Chapter 4

Bioassays for Assessing Jasmonate-Dependent Defenses Triggered by Pathogens, Herbivorous Insects, or Beneficial Rhizobacteria

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Abstract

Jasmonates, together with other plant hormones, are important orchestrators of the plant immune system. The different hormone-controlled signaling pathways cross-communicate in an antagonistic or a synergistic manner, providing the plant with a powerful capacity to finely regulate its immune response. Jasmonic acid (JA) signaling is required for plant resistance to harmful organisms, such as necrotrophic pathogens and herbivorous insects. Furthermore, JA signaling is essential in interactions of plants with beneficial microbes that induce systemic resistance to pathogens and insects. The role of JA signaling components in plant immunity can be studied by performing bioassays with different interacting organisms. Determination of the level of resistance and the induction of defense responses in plants with altered JA components, through mutation or ectopic expression, will unveil novel mechanisms of JA signaling. We provide detailed protocols of bioassays with the model plant *Arabidopsis thaliana* challenged with the pathogens *Botrytis cinerea* and *Pseudomonas syringae*, the insect herbivore *Pieris rapae*, and the beneficial microbe *Pseudomonas fluorescens*. In addition, we describe pharmacological assays to study the modulation of JA-regulated responses by exogenous application of combinations of hormones, because a simultaneous rise in hormone levels occurs during interaction of plants with other organisms.

Key words *Arabidopsis thaliana*, SA, JA, Plant hormones, Bioassay, ISR, Herbivorous insect, Plant immunity

1 Introduction

The use of bioassays to study the effects of treatments on the resistance level of plants against an attacker has been recorded for the first time at the beginning of the twentieth century [1, 2]. Numerous examples were described in which plants were protected against pathogen infection after pretreatment with (attenuated) pathogens or extracts obtained from pathogens [1, 2]. In nature, plants encounter a plethora of harmful and beneficial organisms, including

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bacteria, fungi, oomycetes, viruses, nematodes, and insects. Each of these interacting organisms exploits highly specialized features to establish an intimate relationship with its host plant.

The plant responds differently to various types of ingression by interacting organisms through changes in levels of and sensitivity to plant hormones. Plant hormones play an important role in the organization of the immune signaling network that induces defense responses. The hormones jasmonic acid (JA) and salicylic acid (SA) are recognized as major players in plant immune signaling, whereas other hormones have modulating roles in the JA- and SA-controlled responses [3]. JA-regulated defenses triggered by wounding control resistance to insect herbivores [4] and also to pathogens with a necrotrophic lifestyle. These pathogens first kill the cells and then live on the contents [5]. Here, we describe bioassays with *Arabidopsis thaliana* and the JA-controlled necrotrophic pathogen *Botrytis cinerea* and the herbivorous insect *Pieris rapae*. Biotrophic pathogens, such as *Hyaloperonospora arabidopsidis*, that keep the host cells alive and retrieve nutrients by forming specialized feeding structures (haustoria), are controlled by SA-regulated defense responses [5]. Some plant pathogens display both necrotrophic and biotrophic lifestyles, depending on the stage of their life cycle, and are called hemi-biotrophs. The chapter also provides a description of a bioassay with the hemi-biotrophic bacterial pathogen *Pseudomonas syringae*.

Beneficial soil-borne microorganisms, such as mycorrhizal fungi and plant growth-promoting rhizobacteria, can cause induced systemic resistance (ISR) in distant plant parts [6, 7]. During ISR, a mild, but effective, immune response is activated in systemic tissues that in many cases is regulated by JA-dependent signaling pathways. ISR is associated with priming for accelerated JA-dependent defense gene expression rather than with direct activation of defense responses, and is predominantly effective against a broad spectrum of pathogens and insects that are sensitive to JA-controlled defenses [8, 9]. In *Arabidopsis,* ISR triggered by the rhizosphere-colonizing bacterium *Pseudomonas fluorescens* WCS417 is well studied and bioassays to assess WCS417-ISR are described in this chapter.

In recent years, molecular, genetic, and genomic tools have been used to uncover the complexity of the hormone-regulated induction of the defense signaling network. Besides balancing of the relative abundance of different hormones, intensive interplay between hormone signaling pathways has emerged as an important regulatory mechanism by which the plant is able to tailor its immune response to the type of invader encountered [10, 11]. For example, resistance of *Arabidopsis* to *P.* was shown to depend on activation of SA signaling, and was associated with suppression of JA signaling [12, 13]. JA-dependent resistance to the necrotrophic fungal pathogen *B. cinerea* was found to be synergized by ethylene, but antagonized by abscisic acid (ABA) [14, 15]; opposite

effects of ethylene (antagonistic) and ABA (synergistic) on JA-dependent resistance against insect herbivores were reported [16, 17]. Pharmacological assays in which hormones are applied to the plant have further elucidated some of the molecular mechanisms involved in the communication between different hormone signaling pathways [3, 18–22]. Modulation of JA signaling by other hormones has been reported to occur by interfering with the function of certain JA signaling components, such as the transcription factor MYC2 in the case of ethylene and ABA, but their exact influence on MYC2 is still not clear [14, 23]. For the antagonistic effect of SA on JA signaling, the JA-regulated transcription factor ORA59 has been suggested as target [20]. Pharmacological assays with combinations of defense-related hormones are described in this chapter.

Despite its unquestioned role in the plant's immunity, many aspects of JA signaling are still unresolved. The use of proper bioassays and pharmacological assays, as described here, will help us to piece the JA puzzle together.

2 Materials

2.1 Equipment

1. Growth chambers set at 21 °C, 70 % relative humidity, and 10-h/14-h day/night regime with a light intensity during the day of 200 μE/m^2/s provided by bulb HPI lamps (Philips, Eindhoven, The Netherlands) or LuxLine plus F58W/840 cool white tube lamps (Havells Sylvania, London, UK).
2. Large autoclave (50 L) and autoclavable plastic bags (40 × 60 cm).
3. Containers (30–50 L) for mixing water and solutions through soil.
4. Sieved potting soil mixed with river sand (12:5 v/v).
5. Small trays (100–500 mL; 4 cm high) for seedling cultivation.
6. Pots (60 mL) with holes in the bottom for plant cultivation after the seedling stage.
7. Small 5-cm Petri dishes.
8. Trays (approximately 45 × 30 × 8 cm) to contain small trays or pots that can be covered with transparent lids to achieve 100 % relative humidity.
9. Tweezers with curved beak tip.
10. Table centrifuge.
11. Spectrophotometer.
12. Incubator set at 22 °C, 10-h day/14-h night, Philips TL-D 36 W/33 lamps for fungus growth or at 28 °C for bacterial growth.
13. Hemocytometer.

14. Light microscope.
15. Empty pipette tip box.
16. Needleless 1-mL syringe.
17. One-hole puncher (diameter 6 mm from an office supplier).
18. 96-Deep-well microplate (96-well format boxes containing 12 disposable 8-strip tubes and caps (Greiner Bio-one, Frickenhausen, Germany)).
19. Stainless steel beads (diameter 2.3 mm).
20. Orbital shaker at 28 °C.
21. Plate shaker MM301 (Retsch, Haan, Germany) or a regular paint shaker.
22. 8-Channel pipette (10, 20, 180 μL).
23. 96-Well dilution plates (≥200 μL).
24. Fine paintbrush.
25. A desiccator or other device that can be air-tightly closed.

2.2 Buffers, Media, and Solutions

1. Seeds of *Arabidopsis thaliana* (L.) Heynh.
2. 0.1 % (w/v) agar.

2.2.1 Arabidopsis thaliana Cultivation

3. Half-strength, modified Hoagland nutrient solution: 2 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, trace elements, pH 7 [24], 10 μM Fe-ethylenediamine-di[*o*-hydroxyphenylacetic acid] (Sequestreen; Ciba-Geigy, Basel, Switzerland) (*see* **Note 1**).
4. Plant labels.

2.2.2 Pseudomonas fluorescens ISR Bioassay

1. *P. fluorescens* strain WCS417 [25] or any other biocontrol pseudomonad strain (stocks stored in 25 % glycerol at −80 °C).
2. King's B (KB) medium agar [26]: 20 g proteose peptone no. 3 (Difco™ BD Diagnostics, Franklin Lakes, NJ, USA), 10 g glycerol, 1.5 g $MgSO_4$, 1.2 g KH_2PO_4 per liter demineralized water supplemented with 13 g of granulated agar (Difco™) for the solid medium in Petri dishes (*see* **Note 2**).
3. Sterilized 10 mM $MgSO_4$.

2.2.3 Botrytis cinerea Bioassay

1. Pathogen *B. cinerea* isolate B0510 (stocks stored in 25 % glycerol at −80 °C).
2. Half-strength potato dextrose broth (PDB; Difco™).
3. Half-strength potato dextrose agar (PDA; Difco™), supplemented with 0.75 % granulated agar (Difco™) to obtain a final concentration of 1.5 % agar.

2.2.4 Pseudomonas syringae Bioassay

1. *P. syringae* pv. *tomato* DC3000 [27] or another virulent *P. syringae* strain (stocks stored in 25 % glycerol at −80 °C).
2. KB liquid medium and KB agar supplemented with 25 mg/mL rifampicin to select DC3000 (*see* Subheading 2.2.2 and **Note 2**).
3. Sterilized 100-mL Erlenmeyer flasks with cotton plugs containing 25 mL of liquid KB.
4. Sterilized 10 mM $MgSO_4$.
5. Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands).

2.2.5 Pieris rapae Two-Choice Bioassay

1. First-instar (L1) larvae of *P. rapae*. Request the caterpillars from a collaborator or use caterpillars of your own collection (*see* **Note 3**).
2. *Brassica oleracea* (white cabbage) or *Brassica campestris* (Chinese cabbage) as food sources for the caterpillars.
3. *Lantana* sp. (shrub verbena) plants that supply nectar to the butterflies.

2.2.6 Combinatorial Hormone Application Pharmacological Assay

1. SA (Mallinckrodt Baker, Deventer, The Netherlands) or sodium salt SA (Na-SA; Sigma-Aldrich, St. Louis, MO, USA) (*see* **Note 4**).
2. Methyl jasmonate (MeJA; Brunschwig Chemie, Amsterdam, The Netherlands) (*see* **Note 5**).
3. 96 % Ethanol.
4. Silwet L-77 (Van Meeuwen).
5. Optionally, 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma-Aldrich).
6. For plate assays with seedlings, Murashige and Skoog (MS) medium supplemented with vitamins (pH 5.7; Duchefa, Haarlem, The Netherlands), 5 % sucrose, and plant agar (0.85 %; Duchefa) in 10 × 10 cm square plates.
7. For liquid assays with seedlings, MES buffer (5 mM 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES), 1 mM KCl, pH 5.7) in 24-well plates.
8. For seed surface sterilization: HCl (37 %), household chlorine (original Glorix; Unilever, London, UK).

3 Methods

The introduction of microbes and insects by plant pathologists and entomologists in the plant growth facilities is harmless. The described experiments, with the exception of those with caterpillars, can be done in close proximity to other plant experiments without the risk for cross-contamination.

3.1 Arabidopsis Cultivation

1. Suspend *Arabidopsis* seeds (3× more than the number of plants needed; 100 seeds weigh approximately 1.5 mg) in 0.1 % agar in 1.5- or 15-mL tubes and imbibe at 4 °C for 2–4 days (*see* **Note 6**).
2. Autoclave (moist) river sand in (double) plastic bags (with 5–10 kg sand) for 20 min at 121 °C.
3. Autoclave (moist) potting soil:river sand mixture (12:5) in (double) plastic bags (with 5–10 kg mix) for 1 h at 121 °C. Repeat the next day.
4. Add half-strength modified Hoagland nutrient solution to the sand (250 mL/kg) and supply water until sand is nearly saturated with fluid.
5. Fill up the 4 cm high small trays (100–500 mL) with the sand.
6. With a Pasteur pipette, distribute the seeds (in 0.1 % agar) evenly onto the sand (60 seeds/25 cm^2).
7. Place the sown trays in a large tray covered with a transparent lid (to achieve 100 % relative humidity) and place in a growth chamber for 12 days.
8. In a large container, mix the autoclaved soil mixture with Hoagland nutrient solution (50 mL/kg).
9. Supply water if needed: a filled 60-mL pot should weigh 75 g.
10. Fill 60-mL pots with holes in the bottom with the soil mix, push slightly on the soil top for firmness, and make one hole in the middle of the soil with the conical end of a 15-mL tube.
11. Place the pots on small Petri dishes that function as saucers to allow individual water/nutrient supply and to prevent cross-contamination between different treatments (*see* **Note 7**).
12. Flood the small trays containing 12-day-old seedlings in sand with water and use tweezers to gently transfer single seedlings from the sand to the planting holes in the potting soil.
13. Close the planting hole lightly by pushing the soil back around the root, leaving the above-ground plant parts free of soil.
14. Stick a color plant label in every pot for genotype/treatment indication.
15. Place the seedling-containing pots in a randomized order in large plant trays (30 plants/tray).
16. Cover the trays with transparent lids for 2 days, after which they are removed.
17. Every other day, water the plants with approximately 10 mL per pot during the first 10 days, and up to 20 mL at later growth stages.
18. Once a week, give the plants 10 mL of Hoagland solution (*see* **Notes 7** and **8**).

3.2 Pseudomonas fluorescens ISR Bioassay

1. Start a culture of *P. fluorescens* strain WCS417 or another biocontrol pseudomonad strain by inoculating bacteria from a glycerol stock on two KB agar plates and incubating them for 1 day at 28 °C (*see* **Note 9**).
2. Harvest the bacteria by scraping them off the plates in 10 mM $MgSO_4$.
3. Wash the bacterial cells by spinning down in Eppendorf tubes at 1,500 × *g* for 5 min in a table centrifuge and resuspend in 10 mM $MgSO_4$.
4. Measure the density of the bacterial suspension in a spectrophotometer at the optical density (OD) 660 nm (1 = 10^9 cells/mL).
5. Mix 50 mL of 10^9 colony-forming units (cfu)/mL per kg of soil to obtain 5×10^7 cfu/kg, whereas the control treatment receives 50 mL of 10 mM $MgSO_4$ per kg of soil.
6. Proceed with the plant cultivation (*see* Subheading 3.1) and treat with pathogens/insects/hormones as described below.

3.3 Botrytis cinerea Bioassay

1. To determine the level of disease resistance to *B. cinerea*, use 20 plants per genotype/treatment (*see* **Note 10**).
2. For gene expression analysis, harvest ten inoculated leaves in triplicate of a total of ten plants per time point (e.g., $t = 0$, 1, and 2 days after inoculation) (*see* **Note 10**).
3. Start a culture of *B. cinerea* by inoculating conidia from a glycerol stock on half-strength PDA plates and incubate them for 2 weeks at 22 °C under a 10-h day/14-h night regime.
4. Around 1:00 p.m., harvest conidia by scraping them off the plates in half-strength PDB.
5. Filter the suspension through glass wool.
6. Measure the conidial density in a hemocytometer with a light microscope.
7. Dilute the suspension with PDB to a final concentration of 5×10^5 conidia/mL.
8. Leave the conidia in PDB for 2 h at room temperature.
9. Around 3:00 p.m., inoculate the plants by pipetting a 5-μL droplet of the conidial suspension on approximately five fully grown leaves per plant (*see* **Note 11**).
10. Place two wet towels in the plant trays and tape-shut transparent lids to the trays to create 100 % relative humidity.
11. Record disease symptoms at 3–7 days after inoculation and categorize them in different disease severity classes depending on the size and appearance of the lesions (Fig. 1) (*see* **Note 12**).
12. Determine the percentage of leaves per plant falling in each disease class and by means of the Chi-square test, whether the

Fig. 1 Classification of disease symptoms caused by infection with *B. cinerea*. From *left* to *right*: Stage I, lesion 2 mm; stage II, lesion 2 mm + chlorosis; stage III, lesion 2–4 mm + chlorosis; stage IV, lesion > 4 mm + chlorosis

distribution between the different classes differs between genotypes/treatments.

13. Determine the number of *in planta*-formed spores on *B. cinerea*-infected leaves in three pools of 16 inoculated leaves of four plants per genotype/treatment.
14. Shake the leaves vigorously in a test tube containing 10 mL of water to release the spores from the leaf surface.
15. Use tweezers to remove the leaves, centrifuge the remaining spore suspension at 200 × *g* for 10 min, and resuspend the spores in 500 μL of water.
16. Count the spores in a hemocytometer with a light microscope.
17. Log-transform the data and perform a Tukey's honestly significant difference test to analyze the differences between genotypes/treatments.

3.4 Pseudomonas syringae Bioassay

Basically, the resistance level against *P. syringae* can be determined by two different inoculation methods: (a) dipping and (b) pressure infiltration of the leaves with the bacterial suspension. By dipping, the bacteria enter through the stomata and start colonizing the leaves from there, whereas by infiltration the bacteria are immediately present everywhere in the apoplast of the infiltrated area. The dipping method is commonly used in ISR bioassays, whereas the infiltration method is used in most other experiments with *P. syringae*.

1. Use 20 plants per genotype/treatment for the dipping bioassay and 10–20 plants per treatment for the infiltration bioassay (*see* **Notes 10** and **13**).

2. For gene expression analysis, harvest ten inoculated leaves in triplicate of a total of ten plants per time point (e.g., $t = 0$, 6, and 24 h after inoculation) (*see* **Note 10**).
3. At around 4:00 p.m., start a culture of *P. syringae* by inoculating bacteria from a glycerol stock in an Erlenmeyer flask containing liquid KB and incubate overnight at 28 °C in an orbital shaker (225 rpm).
4. The next morning, wash the bacterial cells by spinning them down in Eppendorf tubes at $1{,}500 \times g$ for 5 min in a table centrifuge and resuspend them in 10 mM $MgSO_4$.
5. Measure the density of the bacterial suspension in a spectrophotometer at OD_{660} ($1 = 10^9$ cells/mL).
6. For the dipping bioassay, dilute the bacteria in $MgSO_4$ until 2.5×10^7 cfu/mL and amend with Silwet L-77 to 0.02 % (v/v) to facilitate entry of the bacteria into the leaves. For the infiltration assay, dilute the bacteria to $OD_{660} = 0.0005$ for bioassays and to $OD_{660} = 0.005$ (thus tenfold higher) for gene expression analyses (*see* **Note 14**).
7. Proceed with **steps 8** and **12** for the dipping and infiltration assay, respectively.
8. For dipping, turn the plant in the pot upside down in the bacterial suspension, so that all the leaves are immersed, for 3 s (*see* **Note 15**).
9. Refresh the inoculum at least once every 30 plants and use separate boxes for differently pretreated plants to prevent cross-contamination.
10. After inoculation, place the transparent lids on the plant trays.
11. After 4 days, score the percentage of leaves with disease symptoms (presence of water-soaked lesions and chlorosis) per plant and analyze the differences between genotypes/treatments with the Tukey's honestly significant difference test.
12. For pressure infiltration, gently turn the leaf so that its adaxial side is pressed on the index finger and gently press the plunger of a needleless 1-mL syringe firmly placed on the abaxial side to release the bacterial suspension into the leaf.
13. First, indicate with a marker pen on the petioles which leaves will be infiltrated (*see* **Note 11**).
14. After 3 days, determine the disease symptoms (*see* Subheading 3.4, **step 11**).
15. Determine the bacterial growth *in planta* by analyzing eight samples containing two leaf discs of two leaves of one plant, which are collected in 96-deep-well plates containing two beads per well (*see* **Notes 16** and **17**).

16. After all the samples for a time point are collected, add 400 μL of 10 mM $MgSO_4$ to each sample with a multichannel pipette and homogenize the tissue in a plate shaker.
17. Make dilution series in 96-well dilution plates by pipetting 20 μL of homogenate into 180 μL of 10 mM $MgSO_4$ (*see* **Note 18**).
18. Plate the serial dilutions on KB agar containing 25 mg/mL rifampicin to select for *P. syringae* pv. *tomato* DC3000.
19. For high-throughput plating, split the plate into two with a stripe on the back of the plate and streak 2.5-cm lines of 10 μL of a dilution of 8 samples with an 8-channel pipette (one treatment) on one half and repeat on the other half of the plate (*see* **Note 19**).
20. Incubate for 2 days at 28 °C and count the cfu.
21. From these data, calculate the ^{10}log-transformed cfu/cm^2 leaf surface area and subject to the Tukey's honestly significant difference test to analyze differences between genotypes/treatments.

3.5 Pieris rapae Two-Choice Bioassay

The caterpillars of *P. rapae* (small cabbage white butterfly) are specialists on cabbage plants and because *Arabidopsis* is also a member of the Cruciferaceae, they can also feed on *Arabidopsis*. As specialists, their performance is hardly influenced by activation of JA-dependent responses, but when given a choice, they prefer to feed on plants that express the ERF branch of the JA signaling pathway that is controlled by the ERF transcription factor ORA59 rather than be deterred by induction of the MYC branch [17]. In case of two-choice assays, the preference of the caterpillars for either one of two genotypes or treatments is tested.

1. For the two-choice bioassay, place four 6-week-old plants, two of each genotype/treatment, close together so that the leaves overlap and the caterpillars can move from one plant to the other (*see* **Note 20**).
2. Create an empty space of at least 30 cm between each plant arena to prevent crossing-over of the caterpillars.
3. To get reliable data, test the choice of the caterpillars in at least 20 arenas.
4. For gene expression analysis, plants can grow in the usual (no-choice) setup.
5. Harvest ten infested leaves in triplicate per genotype/treatment of a total of ten plants per time point (e.g., $t = 0$, 6, 24, 48 h).
6. Collect L1 larvae from the insect-rearing facility by cutting leaves from cabbage plants harboring caterpillars that are 1–2 days old (*see* **Note 21**).
7. Using a fine paintbrush, place two caterpillars on each plant so that in each plant arena eight caterpillars are released.

8. For two-choice assays, allow the caterpillars to feed for 4 days.
9. For gene expression analyses, remove the caterpillars from the plants with a paintbrush, after 24 h of feeding.
10. Cut through the hypocotyl and inspect the rosette carefully to monitor the presence of the caterpillars on the different plant genotypes/treatments in each arena (*see* **Note 22**).
11. Calculate the frequency distribution of the caterpillars over the different genotypes/treatments per two-choice arena and test for statistical difference from a 50 % distribution (equal choice) using the Student's *t*-test.

3.6 Combinatorial Hormone Application Pharmacological Assay

Preparation and application of the hormonal solutions is the same for combinatorial pharmacological assays as for hormonal induction treatments in bioassays with interacting organisms. In most of the hormone combination assays with SA and JA, we use 5-week-old soil-grown plants that are dipped in combinatorial hormonal solutions, but sterile, plate-grown or liquid medium-grown seedlings can be assayed for SA/JA cross talk as well. Usually, in hormone dipping assays, 1 mM SA and 100 μM MeJA are applied to study cross-communication between hormone signaling pathways by means of their effect on gene expression 24 h after treatment. However, other experimental scenarios are suitable as well, because the antagonistic effect of SA on JA signaling is apparent when SA is supplied up to 30 h before the MeJA application and the SA/JA cross talk effects last for at least 96 h [19]. Moreover, SA concentrations as low as 0.1 μM suffice to antagonize the JA-induced signaling.

3.6.1 Soil-Grown Plants

1. To determine the effect of SA and MeJA on each other's action (such as induction of gene expression), use 30 plants per treatment in a dipping assay that allows for sampling at $t = 0$ and $t = 24$ h (*see* **Note 10**).
2. Prepare SA and MeJA solutions (*see* **Notes 4** and **5**). For dipping, add Silwet L-77 to a final concentration of 0.015 % to facilitate entry into the leaves.
3. For the dipping assay, follow instructions as described in Subheading 3.4, **step 8**, except that lids on the trays are not fully closed, but cracked (*see* **Notes 14** and **23**).

3.6.2 Sterile-Grown Seedlings

1. Put <200 seeds in an open Eppendorf tube and place in a desiccator together with a 200-mL beaker containing 97 mL of HCl.
2. Add briefly 3 mL of chlorine to the HCl and mix with a pipette, immediately followed by closure of the desiccator with its lid (*see* **Note 24**).
3. Take out the seeds after 3 h and transfer the seeds to MS plates.

4. Imbibe the seeds for 2 days at 4 °C, after which the sown plates are placed vertically in a growth chamber for 12 days (*see* **Note 25**).
5. Transfer the seedlings either to fresh MS agar medium supplemented with 0.5 mM SA, 20 μM MeJA, or a combination of both chemicals or to 1.5 mL of liquid MES buffer medium in 24-well plates (5 seedlings per well), where they are left to acclimatize for 1 day before addition of SA and MeJA at the final concentrations of 0.5 mM and 100 μM, respectively (*see* **Note 26**).

4 Notes

1. We make 20× concentrated stock solutions and store them at room temperature. Prepare a working solution by filling a 50-L tap can with 25 L tap water; add 25 mL of the stock solutions; fill the can up to 50 L with tap water. Mix solution well. To avoid algae growth, place a black bin over the can.
2. To make 1 L of KB, place a 2-L Erlenmeyer flask containing 500 mL demineralized water and a magnet on a magnetic stirrer. To avoid precipitation, add all the ingredients one by one and dissolve completely before adding the next compound. Adjust with water to obtain 1 L and pour into bottles for autoclaving. Agar has to be added to the bottle and not to the Erlenmeyer because it will not dissolve without heating.
3. Keeping an in-house colony of *P. rapae* is laborious and demands large temperature-controlled growth facilities, as one chamber is used for rearing of *P. rapae* and another to cultivate the plants needed for the rearing of *P. rapae* (see Subheading 2.2.5).
4. SA is acidic and should be buffered to neutral pH. As at a (common) 1 mM SA concentration, the buffering capacity of tap water suffices, we usually prepare SA solutions in tap water. Whereas Na-SA readily dissolves in water, SA does not and has to be boiled. Stock solutions of 100 mM SA can be stored at room temperature, but boiling is required to dissolve the precipitated SA. SA (stock) solutions of a low concentration appear to lose their defense-inducing activity when stored.
5. MeJA is available as a 4.46 M solution. Make a 1,000-fold concentrated stock in 96 % ethanol by adding 10 μL MeJA to 436 μL ethanol, resulting in a 100 mM MeJA solution. To all the solutions without MeJA, a similar volume of ethanol is added (0.1 %).
6. The 0.1 % agar prevents the seeds from sinking to the tube bottom and allows an equal distribution of the seeds in the solution.

7. Water and nutrients are supplied via the saucers by using a bottle dispenser fused to rubber tubing. This prevents contamination between pots and allows the fluids to stream from the bottom up and not from the top down.
8. Assays as described in this chapter can usually be performed when the plants are 5 weeks old.
9. They grow also at room temperature, albeit more slowly.
10. Plants are 5 weeks old at the time of inoculation.
11. Be careful to select leaves that are younger than leaf four, because the round-shaped older leaves tend to be very susceptible, irrespective of the genetic background or treatment of the plant.
12. The rate of disease progression and also the symptom appearance can differ between experiments and, thus, the day of symptom scoring and the criteria of the disease classes might have to be adjusted accordingly.
13. Ten plants are needed when only one time point is harvested and more plants are needed for multiple time points to determine bacterial titer.
14. Inoculation with *P. syringae* and treatment with SA preferably take place in the morning, because then SA-dependent signaling is activated stronger and the difference in disease level between resistant and susceptible plants is greater.
15. A pipette tip box can be used to contain the suspension.
16. The infiltration method is usually coupled with assessment of *in planta* bacterial growth, which is a highly valuable but also laborious method. Therefore, we have tried to automate it as much as possible. A one-hole puncher significantly speeds up the process of cutting leaf discs compared to the classical cork borer.
17. When samples are taken at $t=0$ to determine the number of bacteria that entered the leaves (in practice 1 h after inoculation), then the leaves need to be washed briefly (3 s) in 70 % ethanol and subsequently rinsed with water and dried with a tissue, before leaf discs can be sampled.
18. The $t=0$ samples are diluted 10×; the $t=3$ samples are diluted 10,000×, but when plants are very susceptible 1,000,000× dilutions can be needed.
19. This way, if you plate three dilutions, you use three plates per genotype/treatment.
20. Plants of the same genotype/treatment are placed diagonally to each other.
21. Seven days prior to the experiment, a fresh cabbage plant is introduced into the insect-rearing room on which butterflies are allowed to deposit eggs for 1 day, after which the plants are

placed in a closed cage; 5 days later, the caterpillars hatch from the eggs.

22. The caterpillars are hard to track down: they are small and green and in addition, tend to crawl on the abaxial side of the leaves. Chances of finding them back increase if the rosette is held against the light, which shines through the leaves except where the caterpillars are.
23. Treat plants with hormones in the morning (before 12:00 p.m.) and sample tissue to analyze for JA-induced gene expression around 2:00 p.m., because then the plants show high sensitivity to JA while the basal expression level of genes like *PDF1.2* is low. The basal *PDF1.2* transcript levels are high at the end of the day due to the circadian rhythm.
24. The HCl–chlorine mixture needs to be freshly prepared.
25. There is still chlorine gas in the seed coat after surface-sterilization and this will eventually kill the seeds if it cannot be released. Therefore, the tubes with seeds should be left open in a sterile hood for at least half an hour before they are transferred to plates or stored at 4 °C.
26. To enhance the induction of JA-sensitive genes that are co-regulated by ethylene, like those under control of the ERF branch of the JA signaling pathway, 0.002 mM ACC can be added to the medium. Be careful with increasing the ethylene concentration in the assay, because it is known to suppress the antagonism by SA on JA signaling [20].

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