

Characterizing Jasmonate Regulation of Male Fertility in *Arabidopsis*

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Abstract

Coordination of events leading to fertilization of *Arabidopsis* flowers is tightly regulated, with an essential developmental cue from jasmonates (JAs). JAs coordinate stamen filament elongation, anther dehiscence, and pollen viability at stage 12 of flower development, the stage immediately prior to flower opening. Characterization of JA-biosynthesis and JA-response mutants of *Arabidopsis*, which usually have a complete male sterility phenotype, has contributed to the understanding of how JAs work in these reproductive processes. These mutants have also been fundamental to the identification of JA-dependent genes acting in male reproductive tissues that accomplish fertilization. The list of JA-dependent genes continues to grow, as does the necessity to characterize novel JA mutant and related transgenic plants. It is therefore instructive to place these genes and mutants in the framework of established JA responses. Here, we describe the phenotypic characterization of flowers that fail to respond to the JA signal. We also measure gene expression in male reproductive tissues of flowers with the aim of identifying their role in JA-dependent male fertility.

Key words Jasmonic acid, Flower development, Stamen, Anther, Pollen, Sterility

1 Introduction

Jasmonoyl-isoleucine, the active form of the jasmonate hormone, is an oxylipin signaling molecule derived from α -linolenic acid (18:3). Characterization of the *fad3-2 fad7-2 fad8* mutant, which is defective in the activity of three fatty acid desaturases required for synthesis of this JA precursor, led to the discovery that JA is required for male reproductive processes in *Arabidopsis thaliana* [1]. Other *Arabidopsis* mutants lacking either the ability to synthesize JA or the capacity to perceive JA are male sterile as well [2–6]. Here, we refer collectively to both JA biosynthesis and response/signaling mutants as “JA mutants.” Stamen filaments in these JA mutants do not elongate to place anthers at the stigmatic (female) surface for self-pollination. Furthermore, the anthers do not dehisce and, although pollen develops to the trinucleate stage, it is predominantly nonviable. Whether this nonviability is due to pollen-specific defects or the

inability of anther tissues to provide nutritive support in later stages of pollen development is still an open question.

Flowers in JA mutants develop and mature normally until just before opening, at which point they are designated as “stage 12” [7]. In contrast to the wild-type stage 12 that lasts 24–48 h before the flowers open, flower opening in JA mutants is somewhat delayed and flowers are sterile for the reasons outlined above. Notably, exogenous treatment of stage-12 flower buds on JA biosynthesis mutants with methyl jasmonate (MeJA) rescues this male-sterile phenotype, with full silique development and seed set as a consequence. Importantly, only stage-12 flowers respond to the JA signal, which supports the notion that JA acts as a trigger within this narrow developmental window. Based on previous investigations of JA-responsive genes in stamens during stage 12, three key regulators have been identified that serve as marker genes for JA-dependent male reproductive processes. Among these are two genes encoding transcription factors, *MYB21* (At3g27810) and *MYB24* (At5g40350), which are induced by JA within 30 min of treatment [8]. A third transcription factor-encoding transcript, *MYB108* (At3g06490), begins to accumulate 8 h after JA treatment [9].

Characterization of phenotypes and JA-inducible gene expression patterns in reproductive tissues of wild type and JA mutants continues to provide important clues regarding the role of JAs in *Arabidopsis* flower development. In JA mutants, lack of stamen elongation and anther dehiscence, as well as their nonviable pollen, is accepted as a complete absence of the JA signal in stage-12 flowers. Identification of these mutants and their gene responses has yielded quantifiable aspects of this male-sterile phenotype. A critical tool provided by JA biosynthesis mutants is the ability to synchronize gene expression and developmental events after the JA signal is given [8, 10]. As new JA-responsive genes are discovered, characterization of their corresponding overexpression and knockout lines can help elucidate gene function by fitting sometimes incomplete male-sterile phenotypes into the established framework of the complete JA male-sterile phenotype [9]. Below, we outline methods used to recognize and quantify JA-dependent male sterility in *Arabidopsis* as well as methods for synchronizing and studying gene expression in male reproductive tissues of JA-biosynthetic mutants.

2 Materials

2.1 Characterization of Stamen Elongation

1. Soil (Sunshine Mix #1 [Sun Gro Horticulture, Vancouver, Canada], or other potting soil).
2. Wild-type and JA mutant *Arabidopsis thaliana* (L.) Heyhn. seeds.
3. Growth chamber or greenhouse.
4. Fine-tipped forceps.
5. Crossing goggles (jewelers magnifying goggles).

6. Glass slides.
7. Light microscope equipped with digital camera.
8. Computer with ImageJ software (<http://rsbweb.nih.gov/ij>).

2.2 Characterization of Anther Dehiscence

1. Soil (Sunshine Mix #1, or other potting soil).
2. Wild-type and JA mutant *Arabidopsis* seeds.
3. Growth chamber or greenhouse.
4. Fine-tipped forceps.
5. Crossing goggles.

2.3 Characterization of Pollen Viability

1. Round 5-cm Petri dishes containing fresh pollen-germination medium: 17 % (w/v) sucrose, 1 mM CaCl₂, and 102 mg/L boric acid, pH 7.0, solidified with 0.6 % (w/v) agarose.
2. UV/light microscope equipped with digital camera.
3. Stock solution of 2 mg/mL fluorescein diacetate (Sigma-Aldrich, St. Louis, MO, USA) in acetone. For working solution, add stock solution dropwise to 17 % (w/v) sucrose until the solution becomes milky.
4. Stock solution of 1 mg/mL propidium iodide in water. Working solution is diluted to 100 µl/mL with 17 % (w/v) sucrose.
5. Immediately before experiment, equal amounts of fluorescein diacetate and propidium iodide working solutions are mixed.

2.4 Chemical Treatment of Flower Buds with Methyl Jasmonate

1. Soil (Sunshine Mix #1, or other potting soil).
2. Wild-type and JA mutant *Arabidopsis* plants grown in 4-in. (10-cm) pots.
3. Growth chamber or greenhouse.
4. MeJA (Bedoukian Research Inc., Danbury, CT, USA) supplemented to a final concentration of 0.01–0.03 % in a solution of 0.1 % (v/v) Tween-20. As MeJA does not immediately dissolve when initially added to the 0.1 % Tween-20 solution, it must be mixed with a stir bar until the oily bubbles disappear.

2.5 Collection of Stamens for Measuring Gene Induction by JA

1. Styrofoam float.
2. Fine-tipped forceps.
3. Liquid nitrogen.
4. RNase-free 1.5-mL microcentrifuge tubes.

2.6 Total RNA Preparation

1. RNase-free 1.5-mL microcentrifuge tubes and matching pestles.
2. RNase-free water.
3. TRIzol (Life Technologies, Carlsbad, CA, USA).
4. RNeasy kit (Qiagen, Hilden, Germany).
5. UV/Vis spectrophotometer.

2.7 cDNA Synthesis

1. SuperScriptIII First-strand cDNA synthesis kit (Life Technologies).
2. RNase-free 0.2-mL PCR tubes.
3. Thermal cycler.

2.8 Measurement of JA-Dependent Reproductive Marker Genes by Quantitative PCR

1. First-strand cDNA (*see* Subheading 2.7).
2. qPCR master mix (Invitrogen).
3. 1.5 mM MgCl₂.
4. 0.2 mM dNTPs.
5. SYBR Green mix (Life Technologies).
6. ROX dye (Life Technologies).
7. *Taq* polymerase.
8. Real-time thermal cycler.
9. Gene-specific primers:
 - (a) *MYB21*
 Fw-5'-TAAAACGAACCGGGAAAAAGTT-3'
 Rv-5'-GCGGCCGAATAGTTACCATAG-3'
 - (b) *MYB24*
 Fw-5'-CAAAATGGGGAAATAGGTGGT-3'
 Rv-5'-TCATC TCATCGACGCTCCAATAGTTT-3'
 - (c) *MYB108*
 Fw-5'-AATGGAGAAGGTCGCTGGAACCTCT-3'
 Rv-5'-CGTTGTCCGTTCTTCCCGGTAAAT-3'
 - (d) *ACTIN2*
 Fw-5'-GGTGATGGTGTGTCTCACACTG-3'
 Rv-5'-GAGGTTTCCATCTCCTGCTCGTAG-3'

3 Methods**3.1 Characterization of Stamen Elongation**

1. Grow *Arabidopsis* wild-type and JA mutant plants on soil until plants reach the reproductive phase and produce a primary shoot and flowers.
2. Collect stage-12 flowers (*see* Fig. 1a and Subheading 1) and flowers that are in the process of opening from flower bud clusters on both wild-type and JA mutant plants.
3. With fine-tipped forceps, carefully remove sepal and petal organs from all flowers leaving intact the pistil and all six stamens (two short and four long), including anthers. Use cross-ing goggles to aid with visualization.
4. Arrange the pistils and stamens (anther along with filament) together with a length marker of known size on a glass slide

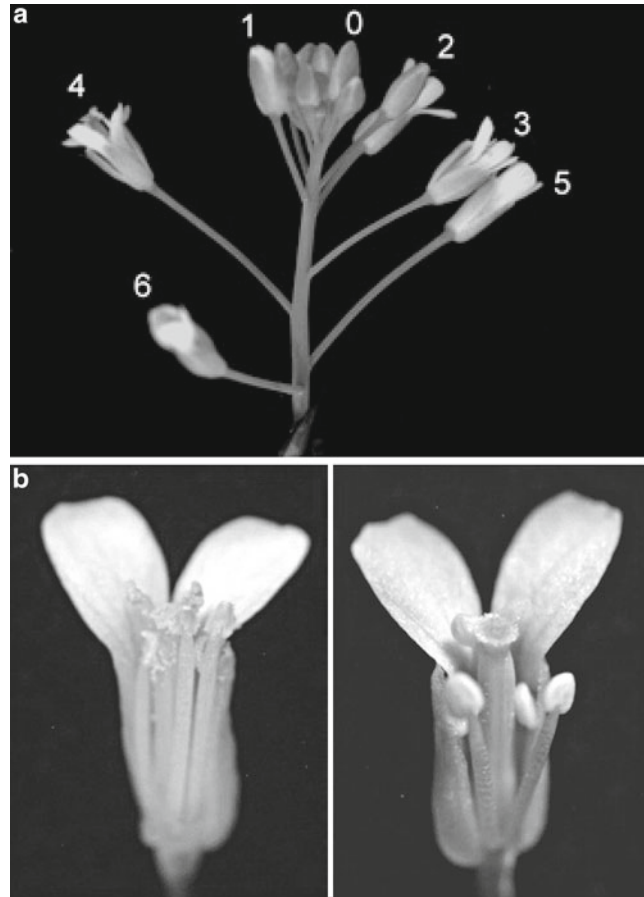


Fig. 1 Flower development and anther dehiscence in *Arabidopsis*. (a) Time course of flower development. The “0” designates stage 12 of development. Numbers continue through the later stages, including fertilization and flower opening (reproduced from [9] with permission © American Society of Plant Biologists; www.plantphysiol.org). (b) Open flowers in the wild type (*left*) and the *opr3* mutant (*right*). In wild-type flowers, stamen filaments elongate to place dehiscent anthers on the stigmatic surface where pollen is deposited. In the *opr3* mutant, stamen filaments do not elongate and anthers do not dehisce so that no pollen is deposited and fertilization does not occur

and view with a microscope. Take a series of digital images of these floral organs, including the length marker.

5. Open images in ImageJ on a computer, calibrate length by using the size marker, and calculate the exact length of all six stamens from the base of the filament to the top of the anther. Likewise, measure the length of the carpel (from the base to the stigma surface) of all the flowers and then calculate the carpel-to-stamen length ratio in the flowers.

3.2 Characterization of Anther Dehiscence

1. Grow *Arabidopsis* wild-type and JA mutant plants on soil until plants reach the reproductive phase and produce a primary shoot and flowers.
2. From a single flower bud cluster, select a set of seven flowers to use as a developmental series as follows. (a) Identify the most mature, but unopened, flower bud, which corresponds to stage 12 of floral development, and designate this as flower “0.” In wild-type plants, this flower typically does not have any dehiscent anthers. Flowers at later stages of development will be farther from the center and/or below the bud cluster and will be in the process of opening. (b) Use flower “0” and the set of six open flowers as a developmental series, starting at stage 12, and designate these flowers “1” to “6” (*see* Fig. 1a). Typically, the first open flowers in the wild type will have six dehiscent anthers.
3. Count the number of dehiscent anthers in each flower from the wild-type and JA mutant plants. In dehiscent anthers, the anther locule folds back and releases pollen. Some of this pollen is deposited on the stigmatic surface. Conversely, anthers that have not dehiscent remain closed and, in the mutant, no pollen is observed on the stigmatic surface (*see* Fig. 1b).
4. Continue the analysis for 3–4 days, or until 50 flowers are analyzed.

3.3 Characterization of Pollen Viability

1. Before starting, make sure that the plates containing pollen germination medium are at room temperature. Harvest pollen from mature flowers by gently using forceps to peel back sepals and petals of flowers that are in the process of opening (*see* **Note 1**). Alternatively, open flowers and anther locules manually by using the forceps. Gently touch the anthers to the surface of the plate to distribute the pollen.
2. Incubate plates in the dark for 16–20 h at room temperature (*see* **Note 1**).
3. Calculate the percentage of pollen germination for wild-type control and mutants as follows. In five or more randomly selected microscope fields, count the number of germinated and ungerminated pollen grains. Use these numbers to calculate the average germination (\pm standard error). Wild-type pollen should have a germination rate greater than 90 %.
4. Pollen viability can also be tested by chemical staining. In this method, place freshly isolated pollen (*see* Subheading 3.3, **step 1**) on a glass microscope slide (*see* **Note 2**).
5. Immediately add fluorescein diacetate/propidium iodide mix dropwise to pollen.
6. Cover pollen with a coverslip and view immediately with the microscope under UV light. Pollen can be viewed immediately after

placing coverslip. This protocol stains viable pollen blue-green and inviable pollen red-brown (*see* **Note 3**). Typically, more than 90 % of the wild-type pollen grains are viable.

3.4 Chemical Treatment of Flower Buds with Methyl Jasmonate

1. Aliquot MeJA solution at room temperature into 2-mL microcentrifuge tubes (*see* **Note 4**).
2. Gently lay plant-containing pots on their side and immerse the primary bud cluster into the MeJA solution for a few seconds (*see* **Note 5**). By laying pots on their side, bending of the shoot is minimized as is handling of the stem and bud cluster. Return pots to their upright position and place back on growth chamber shelf.
3. Observe initial silique elongation 1–2 days after dipping and full elongation up to 3 days after treatment. The rate at which siliques are produced will vary depending on environmental conditions, especially the ambient temperature and photocycle. Healthy flower bud clusters typically contain 2–5 unopened stage-12 flowers, the stage at which this treatment is effective, and, correspondingly, will produce a number of siliques from a single MeJA treatment.

3.5 Stamen Collection for Measuring Gene Induction by JA

1. Grow *Arabidopsis* plants as indicated above (*see* Subheading **3.1, step 1**). Use a JA biosynthesis mutant, such as *opr3*, treated and untreated for a positive and negative JA control, respectively.
2. Treat flower buds with MeJA solution (*see* Subheading **3.4, step 1**) and leave in growth chamber for 30 min, or other duration, as required by the experiment.
3. For stamen collection, prepare the 1.5-mL microcentrifuge tube in a float and place it in liquid nitrogen so that the tube is chilled, but the top is open and accessible.
4. Flowers should be removed one at a time from plants kept in the growth chamber. Remove a single MeJA-treated stage-12 flower bud from the flower bud cluster. Harvest stamens (filaments along with anthers) from one flower before taking another.
5. From the harvested flower, peel back or remove sepals and petals with fine-tipped forceps and expose the stamens. Remove the pistil at the base, which helps increase the yield of male tissues. Lift the stamens from the base with forceps. Dip stamen along with forceps in open 1.5-mL microcentrifuge tubes in liquid nitrogen, making sure that all the stamens are released from the tip of the forceps. Minimize the time between harvest of a flower and transfer of stamens to liquid nitrogen to minimize postharvest changes in gene expression.
6. Continue to harvest flowers one at a time, dissect organs, and harvest stamens until at least 10 mg of tissue is collected, which corresponds to approximately 60 flowers.

7. Alternate treatment and harvest of flowers as needed to ensure that treatment time for each flower is within approximately 5 % of the specified time.
8. Isolate RNA immediately or keep the tissue in liquid nitrogen or at -80°C until further processing.

3.6 Total RNA Isolation

1. Use at least 10 mg of stamen tissue for ease of processing and maximum recovery of RNA. Grind 10–30 mg of stamen (filament + anther) tissue with a plastic pestle in 1.5-mL microcentrifuge tubes in liquid nitrogen.
2. Resuspend the ground tissue in TRIzol according to the manufacturer’s instructions for RNA isolation and use the RNeasy kit for RNA purification.
3. Redissolve RNA in RNase-free water.
4. Measure the RNA concentration and purity with an UV/Vis spectrophotometer. It is desirable for both the 260/230 and 260/280 ratios to be above 1.9.
5. Store RNA at -80°C .

3.7 First-Strand cDNA Synthesis

1. Perform first-strand cDNA synthesis with 2 μg of total RNA with the SuperScript III cDNA synthesis kit according to the manufacturer’s instructions.
2. Set up the first-strand synthesis reaction as follows:

Total RNA (2.0 μg)	<i>n</i> μL
Oligo dT primer	1.0 μL
10 mM dNTPS	1.0 μL
RNase/DNase-free water to make	10.0 μL

3. Incubate in a thermal cycler at 65°C for 5 min, and then place immediately on ice for at least 1 min. Move the contents of the tube to the bottom by brief centrifugation for a few seconds at maximum speed.
4. Add the following to the tube on ice:

10 \times RT buffer	2.0 μL
25 mM MgCl_2	4.0 μL
0.1 M DTT	2.0 μL
RNaseOUT TM	1.0 μL
SuperScript III TM RT	1.0 μL

5. Incubate reaction mix in thermal cycler at 50°C for 50 min.
6. Terminate reaction at 85°C for 5 min and then chill on ice.
7. Store the first-strand cDNA synthesis reaction at -20°C , or proceed directly to quantitative PCR.

3.8 Measurement of JA-Dependent Reproductive Marker Genes by Quantitative PCR

1. Run each reaction in triplicate and use three biological replicates. Use *ACTIN2* as the normalizing gene, and an untreated JA mutant sample as a control.
2. Set up 20- μ L reactions for JA-treated and control samples as follows:
 - 2 μ L of diluted first-strand cDNA.
 - 2.0 μ L 1 \times PCR buffer.
 - 1.5 mM MgCl₂.
 - 0.2 mM dNTPs.
 - 0.2 μ M of each sequence-specific primer.
 - 0.6 μ L of ROX dye (diluted to 1:500).
 - 10 μ L SYBR Green mix with *Taq* polymerase.Reaction conditions are as follows: Denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, at 55 °C for 30 s, and at 72 °C for 30 s. At the end of the run, perform a melt curve analysis of the PCR products.
3. Collect C_T values for the samples and calculate the relative expression of the JA-inducible genes using $\Delta\Delta C_T$ values (*see Note 6*).

4 Notes

1. Pollen germination assays have a reputation for being highly variable and temperature is a major factor in determining viability and reproducibility [11]. Therefore, we suggest that incubation at room temperature is monitored closely, because the building temperature can vary with the time of day. Alternatively, incubate in a temperature-controlled environment.
2. The ideal time to harvest pollen is when stamens have elongated to the point that anthers are placed just level with the stigmatic surface, but have been dehiscent for less than 1 day. If grown in a photoperiod, morning is the best time to harvest pollen.
3. Fluorescein diacetate is taken up by living cells and converted to fluorescein, which emits blue-green light under UV irradiation [12]. Propidium iodide is excluded from living cells, but labels dead cells with red-orange fluorescence under UV irradiation [13].
4. Fresh MeJA solution is best, but it can be stored at 4 °C. When stored solution is utilized, let it warm up to growth chamber temperature before use. Depending on the topic of study,

other JAs or JA precursors may be employed as chemical treatment. For example, 12-oxo phytodienoic acid (Cayman Chemical Company, Ann Arbor, MI, USA) and fatty acids (Nuchek, Elysian, MN, USA) have been used to rescue fertility.

5. Alternative methods used to treat flower buds include (a) spraying with an atomizer or spritzer bottle, or (b) painting. Instead of treating large batches of plants, dipping is the most effective and reproducible method. It is critical to consider the presence and location of plants in the same growth chamber meant to stay untreated. MeJA is volatile and, if present at high enough concentrations, can trigger fertility in untreated JA-biosynthetic mutant plants. Therefore, we favor dipping of flower buds over spraying, because application is much more controlled and plants can be kept in the growth chamber during treatment. Furthermore, by dipping specific flower buds (i.e., those on the primary bud cluster), treatment is more uniform. Healthy, approximately 1-month-old plants respond best to treatment, whereas overly mature and stressed plants will yield lower silique/seed production and the floral tissue will become necrotic. Another advantage of dipping over spraying is that the potentially harmful effects of MeJA are kept off the vegetative tissues. Damage may also occur when the MeJA concentration is too high, which is often the case when flower buds appear red around the base (anthocyanins). Although the outcome remains apparently unchanged, it may be a sign that the upper end of the useful concentration range is being approached.
6. This method assumes that both target and reference genes are amplified with near 100 % efficiency. Perform dilution series with cDNA and each primer pair to ensure that reactions have near 100 % amplification efficiency. Relative quantification of experimental samples is compared to the calibrator or the untreated control. Here, the experimental sample and the control are JA-treated and untreated *opr3* stamens, respectively. JA-inducible genes used are *MYB21*, *MYB24*, and *MYB108*, and *ACTIN* is used as a reference gene. Calculate the ΔC_T value for both the experimental and calibrator samples by subtracting the reference C_T from the target C_T for each (i.e., ΔC_T (experimental) = $C_{T(MYB21)} - C_{T(ACTIN)}$ and ΔC_T (calibrator) = $C_{T(MYB21)} - C_{T(ACTIN)}$). Then, calculate the $\Delta\Delta C_T$ by normalizing the ΔC_T of the experimental sample to the ΔC_T of the calibrator ($\Delta\Delta C_T = C_{T(experimental)} - C_{T(calibrator)}$). Finally, calculate the fold-change (normalized expression = $2^{-\Delta\Delta C_T}$).

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