Chapter 1

Handling *Arabidopsis* Plants: Growth, Preservation of Seeds, Transformation, and Genetic Crosses

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Abstract

Growing healthy plants is essential for the advancement of *Arabidopsis thaliana* (*Arabidopsis*) research. Over the last 20 years, the Arabidopsis Biological Resource Center (ABRC) has collected and developed a series of best-practice protocols, some of which are presented in this chapter. *Arabidopsis* can be grown in a variety of locations, growth media, and environmental conditions. Most laboratory accessions and their mutant or transgenic derivatives flower after 4–5 weeks and set seeds after 7–8 weeks, under standard growth conditions (soil, long day, 23 °C). Some mutant genotypes, natural accessions, and *Arabidopsis* relatives require strict control of growth conditions best provided by growth rooms, chambers, or incubators. Other lines can be grown in less-controlled greenhouse settings. Although the majority of lines can be grown in soil, certain experimental purposes require utilization of sterile solid or liquid growth media. These include the selection of primary transformants, identification of homozygous lethal individuals in a segregating population, or bulking of a large amount of plant material. The importance of controlling, observing, and recording growth conditions is emphasized and appropriate equipment required to perform monitoring of these conditions is listed. Proper conditions for seed harvesting and preservation, as well as seed quality control, are also described. Plant transformation and genetic crosses, two of the methods that revolutionized *Arabidopsis* genetics, are introduced as well.

Key words *Arabidopsis*, Growth conditions, Environmental conditions, Natural accession, Seed germination, Seed quality, Plant transformation, Genetic crosses

1 Introduction

Healthy growth and development of plants is a prerequisite for accurate and reproducible plant research and *Arabidopsis thaliana* (*Arabidopsis*) is no exception. Proper handling and maintenance of *Arabidopsis* plants also enables a high rate of seed production. In this chapter, we describe basic, best-practice protocols needed for handling *Arabidopsis*. The reader should be aware, however, that most of the commonly used growth environmental conditions, particularly in greenhouses, may not be similar to the ones in the native habitats of some natural accessions. This is especially

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important for interpreting phenotypic differences of traits that are known to be strongly influenced by the natural habitat, such as flowering time. Therefore, the protocols described here should be taken only as a guide for the experimental setup and design.

This chapter will address (1) the growth of *Arabidopsis* plants in a variety of environmental settings including growth chambers and greenhouses, as well as in vitro, (2) critical, and optimal conditions to grow healthy *Arabidopsis* plants, including quality control measures, (3) harvesting, seed preservation, and seed quality control, (4) genetic crosses, and (5) transformation with *Agrobacterium tumefaciens* (*Agrobacterium*). Significant emphasis is placed on the equipment required for controlling and monitoring environmental conditions during plant growth. The plant and seed management protocols are given in chronological order.

2 Materials

2.1 Plant Growth and Seed Harvest

1. *Arabidopsis* seeds can be obtained from the public stock centers: Arabidopsis Biological Resource Center (ABRC, abrc.osu.edu), European Arabidopsis Stock Centre (NASC, arabidopsis.info), RIKEN BioResource Center (RIKEN BRC, www.brc.riken.jp/inf/en), French National Institute for Agricultural Research (INRA, cnrgv.toulouse.inra.fr/en), and other laboratory sources [1] and private sources such as Lehle Seeds (arabidopsis.com).
2. Sterile plastic Petri dishes (plates) (10 or 15 cm diameter).
3. Murashige and Skoog basal salt mixture (MS).
4. 2-(*N*-Morpholino) ethanesulfonic acid (MES).
5. Agar granulated.
6. Sucrose.
7. Gamborg's Vitamin Solution.
8. KOH.
9. Distilled water.
10. Magnetic stirring device.
11. Beakers (1 L).
12. Glass bottles (1 L).
13. pH meter.
14. Microcentrifuge tubes.
15. Disposable Pasteur pipettes.
16. Pipetman and pipette tips.
17. Household bleach (5.25 % w/v sodium hypochlorite).
18. Tween® 20.

19. Labeling tape or printable labels.
20. Permanent marker.
21. 3M Micropore surgical paper tape.
22. Thiamine hydrochloride, plant cell culture tested.
23. Double distilled water (ddH_2O).
24. 2,4-Dichlorophenoxyacetic acid (2,4-D), plant cell culture tested, >98 %.
25. Ethanol, absolute, 200 proof, for molecular biology.
26. 0.45-μm filter sterilization unit.
27. Myoinositol, plant cell culture tested.
28. KH_2PO_4.
29. NaOH.
30. Soil mix, e.g., Sunshine® LC1 mix (Sun Gro Horticulture, www.sungro.com) or other peat moss-based potting mix.
31. Fertilizer in slow release pellets, e.g., Osmocote® 14-14-14 (Hummert™ International, www.Hummert.com).
32. Plastic pots with holes in the bottom (e.g., 11 cm diameter, 5.5 cm square) or plastic flats (e.g., 26 cm × 53 cm) with clear domes.
33. Trowel or large spoon.
34. 70 mm filter paper.
35. Pest Trap™ colored sticky cards (Hummert™ International).
36. Enstar® II (Hummert™ International).
37. Conserve® SC (Hummert™ International).
38. Marathon® 1G, granular systemic insecticide (Hummert™ International).
39. Sulfur vaporizer and bulk pelleted sulfur (HID Hut Inc., www.hidhut.com).
40. Spor-Klenz® Ready-To-Use Cold Sterilant (Steris, www.steris.com).
41. Tornado™/Flex cold fog ULV mist sprayer (Curtis Dyna-Fog Ltd., www.dynafog.com).
42. Plastic transparent floral sleeves, e.g., straight sleeve BOPP 60 × 40 × 15 cm (www.zwapak.com) for 11-cm-diameter pots, or other devices for plant isolation such as Aracons™ (Lehle Seeds and Arasystem, www.arasystem.com) or lightweight plastic bags (4–8 L).
43. Hand sieve, e.g., US Standard Stainless Steel Test Sieve No. 40 (Fisher Scientific).
44. Small manila envelopes (e.g., 6 cm × 9 cm) or small glass jars (125 mL) or other containers.

2.2 Control of Environmental Growth Conditions for Optimal Plant Growth

1. Data loggers, e.g., HOBO® U14 LCD (Onset, www.onsetcomp.com).

2.3 Preparation of Seeds for Short- and Long-Term Storage

1. 2-mL polypropylene cryovials with threaded lids and gaskets (e.g., screw cap micro tubes, manufactured by Sarstedt Inc., available from Fisher Scientific) or other sealed containers for permanent seed storage.
2. Permanent marker or printed labels.

2.4 Seed Quality Control

1. Dissecting microscope or magnifying lenses.
2. Plastic Petri plates (10 cm diameter) or other similar containers.
3. Absorbent paper, e.g., filter paper 10 cm diameter.
4. Permanent marker or printed labels.
5. Distilled water.
6. Parafilm or tape.

2.5 Genetic Crosses

1. DV-30 Precision Swiss clamping tweezers (Lehle Seeds).
2. Optical glass binocular magnifier, e.g., OptiVISOR® (Donegan Optical Company, www.doneganoptical.com) or dissecting microscope.
3. 1.5-mL microcentrifuge tubes.
4. Small scissors.
5. Laboratory tape in various colors and permanent marker.

2.6 Transformation of Arabidopsis with Agrobacterium tumefaciens

1. *Agrobacterium* transformed with a construct of interest.
2. LB medium.
3. Selection antibiotics.
4. Sucrose.
5. Silwet L-77®.

3 Methods

3.1 Growth of Arabidopsis Plants and Cultures

3.1.1 Growth of Plants in Sterile Conditions on Solid Media

Growth of *Arabidopsis* in experimental settings such as selection of drug-resistant and transformed plants, examination of early root and shoot phenotypes, and identification of homozygous lethal mutants is typically conducted in sterile conditions on solid media. Liquid bleach sterilization, described here, is a practical method to sterilize a few seed lines at a time. Larger numbers of lines can be sterilized easily and with less manipulation using chlorine gas. Chlorine gas can also be utilized for seeds infested with powdery

mildew or other fungal diseases. Various containers such as Petri plates, Magenta® boxes, or culture tubes are used, depending on the purpose of the experiment. This section describes the use of the most commonly employed medium for sterile growth conditions in Petri plates (1× MS agar media). Adaptation to other sterile formats is straightforward, and most experimental additives can be easily incorporated in the preparation.

1. Add 4.31 g of MS basal salt mixture [2] and 0.5 g of MES to a beaker containing 0.8 L of distilled water and stir to dissolve. Add distilled water to final volume of 1 L. Check and adjust pH to 5.7 using 1 M KOH.
2. Divide the media into two 1 L bottles, 500 mL in each. Add 5 g of agar per bottle. Keep the lid loose.
3. Autoclave for 20 min at 121 °C, 15 psi with a magnetic stir bar in the bottle.
4. Place the bottles on a stir plate at low speed and allow the agar medium to cool to 45–50 °C (until the container can be held with bare hands).
5. Starting from this step, perform all the steps in sterile conditions in a laminar flow hood. Add (optional) 1–2 % sucrose and 1 mL Gamborg's Vitamin Solution, stirring to evenly dissolve (*see* **Notes 1** and **2**).
6. Label the bottom of Petri plates with identification number or name, including the date.
7. Pour enough media into plates to cover approximately half of the depth of the plate.
8. Allow the plates to cool at room temperature for about an hour to allow the agar to solidify. If the plates are not to be used immediately, wrap them in plastic and store at 4 °C (refrigerator temperature) (*see* **Note 3**).
9. Surface-sterilize seeds in microcentrifuge tubes by soaking for 20 min in 50 % bleach with the addition of 0.05 % Tween® 20 detergent.
10. Remove all bleach residue by rinsing five to seven times with sterile distilled water.
11. For planting of individual seeds at low density, adhere one seed to the tip of a pipette using suction, then release seed onto the agar in desired location. For planting seeds at higher densities, mix seeds in sterile distilled water (or 0.1 % cooled top agar), pour onto plate, and immediately swirl to achieve even distribution. Use a sterile pipette tip to adjust the distribution and remove excess water. Allow the water or top agar to dry slightly before placing lid onto plate.
12. Seal with Micropore tape to prevent desiccation, while allowing slight aeration.

13. Place the plates at 4 °C for 3 days (*see* **Notes 4** and **5**).
14. Transfer the plates to the growth environment. Illumination of 120–150 μmol/m² s continuous light and a temperature of 22–23 °C are suitable growth conditions (*see* **Notes 6–8**).

3.1.2 Growth of Plants in Sterile Conditions in Liquid Media

Seedlings of *Arabidopsis* can also be grown in liquid growth media. This method provides large amounts of plant tissue suitable for proteomics and metabolomics or any study that requires a larger amount of starting material. Liquid culture growth is also widely used for high-throughput genomic studies. In this case, growth protocols are adapted to 96-deep-well plates (or other formats) with the MS media supplemented by gibberellic acid.

1. Prepare MS media, as described in Subheading 3.1.1. Do not add agar.
2. After the media has been autoclaved and cooled to room temperature, distribute 75–100-mL MS media into previously sterilized 250-mL Erlenmeyer flasks in a laminar flow hood.
3. Add bleach- or chlorine gas-sterilized seeds to the media (add up to 10 μL of seeds to each flask, which corresponds to approximately 250 seeds).
4. Grow seedlings under continuous light (120–150 μmol/m² s) with gentle rotation in an orbital shaker at 120 rpm for up to 2 weeks.
5. Remove seedlings from the flask. Growth of more than 200–250 seedlings for more than 2 weeks may result in difficulty removing plant material from the flask.
6. Remove excess media from the seedlings using filter paper. Plant material is now ready for downstream applications.

3.1.3 Growth of Arabidopsis Cells in Culture

Cell suspension cultures represent a source of nearly uniform cell material for functional genomics and biochemical, physiological, and metabolomic studies that can be performed under tightly controlled environmental conditions. Several cell cultures derived from *Arabidopsis* tissue explants have been described. Among these, T87 and MM1/MM2d have been most widely used. The T87 cell line originates from the Columbia accession seedlings and can photosynthesize in light [3]. It has been utilized to analyze gene expression changes under stress conditions, hormone signaling pathways, the circadian clock, and plant cell wall biosynthesis [4–6]. Transient and stable transformation protocols for this line have also been established [4, 7]. Unlike T87, MM1 (light grown) and MM2d (dark grown) cell lines, derived from Landsberg *erecta* accession, are synchronous and can therefore be used for cell-cycle studies [8]. Due to limited space, only the protocol describing maintenance of T87 cell culture will be described here.

1. Prepare 10 mg/mL thiamine stock solution by dissolving 0.1 g of thiamine in 10 mL of ddH_2O. Filter-sterilize, aliquot 1 mL into microcentrifuge tubes, and store at –20 °C.
2. Prepare 2,4-D stock solution by dissolving 0.2 g of 2,4-D in 100 mL of 25 % ethanol. Filter-sterilize, aliquot 1 mL into microcentrifuge tubes and store at –20 °C.
3. Prepare 1 L of NT-1 media by adding 4.3 g of MS salt mixture, 30 g sucrose, 0.18 g KH_2PO_4, 100 µL of 10 mg/mL thiamine stock, 220 µL of 2-mg/mL 2,4-D stock, and 100 mg myoinositol to a bottle containing 0.8 L of ddH_2O and stir to dissolve (*see* **Note 9**).
4. Adjust the pH to 5.8 using 5 M NaOH. Add ddH_2O to final volume of 1 L.
5. Distribute 75-mL media into 250-mL Erlenmeyer flasks. Cover flasks with aluminum foil (*see* **Note 10**).
6. Autoclave for 20 min. Let the media cool to room temperature.
7. In a laminar flow hood, transfer 3 mL of 1-week-old T87 cell suspension culture into a flask containing 75 mL of NT-1 medium (*see* **Notes 11** and **12**).
8. Grow the culture at 24 °C under continuous light (40–100 µmol/m^2 s) with gentle rotation in an orbital shaker at 120 rpm.
9. Subculture weekly by transferring cells into fresh NT-1 media, as described in **step 8** (*see* **Note 13**).

3.1.4 Planting Arabidopsis Seeds on Soil

Diverse mixes and media can be used for growing *Arabidopsis*. The term "soil" will be used here for any mix or media utilized for non-sterile growth of plants in pots or similar containers. Commercial potting mixes are popular with *Arabidopsis* researchers due to their convenience and reliability. Potting media often employ peat moss for moisture retention and perlite for aeration. Mixes such as Sunshine® LC1 support healthy *Arabidopsis* growth and include a starter nutrient charge, so that fertilization is not necessary in early growth phases. Seeds can be planted by various methods (*see* **Note 14**). Soil can be autoclaved to eliminate pests, but this is usually not necessary. Preparation of soil for planting in pots can be accomplished as follows:

1. Place soil in a clean container. Add Osmocote® 14-14-14 fertilizer (*see* **Note 15**). Wet thoroughly with tap water and mix well with trowel, large spoon, or hands.
2. Label pots or trays with the stock number or name and date of planting (*see* **Note 16**).
3. Place soil loosely in pots or other containers and level, without compressing, to generate a uniform and soft bed. Pots are then ready for planting (*see* **Note 17**).

4. When planting many seeds in a pot, scatter them carefully from a folded piece of 70-mm filter or other paper; distribute them evenly onto the surface of the soil (*see* **Note 18**). When planting individual seeds, adhere one seed to the tip of a pipette using suction, then release onto the soil. Planted seeds should not be covered with additional soil, since *Arabidopsis* seeds require light for germination.
5. Place pot(s) in a tray, flat, or other container.
6. Cover with a plastic dome or with clear plastic wrap taped to the container (*see* **Note 19**).
7. Place pots at 4 °C for 3 days (*see* **Note 4**).
8. Transfer pots into the growth area.
9. Remove plastic dome or wrap for growth in the greenhouse, but leave them on until germinated seedlings are visible for plants grown in a growth chamber.

3.2 Growth Conditions

The growth and development of *Arabidopsis*, including flowering time, is influenced by a number of environmental conditions in addition to the genetic background. Seeds of most lines germinate 3–5 days after planting under continuous light, 23 °C, adequate watering, and good nutrition. Plants produce their first flowers within 4–5 weeks, and seeds can be harvested 8–10 weeks after planting. High-quality seeds can be produced if watering, light, and temperature are carefully controlled.

For vigorous plant growth, the optimum light intensity is 120–150 μmol/m^2 s (*see* **Notes 6** and **7**) and the optimum temperature is 22–23 °C (*see* **Notes 8** and **20**). Water requirement is strongly influenced by relative humidity. Plants tolerate low (20–30 %) relative humidity well, but depletion of soil moisture may occur in these conditions. Plant sterility may result from very high (>90 %) relative humidity. Mild humidity (50–60 %) is considered optimal for plant growth; however, low humidity (<50 %) is recommended for silique maturation.

The following growth practices are useful for handling plants in any growth context (greenhouse, growth chamber, or growth room):

1. Add water to trays containing pots with perforated bottoms.
2. Maintain approximately 2 cm of water around base of pots during germination, to avoid any soil drying before the first true leaves begin expanding.
3. Reduce the watering frequency to as low as once or twice per week as needed after plants have developed true leaves and until the plants flower, to avoid water stress, but allow proper drainage of the soil (*see* **Note 21**).
4. Water daily during silique filling stage for good seed production. The water requirement of plants increases dramatically during this stage.

5. Keep plants spaced apart with good air circulation to prevent the incidence of powdery mildew.
6. Place several yellow or blue sticky cards (e.g., Pest Trap™) in the growth area to monitor insect populations. Inspect cards and plants daily for pests. Change cards periodically to better judge the pest populations and especially after a pesticide application.
7. Prevent the introduction and spread of pests, which can be transported to the growth area via the soil, seeds, plants, or by humans. Wear a lab coat especially assigned to the growth area, since insects and pathogens can readily be transported on clothing. Plan to have plants of similar age in the growth area, since mature plants are more susceptible to pests than very young plants. Any person who has been in infested growth areas should subsequently abstain from entering noninfested areas; when entering multiple areas, entries should be from the cleanest to the more infested. Keep the area clean and regularly sweep the floors and/or shelves to eliminate or reduce potential sources of pest outbreaks. Mature and dry plants should be harvested and old soil and nonviable dry plant debris should be discarded immediately.
8. Avoid infestation of pests like thrips, aphids, fungus gnats, and white flies by spraying plants with a preventive mixture of Enstar® II, and Conserve® SC. Insecticide mixture is prepared by adding 1.2 mL of each to 12 L of water. This mix can be sprayed lightly on rosettes prior to bolting stage, before placement of any isolation devices (*see* Subheading 3.3.1). Marathon® 1G, a granular insecticide, can also be applied as directed by the label to control aphids, fungus gnat larvae, white flies, psyllids, and thrips (*see* **Notes 22** and **23**).

3.2.1 Maintenance of Plants in Greenhouses

Greenhouses with satisfactory cooling, heating, and supplemental light are suitable for large-scale growth of lines that do not require strict control of environmental conditions, which include most natural accessions (e.g., Col, Ler, Cvi, Ws, Est, Kas, Sha, Kondara, C24) as well as species related to *A. thaliana*. However, conditions are often too hot in temperate climates for *Arabidopsis* growth in greenhouses during the summer. Successful plant growth should start with an empty room, cleaned and maintained as follows (*see* **Note 24**):

1. Remove and properly discard all plants and other materials in the room.
2. Sweep and hose down the entire room interior (benches, floors, window ledges, and windows).
3. Increase the temperature in the room to 40 °C for 3–5 days. The temperature setting may be higher, depending on the

outside environmental conditions and equipment specification. Lights, fans, and cooling pads should be turned off and vents closed during this period.

4. Do not place diseased or older plants in the clean room after the high temperature treatment.
5. Provide supplemental evening and morning light during the winter, since the plants generally require a long photoperiod (at least 12 h) for flowering. In the greenhouse, 16-h photoperiods are typically employed (*see* **Notes 6** and **7**).
6. Use shade cloth during the summer, which helps reduce light intensity and regulate temperature.
7. The recommended growth temperature in the greenhouse is 21–23 °C (*see* **Note 8**). Night temperatures should be maintained 2–4 °C lower than the day temperature.

3.2.2 Maintenance of Plants in Growth Chambers and Growth Rooms

Most of the commercial growth chambers precisely control light intensity, photoperiod, temperature (typically ±1 °C), and often humidity. Custom plant growth rooms provide environmental control similar to that of reach-in chambers. Standard architectural rooms, equipped with supplemental lighting and air conditioning, are popular for reproducing *Arabidopsis* economically. Such rooms must be designed with sufficient light, cooling, and ventilation, but typically afford less rigorous control of growth conditions than custom chambers. Such facilities usually allow better control of temperature and light than is offered by a greenhouse, hence their popularity among *Arabidopsis* researchers. Growth rooms can be maintained within 2–3 °C of a set point, while greenhouse temperatures may spike to higher deviations with rapid changes in sunlight, unexpected hot days, etc. As is the case for greenhouses, it is imperative to start a new planting in a growth facility that has been previously emptied and properly cleaned. Hence, the use of chemicals to control pests and loss of plants due to pest infestation is minimized.

1. Remove and discard all plant residues and related materials (*see* **Note 24**).
2. Sweep and wipe down the interior with wet paper towel.
3. Make sure the intake and exhaust vents are closed.
4. Apply a sterilizing agent, such as Spor-Klenz®, to kill fungal spores if heavy infestation of powdery mildew was present, using a fogger tank (e.g., Tornado™/Flex cold fog ULV mist sprayer) through an external access port of the chamber (*see* **Note 25**).
5. Leave chamber undisturbed overnight, and wipe down the inside of the growth chamber with a wet paper towel the next day.

6. Increase the temperature to 40–45 °C for a period of 3–5 days to eradicate/minimize pests.
7. Do not place diseased or older plants in the cleaned chamber.
8. Use continuous light or a long-day photoperiod if you wish to accelerate the reproductive cycle. Short days (less than 12 h) favor growth of vegetative tissue and delay flowering.

3.2.3 Monitoring the Environmental Growth Conditions

The environmental control systems currently offered for greenhouses, growth chambers, and growth rooms allow for remote monitoring, control adjustment, and alarm notification via Internet connections. These features represent a vital tool for avoiding loss of data during plant production and maintaining control of environmental experiments. Installation of remote sensing is recommended for new growth facilities of all types.

In addition to the control and logging systems in place at the growth facilities, environmental growth conditions can be monitored by placing portable data loggers (e.g., the HOBO® U14) in growth areas. They can act as a complementary, backup, or sole resource for recording environmental data. They can be used to display and record temperature and relative humidity conditions in greenhouses, growth chambers, growth rooms, cold rooms, dry rooms, and laboratories. These data loggers offer reliability, accuracy, convenient monitoring, and documentation of specific environmental conditions. They can be connected to a computer to quickly display and analyze data.

3.3 Seed Handling

3.3.1 Plant Isolation, Harvesting, and Preparation for Storage

Prevention of cross-contamination among adjacent pots and avoiding the loss of seeds due to shattering are equally important. Plants must be isolated from their neighbors without compromising seed quality. Various methods and devices exist to accomplish these objectives, including Aracons™, plastic floral sleeves, plastic bags, and isolation by space on the open bench. Details of each method are described below:

1. Aracons™: Place Aracons™ over single plants soon after bolting.
2. Floral sleeves: Cut four equally spaced holes at the point where the sleeve meets the top of the pot. This will increase aeration and reduce water condensation that may encourage mold growth. Place the sleeve on the pot near the time of bolting, so that all plant inflorescences are maintained within the sleeve (*see* **Note 26**). This method is very effective for achieving high densities while maintaining productivity and purity of single lines of different genetic backgrounds.
3. Plastic bags: If plastic bags are used, train inflorescences of non-erecta lines into a 4–8-L transparent plastic bag before siliques begin to brown. Bags should be kept open to avoid the accumulation of moisture resulting from transpiration.

4. Open bench growth: Plants can be maintained on the open bench for bulk seed production, keeping all lines separated by adequate space. Avoid disturbance of maturing inflorescences. This method is appropriate when growing natural accessions that are late flowering and develop large and dense canopies (e.g., Sij-1, Monte-1, Amel-1, Anholt-1, Appt-1, Bik-1, Bl-1, Do-0).

The simplest procedure is to wait until the entire inflorescence has browned before harvesting. However, some siliques may shatter naturally and seed will be lost. Harvest seeds only after the soil in pots or flats has been allowed to dry. It should be noted that delays in harvesting following physiological maturation of the plant result in seed deterioration, especially under nonoptimal environmental conditions. Seeds from individual siliques can be harvested after the fruits have turned completely yellow, if rapid turnover is required. However, such seeds have high levels of germination inhibitors. Since formation and maturation of siliques occur over time, early siliques can be harvested before later ones mature. Harvest for each of the four isolation methods is as follows:

1. Aracons™: Slide the plastic cylinder off and then cut off the dry inflorescence above the cone device in a threshing sieve.
2. Floral sleeves: While holding the pot, cut away and discard plastic sleeve. Cut the dry inflorescences and place them in a threshing sieve.
3. Plastic bags: Cut the entire plant off at its base. Shake the seeds into the bag; inflorescences can be gently handpressed from the outside, and the seeds will fall to the bottom of the bag. Most of the dry inflorescences can be removed from the bag by hand before seeds are sieved to separate them from chaff.
4. Open bench: Cut off the entire inflorescence at its base, and carefully place into a 4–8 L or larger transparent plastic bag, depending on the size of the bulk of plants.

The major factors influencing seed longevity are (1) genotype; (2) environmental conditions during seed maturation, harvesting, and seed handling; and (3) seed storage conditions. Harvested seeds should be processed promptly (including threshing, cleaning, drying, and packaging) and placed into storage.

Seeds should be threshed when the seed moisture content is approximately 10 %, to minimize seed damage during threshing. This seed moisture content will be reached when all plant material appears to be dry. Hand, rather than machine threshing, is recommended mainly because threshing machines need rigorous cleaning between lines to avoid sample cross-contamination, require very careful adjustment, and do not accommodate the variable size

of *Arabidopsis* seeds well. The hand method is performed as follows:

1. Set a large, clean, white paper on a bench or table for collection of the threshed seeds.
2. Place a clean threshing sieve on top of the paper.
3. Place dry plants directly onto the sieve. If plants are larger than the sieve, they can be cut into pieces that fit the screen.
4. Crush plants using hands to remove all the seeds from siliques. Discard plant material.
5. Sieve seeds through the mesh repeatedly until they are clean and free of chaff. After sieving, the seeds are still likely to be mixed with soil and plant residue. A combination of additional sieving, gentle blowing, and visual inspection can be employed to clean the seeds completely.
6. Clean small samples by hand with the aid of a pointed tool on an opaque glass plate illuminated from below, if needed.
7. Place cleaned seed samples in small labeled manila envelopes or open glass jars to allow seeds to air-dry. Do not use plastic due to static effects.

The ideal moisture content of seeds for storage is 5–6 %. Higher moisture content can cause seed deterioration. There are many methods available for drying seeds. The recommended method is to air-dry the seeds at room temperature and approximately 20 % relative humidity for 1–3 weeks (*see* **Note 27**). Low relative humidity (20–30 %) is necessary for seeds to reach the desired moisture content [9, 10]. Seed moisture content can be determined by several methods [11]. Seed packaging for storage can be accomplished as follows:

1. Use cryovials (with threaded lids and gaskets) for convenient and safe storage. They hold large numbers of seeds, seal tightly, are moisture proof, and can be resealed many times.
2. Label each vial with pertinent information including date of storage.
3. Determine stored seed quantities (approximately 50 μL = 25 mg = 1,250 seeds).

3.3.2 Seed Storage and Preservation

The general conditions for preserving optimal viability of seeds have been well defined [9, 10, 12–14]. Seed storage principles for *Arabidopsis* are similar to those for other plants, with the caveat that the small seeds rehydrate very rapidly if exposed to high humidity. When seeds deteriorate, they lose vigor and eventually the ability to germinate. The rate of this "aging" is determined by interactions of the temperature and moisture content at which seeds are stored, and unknown cellular factors that affect the propensity for damage reactions [9].

Rapid deterioration of seeds has not been observed for the diverse collections currently maintained at ABRC. However, experience regarding the effect of genotype is limited. A large number of genes involved in embryogenesis, reserve accumulation, and seed maturation have been identified. Conspicuously, seeds of the abscisic acid-insensitive mutants fail to degrade chlorophyll during maturation and show no dormancy, leading to low desiccation tolerance and poor longevity [15]. *Arabidopsis* seeds should retain high viability for long storage periods, under proper conditions. With the increase of storage temperature and seed moisture content, the life span of the seeds decreases. Seeds left at room temperature and ambient relative humidity lose viability within approximately 2 years. Seed stored dry at 4 °C or −20 °C should last decades. Below are three storage options for safe seed preservation:

1. For active collections which are accessed often, store seeds at 4 °C and 20–30 % relative humidity. Control of humidity is typically achieved by a dehumidification system in the cold room. Note that the control of relative humidity provides a safety factor in case seed containers are not sealed properly.
2. For long-term or archival storage, the recommended temperature is subzero, preferably −20 °C and also preferably 20 % relative humidity.
3. For open containers such as envelopes, seeds can be stored at 15–16 °C, with a relative humidity maintained very carefully at 15 %. Under this controlled environment, seeds will maintain suitable low moisture content [16]. Storing seeds at relative humidity <15 % will not increase shelf life and may actually accelerate deterioration [10].

When vials are removed from cold storage, condensation of moisture on the seeds and subsequent damage may occur. For vials stored at 4 °C, sealed vials must always be warmed to room temperature before opening. For vials stored at −20 °C, rapid rewarming (placing the sealed vial in a 37 °C water bath for 10 min) is a recognized method to minimize frost damage. If possible, working with seed stocks should take place at low (20–30 %) relative humidity. If accumulation of condensation is suspected, vials should be left open in the dry room until seeds have equilibrated before returning the vials to cold storage.

3.3.3 Seed Quality Control

The purity and physical integrity of seeds and the presence of pests and seed-borne diseases (especially some fungal diseases) can be detected by visual examination with the naked eye, magnifying lenses, or using a dissecting microscope. For a rigorous assessment, spread the seeds on white paper under a well-lit microscope. Generally, gray or white coloration on the seed surface indicates fungal contamination. Discard seeds if possible; otherwise, sterilize seeds with fungicides before planting. Do not discard shriveled,

small, irregular-shaped, and other colored seeds that might correspond to specific mutations, assuming that the seeds were produced under optimal conditions.

Seed viability should be monitored at regular intervals by conducting germination tests under a standard set of conditions. It is recommended that seeds in long-term storage under the optimal preservation standards should be monitored at least every 10 years. Seeds in short-term storage should be monitored at least every 5 years [12, 14].

A germination test for *Arabidopsis* can be conducted in 3–7 days to determine the proportion of seeds in a sample that will produce normal seedlings. Tests should be carried out before seeds are stored, so that poor quality samples can be recognized. *Arabidopsis* seeds may fail to germinate because they are dormant or because they are defective or nonviable. Dormant seeds can be distinguished because they remain firm and in good condition, while nonviable seeds soften and are attacked by fungi. Extending stratification can usually break dormancy (*see* **Note 4**).

Initial germination rate should exceed 80 %, but may be lower for some lines. Mutations in a significant number of genes, mostly involved in biosynthesis and signaling pathways of certain hormones, affect seed germination and/or dormancy. A germination test can be performed as follows:

1. Label the bottom of a 10-cm-diameter Petri plate with name and date.
2. Place two layers of filter paper in the bottom of the plate and moisten with distilled water. Remove excess water.
3. Distribute 100 seeds evenly on the surface of the paper. Seal the plate with Parafilm or clear tape, to prevent drying.
4. Stratify seeds by placing the plates at 4 °C for 3 days.
5. Move the plates to an illuminated shelf or to a growth chamber under standard light and temperature conditions (*see* **Note 28**).
6. Record germination percentage after 3–7 days by dividing the number of seedlings by the total number of seeds and multiplying by 100.

Germination tests can also be performed on solid media, such as MS, described in Subheading 3.1.1.

3.4 Genetic Crosses

Some species of *Arabidopsis*, particularly *A. thaliana*, are mostly self-pollinating, especially in a growth chamber or greenhouse setting where insect populations are minimized [17]. It should be noted that the pollen of *Arabidopsis* does not disperse through the air. Therefore, crossing *Arabidopsis* is mainly conducted through manual emasculation of flowers just prior to flower opening, followed by hand transfer of pollen from the desired male parent to the stigma of the emasculated flower. Although labor intensive,

the manual method remains a reliable technique for achieving cross-pollination.

Species, such as *Arabidopsis halleri* and *Arabidopsis lyrata*, have natural self-incompatibility mechanisms, which prevent the plant from self-pollinating and result in obligate outcrossing [18]. For such species, simple maintenance of a genetic stock cannot easily be accomplished from a single plant, and it is most convenient to start with a small population of founders and perform cross-pollination. The manual techniques for performing genetic crosses of *A. thaliana* can be generalized to the related species. The use of a magnifying visor or dissecting microscope is recommended to visualize floral parts and avoid damage to the pistil. Genetic crosses can be performed as follows:

1. Select the appropriate parent plants. Choose young plants at early stages of flowering. Avoid using the first flowers in the inflorescence, which are usually less fertile, and the smaller flowers produced by mature plants [19] (*see* **Note 29**).
2. Prepare the female parent:
   (a) Select a stem with two to three flower buds, in which the tips of the petals are barely visible and before the anthers begin to deposit pollen on the stigma (*see* **Note 30**).
   (b) Remove siliques, leaves, and any open flowers above and below the selected buds on the chosen stem with a small pair of scissors; avoid damaging the stem.
   (c) Remove the sepals, petals, and all six stamens from the selected flower buds using the precision clamping tweezers, leaving the pistil intact (*see* **Note 31**).
3. Prepare the male parent: Select a newly opened flower with anthers that are dehiscent. These flowers will contain fresh pollen that will contribute to the success of the cross. Remove the flower by squeezing near the pedicel with tweezers.
4. Pollinate the female parent by taking the fully open flower from the male parent and brushing the anthers over the bare stigma of the female parent. Visually confirm that pollen has been deposited on the stigma.
5. Label the crosses, placing tape on the stem of the female plant, noting the male and female parent and the date of the cross.
6. Inspect developing siliques over the next several days. Successful crosses are visible after 3 days when the siliques start elongating. Siliques are ready for harvest once they turn brown, but before they shatter (*see* **Note 32**).
7. Harvest siliques by cutting them with scissors and placing them into a microcentrifuge tube or a small paper envelope.
8. Air-dry seeds at room temperature, preferably at 20–30 % relative humidity, for 1–3 weeks. Thresh seeds if necessary.

3.5 Floral Dip Transformation of Arabidopsis with Agrobacterium tumefaciens

The development of simple and highly efficient stable transformation protocols, without a need for plant regeneration in tissue culture, represented one of the milestones that enabled *Arabidopsis* to become a model that it is today. Transformation of germinating seeds with *Agrobacterium tumefaciens* represented the first breakthrough in this effort [20]. It was followed by a "vacuum infiltration" method, in which *Agrobacterium* was used to infect uprooted flowering plants [21]. This protocol was simplified and streamlined a few years later and became known as the "floral dip" method [22]. In this method, the need for vacuum infiltration was replaced by the use of Silwet L-77®, a surfactant that aids the entry of bacteria into plant tissues. The use of this protocol revolutionized the field of *Arabidopsis* functional genomics, by enabling high-throughput generation of T-DNA mutants and other resources that show stable inheritance of the mutations and other modifications caused by transformation events. Although other methods are still in use in specific cases (e.g., transformation of root explants for transforming sterile mutants [23] or vacuum infiltration for Ler-0 [21]), floral dip has become the most widely used protocol in most of the research labs and for most of the natural accessions (e.g., Col-0, Ws-0, Nd-0, No-0) and will be described here in detail:

1. Grow plants in pots as described in Subheading 3.2 under long-day conditions until bolting (*see* **Note 33**).
2. Remove the first inflorescence stems that bolt to induce growth of secondary shoot inflorescences. Plants will be ready for dipping in 5–7 days.
3. Prepare the starter culture of *Agrobacterium* carrying the construct of interest, by growing the 5-mL culture in LB medium supplemented with appropriate antibiotics at 28 °C for 2 days (*see* **Note 34**). The culture should be started 2–4 days after the first inflorescences have been removed (**step 2**).
4. Use 1 mL of the starter culture to inoculate 200 mL of LB medium supplemented with appropriate antibiotics and grow this large culture for 16–24 h, until the cell growth reaches the stationary phase (*see* **Notes 35** and **36**).
5. Spin down *Agrobacterium* culture at 4,000 × *g* for 10 min at room temperature and resuspend the pellet in 1–2 volumes (200–400 mL) of 5 % sucrose solution (*see* **Note 37**).
6. Immediately before dipping, add the appropriate volume of Silwet L-77® to the *Agrobacterium* cell suspension, to make a final concentration of 0.02–0.05 %; pour the suspension in a beaker.
7. Prepare the plants for dipping by removing the siliques that have already been formed (*see* **Note 38**).

8. Dip the inflorescences for a few seconds by holding the pot with one hand and gently bending the inflorescence shoots to allow them to be completely submerged into the suspension until a film of suspension can be observed on the plants.
9. Moisten paper towel and place it at the bottom of a tray. Lay the pots on their sides in the tray and cover with a lid (*see* **Note 39**).
10. After 1 day, place the pots in their normal upright position and continue growing the plants until they set seeds.
11. Screen the primary transformants on appropriate selection plates.

4 Notes

1. Optional sucrose and vitamins should be added after autoclaving and only after the agar media cools because vitamins are thermolabile and 15–25 % of the sucrose may be hydrolyzed to glucose and fructose at elevated temperatures [24].
2. Plants grow more vigorously and quickly on media containing 1–2 % of sucrose; however, fungal and bacterial contamination must be rigorously avoided by seed sterilization. Note that germination of some mutants might be delayed on sucrose-containing media.
3. Covered plates, boxes, or tubes with solidified agar can be stored for several weeks at 4 °C in a container that prevents desiccation.
4. Most widely used lines have moderate dormancy, and cold treatment, also called stratification, may not be required for germination when planting older seeds of these lines. However, a cold treatment at 4 °C for 3 days will improve the rate and synchrony of germination. The use of an extended cold treatment of approximately 7 days is especially important for freshly harvested seeds, which have more pronounced dormancy. An extended cold treatment is also necessary for certain natural accessions (e.g., Dobra-1, Don-0, Altai-5, Anz-0, Cen-0, WestKar-4). Cold treatment of dry seeds is usually not effective in breaking dormancy.
5. Instead of stratification on plates, seeds suspended in sterile water can also be stratified prior to planting on agar or soil surface.
6. Optimum light intensity is in the range of 120–150 μmol/m^2 s. Higher intensities may result in death of some seedlings, but are tolerated by older plants; purpling of leaves is the first symptom of high-light stress. Very low light intensities may result in weak and chlorotic plants. *Arabidopsis* is a facultative

long-day plant. Plants flower rapidly under continuous light or long-day (>12 h) photoperiods, while under short days (<12 h), flowering is delayed, favoring vegetative growth. Plants grow well under a cycle of 16-h light/8-h dark or under continuous light.

7. Various light sources can be used for optimal plant growth, such as cool-white fluorescent bulbs, incandescent bulbs, very high-output (VHO) lamps, high-intensity discharge (HID) lamps, and shaded sunlight. Cool-white fluorescent bulbs, supplemented by incandescent lighting, are recommended in growth chambers or growth rooms. HID lamps of 400–1,000 W are conventional in greenhouses in temperate climates to supplement the sunlight or prolong the natural photoperiod.
8. The temperature range for *Arabidopsis* growth is 16–25 °C. Lower temperatures are permissible, but higher temperatures are not recommended, especially for germination through early rosette development. Temperatures above 28 °C are better tolerated by more mature plants (past early rosette stage). In general, high temperatures result in a reduced number of leaves, flowers, and seeds. At lower temperatures, growth is slow, favoring the vegetative phase, and flowering is delayed.
9. Thiamine and 2,4-D stock solutions must be added to the media in a laminar flow hood to prevent contamination of stock solutions.
10. Some investigators prefer to use the disposable 250-mL polycarbonate membrane vented-cap flasks that may provide better aeration and result in a better cell growth.
11. Mix the culture well immediately before pipetting, since the cells settle to the bottom of the flask shortly after the orbital shaking has been stopped.
12. The density of cell culture is an important factor for its viability. Too high and too low density can cause cell death and the cessation of cell division, respectively. Adjust the volume of subcultured cells if necessary. If larger clumps of cells are formed, pass the suspension through a sterile 1-mm stainless steel sieve.
13. T87 cell culture can also be propagated and maintained as a callus on solid NT-1 media (on plates or in Magenta® boxes to avoid premature depletion of nutrients). Callus is subcultured once a month by transferring a 1-mm piece to fresh media. Note that growth of the callus after continued passage becomes independent of cytokinin [25].
14. Square pots with a diameter of approximately 5.5 cm can be used to grow one plant, 11-cm-diameter pots are suitable for

growing up to 60 plants, and rectangular flats that are 26 cm × 53 cm can accommodate as many as 200–600 plants grown to maturity. Another option especially suitable for genomic studies is 96-well insets. Higher densities, approximately 3,000 plants per 30 cm^2, can be used if plants are harvested at early stages.

15. Osmocote® 14-14-14 (14 % nitrogen, 14 % phosphate, 14 % potassium) is an extended time-release fertilizer, feeding up to 3 months from planting. Apply in amounts according to the label. Alternatively, nutrient solution can be used to wet the soil [26].
16. Always use clean growth supplies, especially new pots and trays to avoid pest contamination.
17. Prepared pots can be stored in covered trays at 4 °C for several days before planting, although pot preparation and planting should be conducted on the same day if possible.
18. Various methods can be employed to plant seeds. The density of plants varies with genetic circumstances and purpose of the planting. High yields are achieved with 10–20 plants per 11-cm-diameter pot. Generally, low densities increase the yield/plant and are suitable for pure lines. High densities reduce the yield/plant, but are useful when it is necessary to maintain the genetic representation in segregating populations.
19. The plastic wrap should not be allowed to contact the soil surface and should be perforated to provide aeration. If clear plastic domes are used, they should not be tightly sealed.
20. Some winter-annual natural accessions require a period of cold to initiate flowering, a process known as vernalization (e.g., Galdo-1, Monte-1, Cit-0, Dog-4, Istisu-1, Valsi-1, Mir-0, Tamm-2). Young rosettes (2–4 weeks old) of late flowering accessions should be placed at 4 °C for 4–7 weeks to accelerate flowering.
21. Plants should not be overwatered to avoid development of algae, fungi, fungus gnat larvae, and other pests who thrive on overly wet soil. Algae can be manually scraped off and the soil allowed to dry.
22. Preventive application of pesticides is very effective if local regulations allow this and can avert heavy use of chemicals after infestations have developed. Rotation of pesticides is recommended. Biological control agents can also be applied.
23. Marathon® 1G is effective as a preventive insecticide or as treatment following infestation. It can be applied to the soil surface or included in subirrigation watering regime, which reduces damage to the plants.
24. Mature or diseased plants, plant debris, used soil, pots, and other materials can shelter pathogen spores or insects from

former plantings. After removal of the pest and host materials and the sterilization of the growth area, it is very improbable that any pest or pathogen will survive.

25. Read and follow precautionary measures as suggested by the manufacturer of the cold sterilant Spor-Klenz®.
26. Floral sleeves fit snugly around a pot, extend upward, and are wider at the top allowing for expansion of the developing inflorescences. Sleeves made of biaxially oriented polypropylene (BOPP) are very clear, maintain upright stiffness, and tear easily for harvesting. Fold down the tops of the sleeves about 2 cm to ensure they stay open and stable. If plants grow out above the sleeves and are at high plant density, train the top of the plants back down into the sleeve to avoid contamination.
27. The moisture content of *Arabidopsis* seeds stored in open containers corresponds to the room humidity. *Arabidopsis* seeds behave in a similar way to crop seeds with similar chemical composition [12, 13].
28. Environmental conditions for seed germination tests are the same as for growing plants. Two replicates of 100 seeds each provide reliable germination estimates. Cases in which observed germination is <80 % may warrant follow-up testing.
29. Crosses may be performed throughout the duration of the flowering time; however, the crosses will have a higher rate of success during the earlier stages of flowering.
30. Using unopened flowers for the female parent is important in order to avoid self-pollination. Shortly after this stage, stamen/pistil length ratio, as well as the timing of anther dehiscence, favors self-pollination and open flowers have most likely been self-pollinated. All flower candidates for female crossing should be examined for presence of released pollen prior to their use in crossing.
31. If the pistil is damaged, it is highly unlikely that the cross will be successful and the flower should not be used.
32. Siliques should be ready to harvest in about 2–3 weeks after the cross. If siliques are brown, use care, as it is easy to lose all seeds at this stage.
33. Plants can be either grown individually in 5-cm round pots or up to the density of 10–15 plants per 11-cm square pot [27].
34. LB medium can be substituted with Yeast Extract Peptone (YEP) medium to achieve higher *Agrobacterium* density [27].
35. The presence of the construct should be confirmed in the starter culture (e.g., by PCR).
36. The rest of the starter culture can be stored at 4 °C for up to 1 month for future use [28].

37. One to two volumes of sucrose solution used to resuspend the pellet corresponds to the original volume of the large *Agrobacterium* culture. The final OD_{600} of the suspension for dipping should be approximately 0.8.
38. Removing siliques will increase the transformation efficiency.
39. Some investigators prefer using another tray in place of a lid, to avoid the exposure to light and the production of excessive heat around the plants.

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