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# High-throughput Screening for Defense Priming-inducing Compounds in Parsley Cell Cultures

Kathrin Schmitz[#], Linda Werner[#] and Uwe Conrath*

Plant Biochemistry & Molecular Biology Group, Department of Plant Physiology, RWTH Aachen University, Aachen 52074, Germany

[#]Contributed equally to this work

*For correspondence: uwe.conrath@bio3.rwth-aachen.de

**[Abstract]** Defense priming describes the enhanced potency of cells to activate defense responses. Priming accompanies local and systemic immune responses and can be triggered by microbial infection or upon treatment with certain chemicals. Thus, chemically activating defense priming is promising for biomedicine and agriculture. However, test systems for spotting priming-inducing chemicals are rare. Here, we describe a high-throughput screen for compounds that prime microbial pattern-spurred secretion of antimicrobial furanocoumarins in parsley culture cells. For the best possible throughput, we perform the assay with 1-ml aliquots of cell culture in 24-well microtiter plates. The advantages of the non-invasive test over competitive assays are its simplicity, remarkable reliability, and high sensitivity, which is based on furanocoumarin fluorescence in UV light.

**Keywords:** Agrochemicals, Cell culture, Coumarins, Crop protection, Defense priming, High-throughput screen, Induced resistance, Parsley, Plant immunity, Priming

**[Background]** The pressing global issue of food insecurity due to population growth, diminishing arable land, and climate change can only be addressed in agriculture by effective crop protection (UN Food and Agriculture Organization, 2009). Synthetic agrochemicals can effectively protect crops from disease but also raise ecological and health concerns (Lamberth *et al*., 2013; Mascarelli, 2013). Therefore, safe and eco-friendly disease control products are needed (Lamberth *et al*., 2013).

Chemicals that prime the plant immune system for enhanced defense are promising for sustainable crop protection (Beckers and Conrath, 2007; Conrath *et al*., 2015). When primed, plants respond to very low pathogen stimuli with more robust activation of defense than unprimed plants. This frequently reduces disease susceptibility of the primed plant and lowers the risk of pathogen adaptation (Conrath *et al*., 2002, 2006, and 2015; Beckers and Conrath, 2007; Frost *et al*., 2008). Thus, inducing priming by harmless chemicals represents a promising means for sustainable crop disease control. Previous work with several chemical compounds provided proof of this concept (summarized in Conrath *et al*., 2015). However, the economic success of these compounds was limited, mainly because farmers preferred the strong curative performance of standard pesticides.

Today, the commercial success of crop protectants often relies on their ability to combine antimicrobial activity with defense priming in the target crop (Beckers and Conrath, 2007; Conrath *et al*., 2015). However, straightforward test systems for identifying priming-inducing chemistry are rare. Here, we describe a high-throughput screen for identifying chemical compounds that prime microbial pattern-

induced furanocoumarin (phytoalexin) secretion in suspension-cultured parsley (*Petroselinum crispum*) cells. The test measures the enhancement by priming-activating chemicals of furanocoumarin secretion provoked in parsley culture cells by a moderate concentration of Pep13 (Kauss *et al*., 1992), a molecular pattern peptide in the plant pathogen *Phytophthora sojae* (Brunner *et al*., 2002). Over the past 25 years, the test helped to identify novel priming-inducing chemistry (Katz *et al*., 1998; Siegrist *et al*., 1998; Schillheim *et al*., 2018). For higher throughput, we now perform the test with 1-mL aliquots of cell culture in 24-well microtiter plates (Schillheim *et al*., 2018). Other advantages of the test over competitive assays are the extraordinary reliability, simplicity, high sensitivity of fluorescence detection, and the need for only two simple treatments (activation of priming and Pep13 challenge) before final analysis. In principle, the assay should be feasible also with cell cultures of other plant species that synthesize furanocoumarins (*e.g*., giant hogweed, common hogweed, and garden angelica), provided they respond to treatment with a given molecular pattern with furanocoumarin secretion to the culture medium. If that is not the case, other test readouts need to be chosen [*e.g*., the production of reactive oxygen species, as described by Kauss *et al.* (1994)]. A recently introduced competitive assay to the screen introduced here evaluates the enhancement of *Pseudomonas syringae* pv. *tomato avrRpm1*-induced cell death in an Arabidopsis cell culture by priming agents (Noutoshi *et al*., 2012). The identification of several immune-priming compounds verified the power of the assay for discovering priming-inducing chemistry. However, because of the requirement of bacterial challenge, cytochemical staining, washing, dye extraction, and absorbance measurement, the screen is highly elaborate. The same holds true for a more recent respiratory activity-monitoring system for discovering immune-priming chemistry (Schilling *et al*., 2015), which is highly innovative but suffers from low throughput.

## Materials and Reagents

1. Sealing film (Roth, catalog number: H666.1)
2. Sealing foil for cell cultures (Roth, catalog number: EN85.1)
3. Serological pipettes 10 ml (Sarstedt, catalog number: 86.1254.025)
4. Serological pipettes 10 ml without tip (Sarstedt, catalog number: 86.1688.010)
5. Suction bottle 1,000 ml (Roth, catalog number: NY68.1)
6. Syringe filters, 0.22 µm (Roth, catalog number: KH54.1)
7. Aluminum foil (Roth, catalog number: 2596.1)
8. Büchner funnel, 70 ml (Roth, catalog number: XX45.1)
9. Cell culture 24-multiwell plates (Greiner, catalog number: 662160)
10. Cellulose plugs “Zellstoppers” (Diagonal, catalog number: ZE153)
11. Circular filter papers, Type 113A, 55 mm (Roth, catalog number: AP74.1)
12. Disposable syringe 20 ml with Luer-Lock fitting (Roth, catalog number: T550.1)
13. Flasks, 250 ml with a baffle (Diagonal, catalog number: 2121636S1)
14. Flasks, 500 ml with a baffle (Diagonal, catalog number: 2121644S1)
15. Sealing gaskets (Roth, catalog numbers: 9757.1 [33 mm]; 9758.1 [41 mm])

16. Nunc™ MicroWell™ 96-well, Nunclon Delta-Treated, flat-bottom microplate (ThermoFisher Scientific, catalog number: 137101)
17. Pasteur pipettes, 2 ml (Roth, catalog number: 4522.1)
18. Pep13 peptide (Thermo Fisher Scientific, amino acid sequence: VWNQPVRGFKVYE)
19. Petri dishes, 94/16 mm (Greiner, catalog number: 633180)
20. Pipette tips Gilson (Greiner, catalog numbers: 740290 [1,000 µl]; 739290 [200 µl])
21. Pipette tips Starlab (TipOne®, catalog number: 1110-3000 [10/20 µl])
22. Reaction tubes 1.5 ml (Sarstedt, catalog number: 72706)
23. Reaction tubes 50 ml (Greiner, catalog number: 227261)
24. *Petroselinum crispum* PC794 callus culture (DSMZ - Leibniz Institute, catalog number: PC794)
25. Plant agar (Duchefa, catalog number: P1001.1000)
26. Potassium hydroxide, 1 M KOH (Roth, catalog number: 9522.1)
27. Salicylic acid, $C_7H_6O_3$ (TCI, catalog number: H1342)
28. Test compounds
29. Gamborg's B5 medium including vitamins (Duchefa, catalog number: G0210.0050)
30. D-Sucrose, $C_{12}H_{22}O_{11}$ (Roth, catalog number: 4621.2)
31. Hand sanitizer (Roth, catalog number: EH72.2)
32. Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$ (Roth, catalog number: P027.1)
33. 2,4-Dichlorophenoxyacetic acid, $C_8H_6Cl_2O_3$ (Merck, catalog number: 8204510005)
34. Dimethyl sulfoxide, $C_2H_6OS$ (Roth, catalog number: A994.1)
35. Ethanol, $C_2H_5OH$, ≥99.5 %, extra pure (Roth, catalog number: 5054.4)
36. 70% ethanol (see Recipes)
37. 2,4-Dichlorophenoxyacetic acid, 0.2 mg/ml stock solution (see Recipes)
38. Magnesium sulfate, 0.05 mg/ml stock solution (see Recipes)
39. Modified Gamborg's B5 Medium (see Recipes)
40. Salicylic acid in water (SA in water), 10 mM stock solution (see Recipes, store aliquots at -20°C)
41. Salicylic acid in DMSO (SA in DMSO), 80 mM stock solution (see Recipes, store aliquots at -20°C)
42. Pep13, 0.005 µM stock solution (see Recipes, store aliquots at -20°C)
43. Callus medium (see Recipes)

## Equipment

1. 10-µl, 100-µl and 1,000-µl micropipettes (ErgoOne, Starlab)
2. Magnetic bars (Roth, catalog number: 2153.2)
3. Scalpel (Roth, catalog number: X003.1)
4. Scissors (Roth, catalog number: 5489.1)
5. Measuring cylinder, 1,000 ml (Roth, catalog number: P153.1)
6. Measuring cylinder, 100 ml (Roth, catalog number: P150.1)

7. Analytical balance (Mettler Toledo, model: MS204)
8. Autoclave (Systec, model: DX65)
9. Darkroom, temperature controlled (25°C), with two shaker incubators and clean bench with gas burner (see below)
10. Clean bench (IBS Tecomara, model: NU-543-4000EC)
11. Large shaker incubator, 90 rpm, 25°C (Infors HT, model: Multitron Standard)
12. Safety laboratory gas burner (Roth, catalog number: AN82.1) with propane gas bottle (Westphalengas; 11 kg)
13. Small shaker incubator, 130 rpm, 25°C (Infors HT, model: Ecotron)
14. Laboratory vacuum system (Fisher Scientific, model: Welch Ilmvac™ LVS 210T, catalog number: 11882253)
15. Magnetic stirrer (Roth, catalog number: AAN2.1)
16. Microplate Reader (BMG Labtech, model: CLARIOstar Plus)
17. pH meter (Mettler Toledo, model: SevenMulti™)
18. Pipette bulb (Roth, catalog number: YX53.1)
19. Pipette controller (BRAND, model: accu-jet® pro)
20. Ultrapure water system (Satorius, model: arium® pro VF)
21. UV lamp (Thermo Fisher Scientific, catalog number: 12813029)

## Software

1. CLARIOstar Reader Control Software (obtained with microplate reader)
   Plate: NUNC96
   Excitation: 335 nm (Bandwidth: 20)
   Dichroic filter: 365.2
   Emission: 398 nm (Bandwidth: 20)
2. MARS Data Analysis Software (obtained with microplate reader)
3. Statistical software (*e.g.*, GraphPad Prism)

## Procedure

A. Growth and maintenance of parsley cell culture
   1. Setting up the parsley cell culture
      a. *Work aseptically.* At the clean bench and in the darkroom, use a scalpel to transfer 2-3 cm of callus culture (obtained from DSMZ) to 50 ml of freshly prepared modified Gamborg's B5 medium in a 250-ml flask. In addition, transfer a 1 × 1-cm piece of callus to a Petri dish with freshly prepared modified Gamborg's B5 agar. The continuously kept cell callus serves as a backup for future cell culture restarts.

 

b. Incubate the 250-ml flask with cell culture on a shaker incubator (25°C, 90 rpm) in the darkroom for 10 days.
c. *Work aseptically.* At the clean bench, add 25 ml of modified Gamborg's B5 medium to the culture, and repeat this step after 7 and 14 days.
d. After another 7 days, use a 50-ml plastic tube to transfer 35 ml of cell culture to 120 ml sterile (autoclaved) modified Gamborg's B5 medium in a 500-ml flask.
e. Incubate the cell culture on a shaker incubator (25°C, 90 rpm) in the darkroom for 7 days.
f. Determine the cell fresh weight by filtrating 10 ml of cell culture as follows:
    i. Weigh an empty Petri dish containing a circular filter paper.
    ii. Assemble a suction bottle, sealing gasket, and funnel and connect to a vacuum pump.
    iii. Slightly hand-shake the flask containing the cell culture to achieve an as even as possible dispersal of the cells in their culture medium.
    iv. Use the pipette controller and a 10-ml serological pipette without tip to slowly transfer 10 ml of cell culture onto the circular filter paper in the funnel. Apply vacuum (150-200 pascal) and filtrate for 1 min.
    v. Place the filter paper with the cells onto a Petri dish, weigh, and determine the cell fresh weight by subtracting the tare weight (empty Petri dish with a circular filter paper).
    If the cells multiplied to 0.2 g fresh weight/ml within a week, initiate weekly propagation of the cell culture (Step A2).
    *Note: If weight differs much, go back to Step A1d and transfer more (if cell fresh weight is too low) or less (if cell fresh weight is too high) than 35 ml of cell culture.*

2. Weekly propagation of the cell culture
    a. *Work aseptically.* Using a 50-ml reaction tube, transfer 35 ml of a 7-day-grown parsley cell culture to a 500-ml flask equipped with baffle and filled with 120 ml sterile modified Gamborg's B5 medium prewarmed to 25°C.
    *Note: Do not leave the cell culture unshaken for more than 5 min.*
    b. Optional: Repeat Step A2a with more flasks.
    *Note: In our lab, we use 4 flasks a week: one for propagation and three for performing the high-throughput screening.*
    c. Incubate the cell culture on the large shaker incubator (25°C, 90 rpm) in the darkroom. Use 3-day-grown cultures for the screening experiments and 7-day-old cultures for weekly propagation.
3. Propagation of callus for cell culture restarts
    *Work aseptically.* Every four weeks, transfer a 1 × 1-cm piece of grown callus to the center of a fresh Petri dish containing Gamborg's B5 agar using a scalpel. Seal the Petri dish with sealing film and incubate at 25°C in a darkroom.

B. High-throughput screening for defense priming-inducing compounds using parsley cell cultures

1. Compound treatment of cells

a. Powders or crystals of chemical compounds are to be dissolved in DMSO or sterile double-distilled water (depending on the compound‘s solubility in these solvents). If the substance is already in solution, continue with the next step.

b. *Work aseptically.* Prepare a dilution series with the desired stock concentrations.
*Note: For substances dissolved in DMSO, a maximum of 2.5 µl should eventually be added per ml of cell culture. For substances dissolved in water, the volume to be added to 1 ml of cell culture should not exceed 20 µl.*

c. *Work aseptically.* Using a 1,000 µl micropipette and a truncated pipette tip transfer 1-ml aliquots of a 3-day-old parsley cell culture from Step A2c (cell fresh weight ~0.1 g/ml) to a 24-multiwell plate (Figure 1). Seal the plate with foil and put it into the small shaker incubator (25°C, 130 rpm) in the darkroom until further treatment.
*Note: Cut off ~1.3 cm of the tip with scissors to widen the opening and autoclave them. This ensures the easy and non-invasive transfer of cells.*

d. *Work aseptically.* Supply the cell culture with either DMSO or water as an appropriate negative control. The maximal volume of the adequate solvents should be the same as for the compound treatment samples. Use either 20 µl of a 10 mM solution of salicylic acid (SA) in water (see Recipes) or 2.5 µl of 80 mM SA in DMSO (see Recipes, final concentration: 200 µM) as a positive control for defense priming. For the candidate compounds, treat aliquots of cell culture with the desired concentration of compound (Figure 2). Seal the multiwell plate and place it back in the shaker incubator.

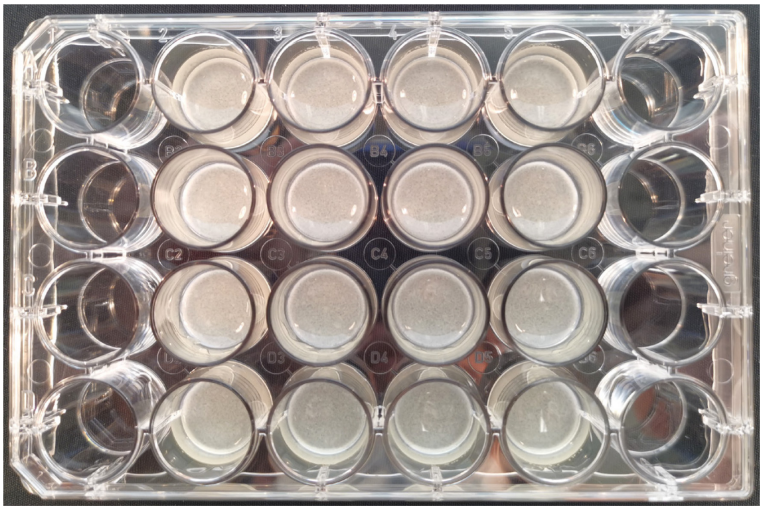

**Figure 1. Aliquots of parsley cell culture in a 24-multiwell plate.** Three-day-old parsley cell culture 1-ml aliquots were transferred to a 24-multiwell plate using a truncated pipette tip.

2. Treatment with Pep13
   a. *Work aseptically.* After 24 h of incubation in the presence of test compound or solvent, add 10 µl of 0.005 µM Pep13 (final concentration: 50 pM) or water (as a control for the Pep13 treatment) to the adequate cell culture (Figure 2).
      *Optional: To further improve the identification of priming-specific chemicals, include a sample in which test compound and solvent are added together after the 24-h preincubation period. This may enable discrimination of priming responses from synergistic effects.*
   b. Seal the plate and put it back in the shaker incubator.
3. Furanocoumarin fluorescence measurement
   a. *From this point on, working in the darkroom is no longer required.* After another 24 h of cultivation, place the plate on a work bench and let cells settle down (approx. 30 s). Transfer 100 µl cell-free supernatant of each sample to a 96-well microtiter plate and determine the relative fluorescence of secreted furanocoumarins in the Microplate Reader at 335 nm excitation and 398 nm emission (Figure 2).
      *Optional: For visualization of the fluorescence, take a picture of the 24-multiwell plate in UV light [e.g., when placed under a UV lamp (Figure 3)].*

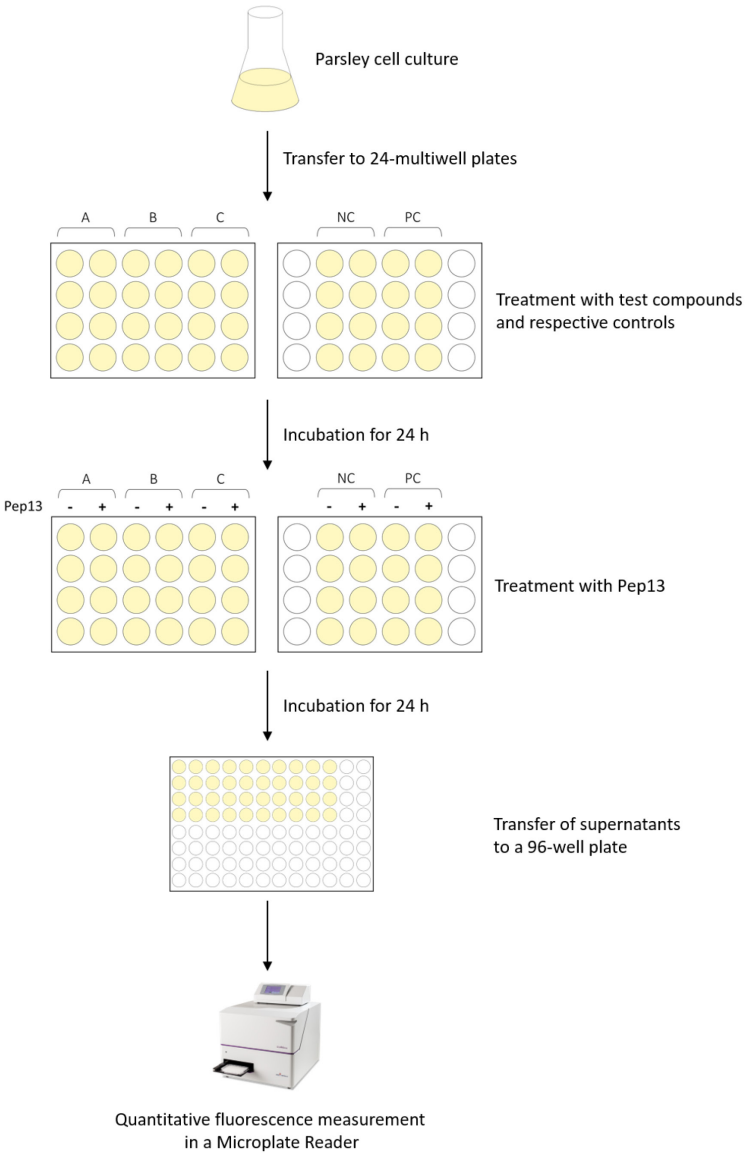


**Figure 2. Workflow of high-throughput screening for defense priming compounds using parsley cell culture.** Transfer 1-ml aliquots of a 3-day-old parsley cell culture to 24-multiwell plates. Treat the cell cultures with the test compounds dissolved or diluted in DMSO or sterile double-distilled water at different concentrations (A, B, C). Use DMSO or sterile double-distilled water as a negative control (NC) and SA in DMSO or SA in sterile double-distilled water as positive (priming) control (PC). After 24 h of incubation in a shaker incubator (25°C, 130 rpm) in a darkroom, add 10 µl of 0.005 µM Pep13 to the respective aliquots of cell culture. After shaking for another 24 h, transfer the supernatants to a 96-well microtiter plate and determine the relative furanocoumarin fluorescence in the Microplate Reader. SA, salicylic acid. Figure modified from Schillheim *et al*. (2018).

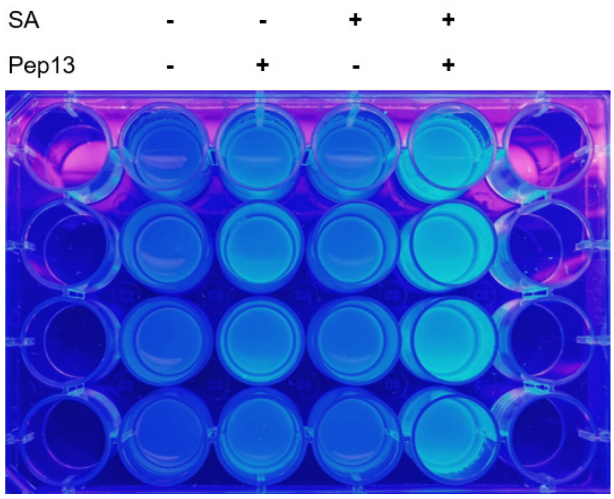


**Figure 3. Fluorescence of secreted furanocoumarins in aliquots of parsley cell culture in a 24-multiwell plate and in UV light.** Parsley cell culture 1-ml aliquots in a 24-multiwell plate were left untreated (-) or were treated with SA (+; final concentration: 200 µM). After 24 h, cell culture aliquots in the absence or presence of SA were left untreated (-) or supplemented with Pep13 (+; final concentration: 50 pM) to activate furanocoumarin synthesis and secretion. After another 24 h, the multiwell plate was exposed to UV light and a photo taken. SA, salicylic acid.

## Data analysis

For each experiment, determine the relative furanocoumarin fluorescence of four technical replicates for each treatment and repeat the experiment three times. For each treatment, calculate the mean values and standard deviation for all the experiments done. Statistically analyze the data by performing one-way analysis of variance (one-way ANOVA) followed by posthoc Student's *t*-test using appropriate statistical software (*e.g.*, GraphPad Prism).

## Notes

It is very important to work aseptically wherever indicated. Before each step, disinfect your hands and clean and sterilize all materials with 70% ethanol before you work at the clean bench. For all cultivation and propagation steps, sterilize flasks, cellulose plugs, and scalpels using the flame of the gas burner. Avoid physical contact of reaction tubes and flasks while transferring the cell culture.

## Recipes

1. 70% ethanol
   Mix 350 ml ethanol (≥99.5 %, extra pure) and double distilled water up to 500 ml.
2. 2,4-Dichlorophenoxyacetic acid (0.2 mg/ml stock solution)
   Dissolve 40 mg 2,4-dichlorophenoxyacetic acid in 1 ml ethanol (≥99.5 %, extra pure).

Add double distilled water up to 200 ml.

3. Magnesium sulfate (0.05 mg/ml stock solution)
   Dissolve 10 g magnesium sulfate heptahydrate in 200 ml double distilled water.
4. Modified Gamborg's B5 medium
   3.16 g Gamborg's B5 medium including vitamins
   20 g D-sucrose
   10 ml of 0.2 mg/ml 2,4-dichlorophenoxyacetic acid stock solution
   5 ml of 0.05 mg/ml magnesium sulfate stock solution
   Add double distilled water up to 1 L.
   Adjust pH to 5.5 with 1 M KOH (approx. 3 droplets).
   Autoclave at 121°C for 15 min (*Note: End temperature set to 80°C*).
   For solid medium, add 10 g plant agar before autoclaving.
5. SA in water (10 mM stock solution)
   345.25 mg SA
   Add double distilled water up to 200 ml.
   Adjust pH to 5.5 with 1 M KOH.
   Fill up to 250 ml with double distilled water.
   Sterile-filter and aliquot in 1.5 ml reaction tubes.
   Store at -20°C until use.
6. SA in DMSO (80 mM stock solution)
   276.2 mg SA
   Add DMSO up to 25 ml.
   Aliquot in 1.5-ml reaction tubes.
   Store at -20°C until use.
7. Pep13 (0.005 µM stock solution)
   4.055 mg Pep13
   Add double distilled water up to 50 ml.
   Dilute 1:1,000 with double distilled water.
   Filter sterilize and distribute to 1.5-ml reaction tubes.
   Store at -20°C until use.

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We would like to thank our collaborators, whether public or private, with whom we used the parsley cell culture to spot priming-inducing chemistry over the past 25 years and who helped us optimize the screen continuously. This protocol is based on our previous publication (Schillheim *et al.*, 2018; doi: 10.1104/pp.17.00124).

## Competing interests

We declare we have no financial or non-financial competing interests.

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