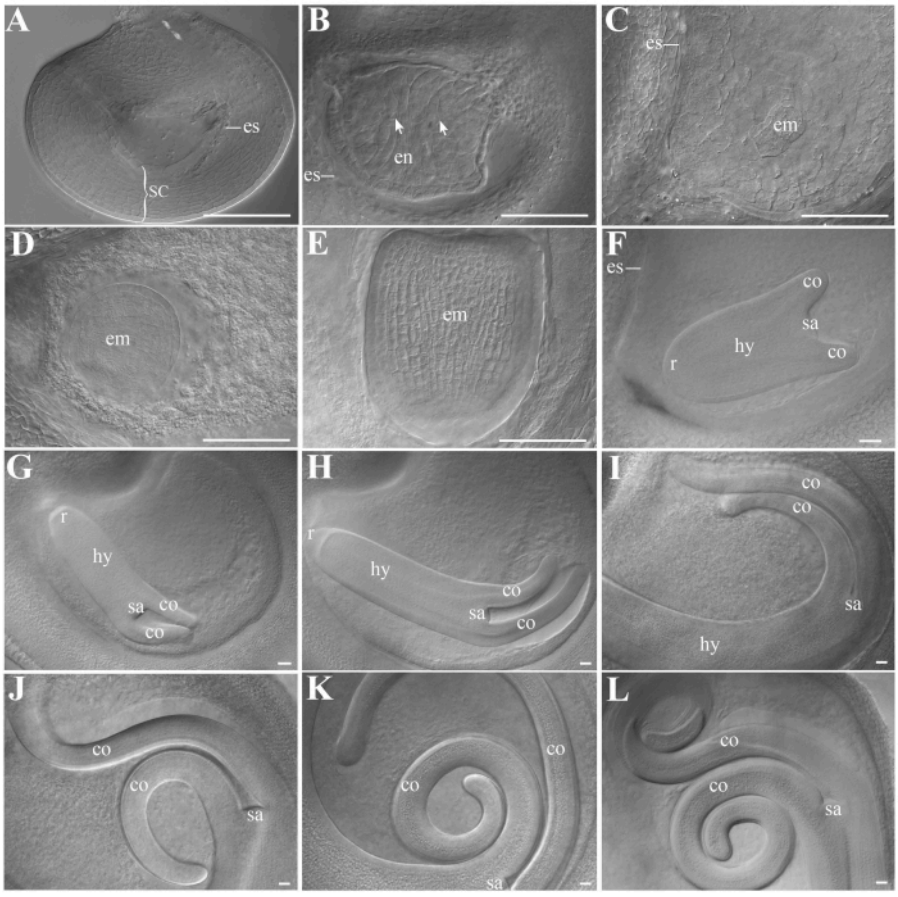

Figure 5: Differential interference contrast images of *S. lycopersicum* embryos cleared using the optimized protocol. (**A-L**) DIC microscopy images of the tomato ovule with visible embryo sac at (**A**) 3 DAF and (**B**) 5 DAF, (**C**) a stick-shaped embryo at 7 DAF, (**D**) a globular embryo at 9 DAF, (**E**) a heart embryo at 11DAF, (**F**) an early torpedo embryo at 13 DAF, (**G**) a middle torpedo embryo 14 DAF, (**H**) a late torpedo embryo at 15 DAF, (**I**) an early-cotyledon embryo at 16 DAF, a bent-cotyledon embryo at (**J**) 19 DAF and (**K**) 21 DAF, and (**L**) a mature embryo at 23 DAF. Scale bars = 50 µm; em = embryo; sc = seed coat; en = endosperm; es = embryo sac; hy = hypocotyl; co = cotyledon; r = radicle; sa = shoot apical. Please click here to view a larger version of this figure.

	Tissue processing	Embryo development					
		3-7 DAF	8-10 DAF	11-13 DAF	14-16 DAF	17-20 DAF	21-23 DAF
1	Number of fresh seeds	50	40	30	20	15	10
2	Disinfection	1.5 mL, 3 min	1.5 mL, 10 min	1.5 mL, 20 min	1.5 mL, 30 min	1.5 mL, 40 min	1.5 mL, 50 min
3	Peeling off the mucilage	No	No	Yes	Yes	Yes	Yes
4	Washing	5x 10 s					
5	Clearing	2x the volume of seeds					
6	Vacuuming	No	1 x 10 min	2 x 10 min	3 x 10 min	4 x 10 min	5 x 10 min
7	Incubation	30 min	2 h	3 h	8 h	1–4 days	3–7 days

Table 1: Optimization of the protocol for improving clearing efficacy in tomato seeds. 1 = Batch operations, clearing observation of a large number of seeds at once; seeds were placed in a 2 mL centrifuge tube. 2 = Disinfection using 6% NaClO:dH_2O:Tween-20, in proportions of 200:800:1 (v/v); all the steps were performed on an orbital shaker with 30 rpm shaking. 3 = All the steps were performed under a dissecting microscope with forceps and dissecting needles unless otherwise specified. 4 = Seeds were washed with distilled water after each step. 5 = Clearing using 100% glycerol:chloral hydrate:dH_2O, in proportions of 1:8:2 (v/w/v). 6 = Each vacuum treatment lasted 10 min and was spaced at 10 min intervals. 7 = Replace with fresh clearing solution and store seeds away from light at room temperature; for seeds from 17 DAF to 23 DAF, replace clearing solution with fresh solution and vacuum for 10 min everyday throughout the incubation period.

Discussion

Compared to mechanical sectioning, the clearing technology is more advantageous for three-dimensional imaging as it retains the integrity of plant tissues or organs[16]. Conventional clearing protocols are often limited to small samples due to easier penetration of chemical solutions. Tomato seed is a problematic sample for tissue clearing because it is about 70 times larger than an *Arabidopsis* seed in size and has more permeability barriers. The *Arabidopsis* seed coat is composed of four to five living cell layers derived from the outer integument and inner integument[24]. However, compared to *Arabidopsis*, there are 22-27 parenchymal cell layers (in the stage of the globular embryo) in the tomato seed coat[6]. The innermost layer of the tomato seed coat is composed of suberin and is known as the semipermeable layer, which has been shown to be a barrier to the movement of water-soluble compounds[38]. In addition, the polysaccharide-rich seed mucilage showed a high viscosity and a significantly low permeability, which hampered the penetration of the clearing

solution. The pigment-rich seed coat of tomato also reduces image quality[35,36,37]. Historically, the removal of pigments is solved by the application of oxidants accompanied by heating or prolonged treatment[37,39,40,41]. However, hardly any application of oxidants in the clearing method based on chloral hydrate has been reported.

In this study, the seeds were treated before the clearing process with a disinfectant solution containing sodium hypochlorite, supplemented with a surfactant Tween 20. Surprisingly, after treatment with the disinfection solution (3 to 50 min), the mucilage of tomato seeds could be easily removed, greatly improving the permeability of the seeds. The bleaching process of the seed coat can be observed in real-time and the duration of seed disinfection is adjusted accordingly. The bleaching process also maintains a relatively sterile environment, thereby allowing subsequent storage of the treated seeds for 8 months without contamination.

The optimized protocol developed in the study achieved overall transparency of tomato seeds from 3 DAF to 23 DAF. Advantages of this protocol are mainly reflected in the following aspects: First, the elimination of FAA fixation and subsequent ethanol gradient dehydration shortened the treatment time by at least 7 h. The use of sodium hypochlorite treatment combined with clearing solution significantly improved the permeability of the seeds and visualization of the innermost layer of the seed coat. Finally, for middle embryos, high-quality images of embryos can be acquired after 10 h, while it takes at least 3 days with traditional protocols.

Despite the advantages of this protocol, it does have two important limitations. First, at 5 DAF, the early proembryo at the two- to sixteen-celled stage was not observed, which was probably due to the dense and larger cellular-type endosperm cells hindering the visualization of embryos. Second, though the morphology of the tomato embryo is nearly complete at around 23 DAF, distinct images for seeds after 24 DAF could not be obtained with this optimized method. It is possible that as the embryo approaches its full size, the cuticle on the surface of the endosperm significantly thickens, the remnants of dying integument parenchyma cells form dense layers and the outer epidermis cells of the integument dramatically increase in lignification[6]. These suggest a drastic reduction in seed penetration capacity, making it impossible to visualize the internal tissue with the existing methods.

In conclusion, this optimized protocol provides an effective means to find the abnormal period of embryonic development, providing the basis for the observation of finer cell structures using slicing techniques. Moreover, to some extent, this method is likely to provide a reference scheme for other commercially important crops in Solanaceae, such as potatoes, eggplant, peppers, and tobacco.

Disclosures

The authors have no conflicts of interest to disclose.

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