Chapter 5

Techniques to Study Common Root Responses to Beneficial Microbes and Iron Deficiency

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Abstract

Iron (Fe) plays a central role in the vital processes of a plant. The Fe status of a plant influences growth and immunity, but it also dictates interactions of roots with soil microbiota through the production of Fe mobilizing, antimicrobial fluorescent phenolic compounds called coumarins. To adapt to low Fe availability in the soil, plants deploy an efficient Fe deficiency response. Interestingly, this Fe deficiency response is hijacked by root-colonizing microbes in the root microbiome to establish a mutually beneficial relationship. In this chapter, we describe how we cultivate plants and microbes to study the interaction between plants, beneficial rhizobacteria, and the plant's Fe deficiency response. We describe (a) how we study activity and localization of these responses by assessing gene-specific promoter activities using GUS assays, (b) how we visualize root-secreted coumarins in response to Fe deficiency and colonization by beneficial rhizobacteria, and (c) how we prepare our samples for metabolite extraction and reverse-transcriptase quantitative PCR to analyze the expression of marker genes.

Key words Iron deficiency, Coumarins, *Pseudomonas simiae* WCS417r, Induced systemic resistance, Plant-microbes interaction

1 Introduction

Iron (Fe) is a redox catalyst for essential cellular processes in almost every organism. Although it is an abundant element in the Earth's crust, its bioavailability is hampered by the fact that it mostly occurs as ferric oxide, which is poorly soluble at neutral and high pH [1–3]. Plant immunity and Fe status are tightly linked: plants can use Fe withholding strategies as well as Fe accumulation to combat infectious pathogens, and it has been shown that Fe deficiency directly influences plant resistance [3–6]. Indirectly, Fe status also mediates beneficial interactions with the microbial community in

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the rhizosphere. A well-known example is the plant-protective effect of *Pseudomonads* in disease-suppressive soils, where bacterially produced Fe-chelating siderophores limit Fe availability and therewith pathogen growth [7]. Some of these *Pseudomonas* strains can also directly stimulate plant broad-spectrum foliar resistance when colonizing its roots [8]. This response is termed induced systemic resistance (ISR) and has been described in many species, but has been mainly studied in the *Arabidopsis thaliana* (Arabidopsis) and *Pseudomonas simiae* WCS417 (WCS417) model system [8]. This ISR response is known to be dependent on the hormones jasmonic acid and ethylene, but how root colonization translates to shoot resistance and associated long-distance signals has remained elusive [9, 10].

Aside from stimulating plant defense and the production of siderophores, WCS417 also activates parts of the canonical Fe deficiency response in plants [11, 12]. This involves induction by WCS417 of many genes upregulated in roots following Fe deficiency, such as Fe transporter gene *IRT1* and the ferric chelate reductase gene *FRO2* [12]. Part of this Fe-deficiency module that is activated in the roots, namely the transcription factor MYB72 and the β-glucosidase BGLU42, were shown to be integral to the induced resistance response described above [13, 14]. MYB72 and BGLU42 participate in the roots in the production and secretion of coumarins, fluorescent phenolic compounds that have been extensively studied in the last 10 years for their role in Fe acquisition, especially in alkaline soils [14–18]. As such, these compounds might act downstream of MYB72 and BGLU42 as long-distance signals that participate in the induction of foliar defenses [10].

Next to their potential role in ISR, these Fe deficiency-induced compounds can affect plant-microbiota interactions in the rhizosphere [17, 19, 20]. Arabidopsis mutants for the key enzyme in coumarin biosynthesis F6’H1 assembled distinct microbiomes on their roots compared to wild-type Arabidopsis [17, 20]. Coumarins can shape the root-associated microbiota and display selective antimicrobial activity, evidenced by beneficial bacteria such as WCS417 being resistant to their antimicrobial effects, while some soil-borne pathogens are sensitive to them [17]. All in all, the plant’s Fe-deficiency response plays a significant role in microbially induced resistance and the direct interaction with beneficial microbes. Here, we describe the protocols that our lab routinely uses to describe and compare different aspects of the plant’s response to Fe-deficiency as well as to inoculation with beneficial rhizobacteria. Firstly, we provide a detailed explanation of our plant and bacterial culturing methods, and describe how we prepare tissue for both gene expression as well as targeted metabolomics analysis. Then, we explain how we utilize qPCR and GUS staining to quantify and localize the expression of Fe deficiency and ISR-related genes in response to both WCS417 and Fe deficiency,

and during different time periods. We describe the different setups we use to visualize coumarins in liquid media as well as in an agar plate system. These methods provide reproducible results that give us insight into how beneficial bacteria interact with plant physiology, and how this can result in adaptive plant responses.

2 Materials

2.1 Equipment

- Flow cabinets.
- 100–1000 μL (P1000), 20–200 μL (P200), and 2–20 μL (P20) pipettes and corresponding pipette tips.
- Square Petri dishes (120 × 120 mm).
- Petri dishes (94 mm diameter).
- pH meter.
- Tweezers.
- Spreader.
- Parafilm M.
- Bunsen burner.
- VAPOUR-Line Lite Autoclave.
- 96% ethanol.
- Demineralized water.
- Cutting blade (Fisherbrand™ Razor Blades).
- Bio Dancer Benchtop Shaker (New Brunswick).
- Centrifuge that can hold 50-mL Falcon tubes.
- 50-mL Falcon tubes.
- Glass beaker (250 mL).
- 1.5-mL microcentrifuge tubes.
- Plastic containers.
- Fume hood.
- Desiccator.
- CELLSTAR® 12-well multiwell culture plates.
- CELLSTAR® 96-well flat bottom plates.
- Glass beads.
- Vortex mixer.
- TissueLyser II (Qiagen).
- Intelli-Mixer™ RM-2 M (ELMI).
- NanoDrop™ 2000/2000c (Thermo Fisher).
- ViiA7 real-time PCR system (Thermo Fisher).

- S1000™ Thermal Cycle (Bio-Rad).
- Incubators (65 °C, 28 °C, 37 °C).
- Multichannel Pipette (5–10 μL, 200 μL).
- Strip tubes for PCR and cap strips for PCR tubes.
- Microcentrifuge.
- Plate centrifuge.
- QPCR plate (384-Well 0.02 mL) and optical adhesive film.
- Filter Tip, 10 μL.
- Glass microscope slides.
- Glass cover slips.
- UV Transilluminator.
- Plant growth chamber (22 °C; day/night photoperiod: 10 h/14 h; 100 μmol · m^{-2} · s^{-1} light intensity; 70% humidity).
- Plate reader with a fluorescence detector.

2.2 Buffers, Media, and Solutions

2.2.1 Plant Cultivation

1. Seeds of *Arabidopsis thaliana* (accession Col-0) or Arabidopsis *pGENE::GUS* lines (in the Col-0 background) of your gene of interest. In our case, we used GUS lines of coumarin biosynthesis-related genes such as *MYB72* and *F6'H1*.
2. Bleach.
3. 37% (v/v) hydrochloric acid (HCl) in demineralized water.
4. *Murashige and Skoog* [21] *nutrient solution*: 0.44% (w/v) Murashige and Skoog medium including vitamins, 4.7 mM MES Monohydrate 0.5% (w/v) sucrose per liter of demineralized water, supplemented with 10 g of Plant agar. Adjust the pH to 5.5 using 1M KOH.
5. *Hoagland* [22] *nutrient solution*: 2 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 2.5 mM KH_2PO_2, 70 μM H_3BO_3, 14 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 10 μM NaCl, Na_2MoO_4, 4.7 mM MES monohydrate, 0.5% (w/v) sucrose and for available Fe treatments 50 μM Fe-(III) EDTA per liter of demineralized water, supplemented with 10 g of Plant agar when plants need to be grown on square Petri dishes. Adjust the pH to 5.5 or pH 7.3 using 1M KOH for normal and alkaline conditions, respectively.

2.2.2 Preparing the WCS417 Inoculum

1. Sterile 10 mM $MgSO_4$ in demineralized water.
2. *King's medium B solution* (KB): 20 g Protease Peptone N°3, 1.5 g of $MgSO_4$, 1.2 g of K_2HPO_4, and 10 g of glycerol per liter of demineralized water, supplemented with 15 g of Difco Agar Granulated.
3. Rifampicin stock: 100 mg · mL^{-1} Rifampicin in DMSO.

2.2.3 Harvesting Plant Material for Metabolomics or Gene Expression

1. Sterile MQ water.
2. Liquid nitrogen.

2.2.4 GUS Staining

1. *GUS buffer solution*: 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA (pH 8.0), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 0.5 mM X-GlcA and 0.1% (v/v) Triton-X per 50 mL of demineralized water.
2. 20% (v/v) glycerol in demineralized water.

2.2.5 RNA Extraction and cDNA Synthesis

1. Sterile MQ water.
2. 10% SDS stock (pH = 7.2, adjust pH with 1 M HCl).
3. *Cell lysis solution*: 2% SDS, 68 mM sodium citrate, 132 mM citric acid, 1 mM EDTA. The pH of the solution should be between 4 and 4.5, adjust pH with 1 M HCl.
4. *Protein-DNA precipitation solution*: 4 M NaCl, 16 mM sodium citrate, 32 mM citric acid.
5. Isopropanol.
6. DNAse I 1 U · μL^{-1}.
7. 10× Reaction Buffer with $MgCl_2$ for DNase I (Thermo Scientific™).
8. 50 mM EDTA.
9. Oligo dT primer (200 nM).
10. 5× RT buffer (Thermo Scientific™, EP0451).
11. 10 mM dNTPs.
12. RevertAid H minus reverse transcriptase 200 U · μL^{-1} (Thermo Scientific™, P0451).

2.2.6 Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

1. Primers of interest.
2. Sterile MQ water.
3. SYBR® Green Master Mix (Applied Biosystems).

3 Methods

3.1 Seed Sterilization and Sowing

1. Put the desired amount of Arabidopsis seeds into a 1.5-mL Eppendorf tube and vapor-sterilize the seeds by placing them, with the lid of the Eppendorf tube open, in a desiccator together with a glass beaker containing a mix of 100 mL of bleach and 3.2 mL of 37% (v/v) HCl for 3.5 h (*see* **Notes 1** and **2**). This must be done in a fume hood.

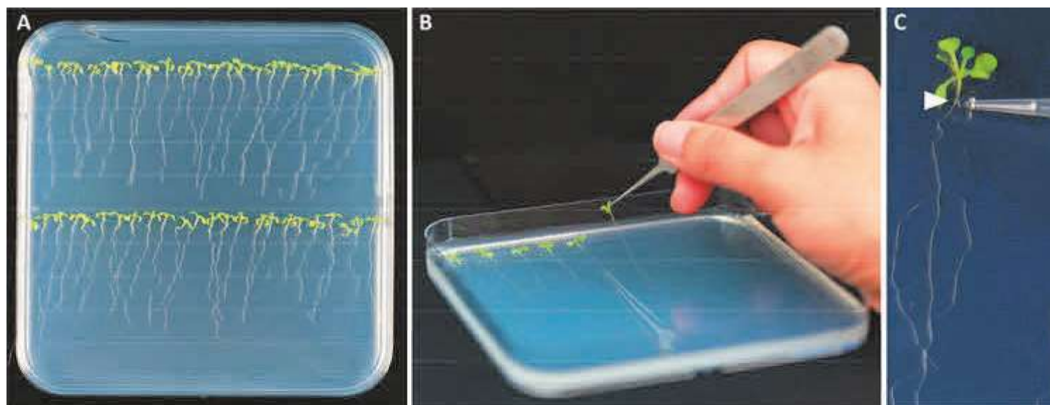

Fig. 1 Square Petri dishes with Arabidopsis plants during different stages of growth and treatment. The sowing pattern of seedlings (**a**), transplantation to a new square Petri dish using sterile tweezers (**b**) and inoculation with *P. simiae* WSC417r at the root-shoot junction (**c**)

2. Close the Eppendorf tubes containing the seeds and move them to a clean flow cabinet. Open the cap of the Eppendorf tube for 30 minutes to allow the excess chlorine gas vapors to disperse out of the tube.
3. *Sowing*:
   (a) For all assays except the coumarin fluorescence quantification assay: sow the seeds on agar-solidified Murashige and Skoog (MS) medium in a square Petri dish (120 × 120 mm) in two rows of 25 seeds (Fig. 1a) and seal the square Petri dish with Parafilm.
   (b) For the coumarin fluorescence quantification assay: sow the seeds on a 96-well plate containing 200 μL of agar-solidified Hoagland medium (with available Fe at pH 5.5 or without available Fe at pH 7.3) in each well, resulting in 1 plant per well. Seal the plate with Parafilm.
4. After sowing, stratify the seeds in darkness at 4 °C for 48 h.
5. Transfer the square Petri dishes and the 96-well plates into the short-day growth chamber and position them vertically and horizontally, respectively.

3.2 Transplanting

1. Take the square Petri dishes containing plants from the growth chamber.
2. *Transplanting*:
   (a) To transfer to square Petri dishes containing agar-solidified medium: open the square Petri dishes containing the plants grown on MS medium and move the plants with sterile tweezers gently to agar-solidified Hoagland medium in square Petri dishes (120 × 120 mm) by lifting the hypocotyls, resulting in 10 plants per square Petri dish (Fig. 1b) (*see* **Note 3**).

Table 1
Overview of plant age for the transplanting and the treatment steps per experiment

Experiment	Transplanting age (days)	Treatment age (days)	Harvest age (days)
GUS assisted localization of gene expression	5	5	10
Preparing plant material for qPCR and coumarin metabolite profiling	10	12	14
Coumarin fluorescence visualization	10	10	17
Coumarin fluorescence quantification	–	–	7

(b) To transfer to 12-well plates containing liquid medium: open the square Petri dishes containing the plants grown on MS medium and move the plants with sterile tweezers gently into a well of a 12-well plate containing 1.5 mL of Hoagland medium per well by lifting the hypocotyls, resulting in 2 plants per well (Fig. 3a).

3. Transfer the square Petri dishes and the 12-well plates to a short-day growth chamber and store them vertically and horizontally, respectively.

See Table 1 for more details.

3.3 Preparing the Bacterial Inoculum and Plant Inoculation

1. Streak bacteria from a frozen glycerol stock on Petri dishes containing agar-solidified KB with 50 μg · mL^{-1} rifampicin using a sterile inoculation loop, and incubate them upside-down, overnight at 28 °C and in darkness.
2. In a sterile flow, add 2–5 mL of sterile 10 mM $MgSO_4$ to the bacterial cultures grown overnight and suspend the bacteria in the solution by scraping them off the agar medium using a sterile spreader.
3. Pipet 50 μL of bacterial suspension to a fresh Petri dish with agar-solidified KB containing 50 μg · mL^{-1} rifampicin and spread them over the medium using a sterile spreader. Store the Petri dish upside-down, overnight at 28 °C and in darkness.
4. After 24 h, add 5 mL of 10 mM $MgSO_4$ to the Petri dish and gently scrape the bacteria from the agar into a suspension using a sterile spreader.
5. Pour the suspension into a 50-mL Falcon tube, then centrifuge at 4500 *g* for 5 min.
6. Pour off the supernatant, then add 25 mL of 10 mM $MgSO_4$ to the tube, and resuspend the pellet using a vortex. Finally, centrifuge the tube at 4500 *g* for 5 min.
7. Repeat **step 6**.

8. Pour off the supernatant and resuspend the pellet in 20 mL of fresh 10 mM $MgSO_4$.
9. Measure the optical density (OD) at $\lambda = 600$ nm of the resulting bacterial suspension (*see* **Note 4**).
10. Then dilute bacterial suspension down to $OD_{600} = 0.1$.
11. Take the square Petri dishes with plants from the growth chamber at your growth stage of interest (Table 1) and open them in a sterile flow cabinet.
12. Using sterile tips, pipet 5–10 μL of bacterial solution or 10 mM $MgSO_4$, as a mock treatment, to the shoot-root junction (Fig. 1c, white arrowhead).
13. Leave the square Petri dishes open to dry but close them immediately as the liquid is evaporated to protect the plants from drying out.
14. Seal the square Petri dishes using Parafilm and store them upright in a short-day growth chamber.

3.4 Harvesting Plant Material for qPCR and Coumarin Metabolite Profiling

1. Sample size for RNA extraction or coumarin metabolomic profiling: for one biological repeat, take 30 roots of plants from 3 independent square Petri dishes (the weight of 30 roots of 14-days-old Arabidopsis is around 50–65 mg) (*see* **Note 5**).
2. Prepare liquid nitrogen, Eppendorf tubes, tweezers (clean with 70% ethanol before use), two square Petri dishes filled with sterile MQ water, and clean tissues.
3. Cut the plants with a razor blade at the shoot-root junction to separate the root from the aerial part of the plant.
4. Carefully collect all roots from three square Petri dishes using tweezers and wash them twice by submerging them in MQ water-filled square plates (*see* **Notes 6** and **7**).
5. Gently dry the roots on clean tissues.
6. Transfer the root material into the Eppendorf tubes.
7. Add 3 sterile glass beads into each sterile 1.5 mL Eppendorf tube containing the plant samples.
8. Snap-freeze the samples in liquid nitrogen and store them at −80 °C until RNA isolation or extraction for metabolomic analysis.

3.5 GUS-Assisted Localization of Gene Expression

1. Use 10-day-old plants that have been transplanted after 5 days and treated for 5 days.
2. Prepare a 12-well plate, adding 1 mL of GUS buffer solution to each well.

3. Gently lift the plants off the agar using tweezers and submerge them in sterile MQ. Swivel them around gently to make sure the plants are clean (*see* **Note 7**).
4. Gently submerge the plants in the GUS buffer solution, with a maximum of 5 plants per well. Take care to separate plants of different treatment conditions in separate wells and mark the wells carefully. Make sure no roots and leaves are sticking to the sides of the wells.
5. *Optional*: The 12-well plate can be put in a desiccator and under vacuum for 30 s. This can enhance the GUS staining effectivity as it helps the GUS staining buffer to penetrate the tissue, especially in the leaves. For root tissue of Arabidopsis, it is not necessary, so we often skip this step.
6. Seal the 12-well plate using Parafilm, wrap it in aluminum foil, and store at 37 °C for the required amount of time (*see* **Note 8**).
7. When differences between treatments can be clearly observed, remove the 12-well plate from the stove and carefully remove the GUS buffer solution using a P1000 pipette. A good practice is to tilt the 12-well plate and pipet in a place where no tissue is present.
8. To decolor the plant tissue, add 1 mL of 96% ethanol to each of the wells, seal the 12-well plate using Parafilm, and wrap in aluminum foil. Then place the 12-well plate on a Bio Dancer (New Brunswick) or an equivalent plate shaker. Refresh the ethanol 2 or 3 times (every few hours), the 12-well plate can also be left overnight on the shaker.
9. When the plant tissue has lost all chlorophyll, the plants are ready for imaging. If the plants need to be kept for later analysis, replace the 96% ethanol with 70% ethanol and store them at 4 °C and in darkness.
10. Take the plants out of their wells and submerge them in a container with MQ water and gently untangle them using tweezers.
11. Prepare a microscope slide by adding 100 μL of 20% (v/v) glycerol in MQ to the slide. Place one plant on the slide and take care to spread the root system out, so all roots are clearly visible.
12. Add a cover slip on top by placing its edge at one side of the microscope slide and slowly letting the other edge come down upon the slide. Be careful to avoid formation of air bubbles.
13. Now the slides are ready for microscope imaging (*see* **Note 9**) (Fig. 2).

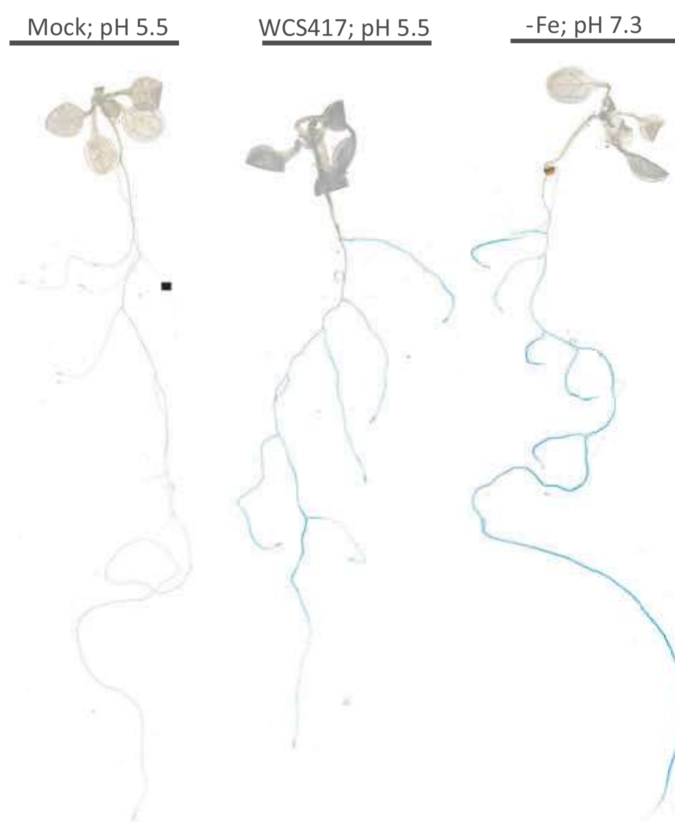

Fig. 2 GUS-stained Arabidopsis *pF6′H1::GUS* plants treated with mock, WCS417 or Fe deficiency conditions. These are composite images of photos that have been stitched using the MosaicJ plugin of ImageJ (*see* **Note 9**)

3.6 Coumarin Fluorescence Visualization

1. Take plants that were transplanted when 10 days old and treated for 7 days from the growth chamber. Remove the lids of the square Petri dishes containing plants of interest and place them on a UV transilluminator (365 nm) in a dark room.
2. Take pictures in only UV light using a photo camera (Fig. 3b, c) (*see* **Note 10**).

3.7 Coumarin Fluorescence Quantification

1. Take the 96-well plates containing 7-day-old plants from the growth chamber.
2. Gently remove the plants from the wells using a tweezer, being careful not to break the roots.
3. Measure the fluorescence of the wells using a microplate reader with a fluorescence detector (excitation at 360 nm; emission at 528 nm).

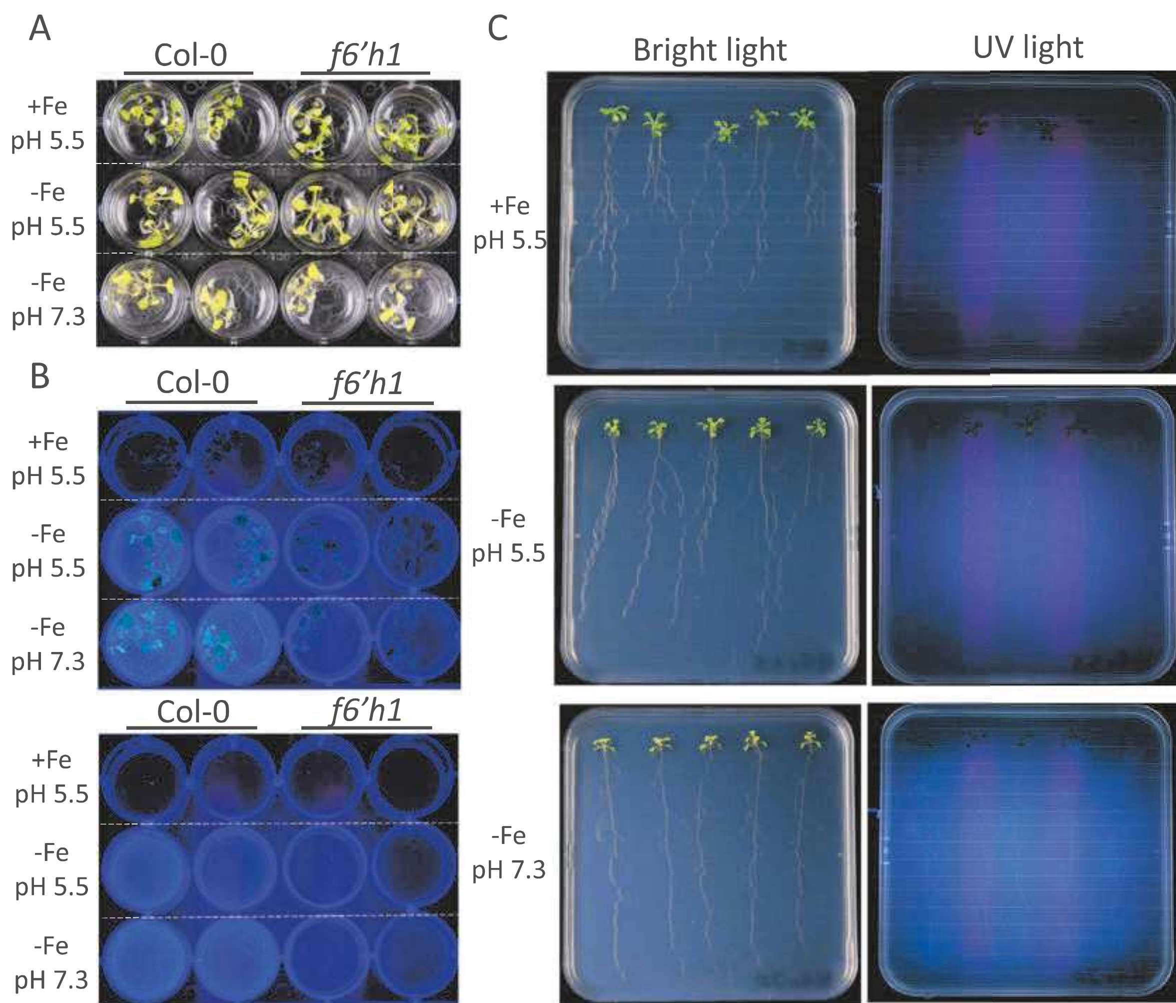

Fig. 3 Visualization of fluorescent coumarins secreted by Arabidopsis in liquid and agar-solidified medium system. (**a**) 10-days-old Col-0 and coumarin biosynthesis deficient mutant *f6'h1* plants growth for 7 days in Hoagland's liquid medium with Fe at pH 5.5 (nonlimited condition), without Fe at pH 5.5 (Fe limited) or without Fe at pH 7.3 (Fe limited plus alkaline stress) (bright light). (**b**) Secretion of fluorescent coumarins of Col-0 and *f6'h1* plants under different Fe-availability treatments (UV light, up: with plants; down: without plants). (**c**) Secretion of fluorescent coumarins of 17-days-old Col-0 on agar-solidified medium system under different Fe-availability treatments (left: bright light; right: UV light)

3.8 RNA Extraction and DNase Treatment

1. Harvest plant materials following the steps shown in Subheading 3.4.
2. The following method is adapted from [23] with slight modifications for use in our system.
3. Pre-cool the rack for TissueLyser II (Qiagen) at −80 °C for at least 5 min.
4. Grind the samples with glass beads using a TissueLyser II (Qiagen) at a frequency of 28 Hz for 30 s (put the samples back into liquid nitrogen immediately after this step, *see* **Note 11**).

5. Take each sample carefully out of the liquid nitrogen and tap the bottom of the tube strongly on a table to avoid ground sample adhering to the lid of the tube.
6. Add 300 μL of cell lysis solution, homogenize rapidly (vortex, tap, and invert the tube); incubate at room temperature for 5 min on rotating Intelli-Mixer™ RM-2 M (*see* **Note 12**).
7. Add 100 μL of protein-DNA precipitation solution (pre-cooled on ice) and homogenize (tap and invert the tube); incubate at 4 °C (on ice) for 10 minutes (*see* **Note 13**).
8. Centrifuge at 16,000 *g* and 4 °C for 15 min; transfer supernatant (300 μL) to a new tube. If some debris/tissue remains in the supernatant, spin down again (16,000 *g*, 5 min) and take the supernatant to a new tube.
9. Add 300 μL of isopropanol and homogenize (invert the tube), then centrifuge at 16,000 *g* and 4 °C for 5 min and pour off the supernatant (*see* **Note 14**).
10. Wash pellet with 300 μL of 70% ethanol (invert the tube); centrifuge at 16,000 *g* for 1 min and pipette off all supernatant, using a pipette (P200) to remove the smallest drops. If necessary, spin down. Wash again as above.
11. Open the lid of the tube, and air dry the pellet for 10 min until it becomes transparent.
12. Resuspend the pellet in 25 μL MQ.
13. Determine the RNA concentration by using NanoDrop™ 2000/2000c.
14. Take 2.2 μg of RNA to a new tube.
15. Add 2 μL 10× DNAse buffer and 1 μL DNAse I (Thermo Scientific™); add RNAse-free MQ to a total volume of 20 μL. Incubate for 30 min at 37 °C.
16. Add 1 μL of EDTA (50 mM); incubate at 65 °C for 10 min to inactivate DNAse.
17. Store the DNAse-treated RNA sample at −80 °C until further use.

3.9 cDNA Synthesis

1. Take 12 μL of DNAse-treated RNA (about 1 μg of RNA) to a new tube of a PCR tube strip (*see* **Note 15**).
2. Add 1 μL of oligo dT primer.
3. Incubate the samples at 65 °C for 5 min.
4. Place the samples on ice immediately.
5. Add 4 μL of 5× buffer, 2 μL of 10 mM dNTPs, and 1 μL RevertAid H minus Reverse Transcriptase, and mix gently (*see* **Note 16**).

6. Incubate the samples at 42 °C for 60 min, then at 70 °C for 15 min.
7. Store the samples at −20 °C until further use.

3.10 Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

1. This protocol is for a 384-well plate loading design with 5 μL total volume of reaction per well.
2. Prepare the sample loading design according to the 384-well plate (with two technical replicates for each sample).
3. Make primer working solution: 25 μL Forward primer (100 μM), 25 μL Reverse primer (100 μM), and 450 μL sterile MQ water (end concentration of primer is 500 nM).
4. Make SYBR® Green Master Mix: for one reaction, use 2.5 μL SYBR® Green and 0.5 μL primer working solution. Prepare the master mix for 10% extra reactions to account for pipetting errors. Divide the master mix into the PCR tube strip. This facilitates the loading of the plate using a multichannel pipette (5–10 μL).
5. Dilute the cDNA 4× in a new PCR tube strip using MQ water (end dilution in the wells is 10×).
6. Before sample loading, spin down all the master mix and cDNA solution and make sure there are no bubbles in the tubes.
7. Take a 384-well plate and place on the bench table (use dark paper underneath the plate for contrast, it helps to load small volumes in each well).
8. Add 3 μL of master mix to the well using the multichannel pipette and low-retention filter tips.
9. Add 2 μL cDNA template to each well using the multichannel pipette and low-retention filter tips.
10. Seal the plate after loading all samples.
11. Spin down using the well-plate centrifuge (1000 *g*, 1 min).
12. Load the plate on a ViiA7 real-time PCR system.
13. qRT-PCR program:
    (a) Initial denaturation: 50 °C for 2 min, 95 °C for 3 min.
    (b) PCR cycle (40 cycles): 95 °C for 15 s, 60 °C for 1 min.
    (c) Melt curve cycle (1 cycle): 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s.
14. Calculate transcript levels related to reference gene *At1g13320* using the $2^{-\Delta Ct}$ ($\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})$ method [24].

4 Notes

1. The optimal duration of vapor sterilization with chlorine gas is between 3 and 4 h. An exposure time above 4 h will be harmful to Arabidopsis seed germination rate.
2. Since the chlorine gas forms immediately after adding the HCl together with the bleach, it is paramount to close the air-tight desiccator immediately after mixing HCL and bleach.
3. When transplanting, make sure the roots of the plants touch the new plate first, after which you will slide the plant upwards to the final resting position of the shoot. This way the roots will be straightened and aligned parallel to each other.
4. Before measuring the OD_{600} we dilute the bacterial suspension 10×. This is because at higher OD values the spectrophotometer may become less accurate.
5. For detection of coumarins using HPLC a minimum of 50 mg of root tissue per sample is advised (although you can go as low as 10 mg). For shoots, a minimum of 100 mg is advised (although you can go as low as 50 mg).
6. Try to collect root samples using tweezers by lifting up root materials instead of clipping, and use new washing plates between treatments.
7. Make sure to especially clean the plants treated with bacteria thoroughly, due to biofilm formation bacteria tend to stick quite strongly to the root.
8. The required amount of time for GUS staining can vary strongly depending on promoter activity and GUS line (15 min to a few hours). Since GUS staining is a cumulative method, all roots will turn blue after sufficient time. Each GUS line should be tested to find the optimal time that allows for detection of differences between treatments in the conditions of interest.
9. Some microscopes have a tile function with which you can make high-resolution pictures of a large area of the root system, but if this equipment is lacking there exists a plugin for ImageJ (available for download at https://imagej.nih.gov/ij/) called MosaicJ (available for download at http://bigwww.epfl.ch/thevenaz/mosaicj/) which allows you to manually stitch images together to approximate the results from tiling [25, 26].
10. When taking pictures, make sure you use a prolonged exposure time. Additionally, as seen in Fig. 3b, c, fluorescent coumarins appear in –Fe conditions in liquid medium at both high and low pH, but on agar plates only at high pH. This could be due to traces of Fe in the plant agar. To account for this, 300 μM of

ferrozine (Sigma) could be added to the medium to make all traces of Fe unavailable to the plant.

11. To inactive RNase, plant materials for RNA extraction need to be stored at −80 °C before extraction and use liquid nitrogen to transport samples before cell lysis solution is added.
12. The pH of cell lysis solution should be between 4 and 4.5; the low pH is important to inactivate RNase.
13. The "cell lysis solution" and "protein-DNA precipitation solution" can be stored at room temperature for up to 1 month.
14. After centrifuging, you will see the pellet at the bottom of tube, use pipette (P1000) to remove supernatant carefully.
15. For faster sample loading later in qRT-PCR, use PCR tube strips (8×) for cDNA synthesis.
16. Prepare master mix containing 5× buffer, 10 mM dNTPs according to the amount of sample before **step 3**, and add transcriptase to master mix during **step 3**.

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