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Quantifying MAMP-induced Production of Reactive Oxygen Species in Sorghum and Maize

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[Abstract] Plants have the ability to recognize microbe-associated molecular patterns (MAMPs) and mount a defense response. The level of the MAMP response can vary depending on genetic and environmental factors. The most commonly studied MAMPs are flg22, a peptide epitope from bacterial flagellin, and chitin, a component of the fungal cell wall. Protocols for measuring reactive oxygen species (ROS) production elicited by flg22 and chitin in maize and sorghum are described.
Keywords: Maize, Corn, Sorghum, MAMP, PAMP, ROS

[Background] In plants, pattern recognition receptors (PRRs) at the plasma membrane recognize microbe-associated molecular patterns (MAMPs, also known as pathogen-associated molecular patterns or PAMPs). MAMPs are molecules that are generally highly conserved among large groups of microbes and are not directly associated with pathogenesis (Segonzac and Zipfel, 2011). The most widely studied MAMP is flg22, a 22-amino acid epitope of bacterial flagellin (Zipfel *et al.*, 2004; Sun *et al.*, 2013). Chitin, a component of the fungal cell wall, has also been studied extensively (Newman *et al.*, 2013). MAMP recognition by PRRs leads to a defense response termed the MAMP response or the MAMP-triggered immunity (MTI) response. The MAMP response can include phenomena such as cell wall reinforcement by callose and lignin deposition, changes in ion flux across the plasma membrane, changes in phytohormone concentrations, induction or repression of plant defense-related genes, and production of reactive oxygen species (ROS) and nitric oxide (NO) (Thomma *et al.*, 2011). Several methods have been used to measure the MAMP response, these include measurement of: ROS production, NO production, growth inhibition, gene expression, MAP Kinase phosphorylation, callose deposition and lignification, seedling growth inhibition, and induced disease resistance (Vetter *et al.*, 2012; Valdés-López *et al.*, 2014; Lloyd *et al.*, 2017; Zhang *et al.*, 2017).

Studies have identified genetically-controlled variation in the MAMP response in a number of species including *Arabidopsis thaliana* (Vetter *et al.*, 2012; Vetter *et al.*, 2016), maize (Zhang *et al.*, 2017), soybean (Valdés-López *et al.*, 2011), tomato (Veluchamy *et al.*, 2014) and sorghum (authors' unpublished results) and in many cases quantitative trait loci (QTL) associated with variation in these responses have been identified. It is also becoming clear that quantitation of the MAMP response is

Bio-101: e3304.
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complex. Relative rankings of lines in a population can vary substantially depending upon the assay (Lloyd *et al.*, 2014; Zhang *et al.*, 2017) and the MAMP used (Veluchamy *et al.*, 2014; Vetter *et al.*, 2016; Lloyd *et al.*, 2017), the environmental conditions (Cheng *et al.*, 2013) as well as the condition and growth stage of the plants (Singh *et al.*, 2014; Zou *et al.*, 2018).

Measurement of ROS production induced by the MAMP flg22 and chitin is probably the most commonly-used method of measuring the MAMP response. Details of the procedure used for measuring MAMP induced ROS production in corn and sorghum seedlings are provided below along with discussion of various considerations and caveats to optimize measurements when using this technique.

Materials and Reagents

1. 200 µl and 1,000 µl pipette tips
2. 2 ml and 5 ml sterile Eppendorf tubes
3. Rubber cork
4. Multi-channel solution reservoir
5. 96-well Black Polystyrene Plate (Corning™ Costar, catalog number:3915)
6. Aluminium seal (AlumaSealII, Excel Scientific, catalog number: 12-169)
7. Aluminum foil
8. Soil

   33% Sunshine Redi-Earth Pro Growing Mix (Canadian Sphagnum peat moss 50-65%, vermiculite, dolomitic lime, 0.0001% silicon dioxide) and 66% pea gravel.
9. Flats and inserts for growing plants
10. L-012 (Wako, catalog number: 120-04891), a chemiluminescent probe that responds to ROS
11. Horseradish peroxidase (Type VI-A, Sigma-Aldrich, catalog number: P6782)
12. Chitin (from shrimp shells, Sigma, catalog number: C9752)
13. Chitooctaose (Accurate Chemical and Scientific Corporation, catalog number: BCR57120010), a chitooligosaccharide composed of eight acetamido-glucose units can also be used (Zhang *et al.*, 2017) (see Note 6)
14. Flg22 (Genscript, catalog number: RP19986)
15. Dimethyl Sulphoxide (DMSO)
16. dH_2O
17. L-012 solution (see Recipes)
18. Horseradish peroxidase solution (see Recipes)
19. MAMP solutions (see Recipes)

Equipment

1. Multichannel pipette
2. -20 °C freezer

Bio-101: e3304.
DOI:10.21769/BioProtoc.3304

3. Biopsy Punch with plunger (Integra Miltex, 32-33-P/25)
4. BioTek™ Synergy™ 2 Multi-Mode Microplate Readers (BioTek, catalog number:11-120-516)
5. Vortex (Vortex-Genie 2, Scientific Industries, catalog number: SI-0236)

Software

1. Microsoft Excel or similar
2. Gen5 (This is the software provided with the Synergy™ 2 plate reader)

Procedure

A. Plant growth conditions
   1. Plant six seeds in each pot about 2 cm depth in standard soil. Grown them in a 16/8-h light/dark cycle at 25/18 °C.
   2. After germination, remove seedlings until two remain in each pot. Grow them until they are ready for the ROS assays: ten days for maize and 15 days for sorghum.

   Note: We use growth chambers so that the conditions are as constant as possible. If sufficient growth chamber space is not available we have used greenhouse facilities. Sorghum and maize are treated in essentially similar ways.

B. Experimental setup and design

   A 96-well plate format is used in the following experimental design. Various experimental setups can be used depending on the requirements of the assay, however certain aspects are important.
   1. Several blank wells including only distilled water.
   2. At least one “mock” well should be included for each line, which includes leaf tissue and all reagents except the MAMP.
   3. Multiple replications in separate wells should be measured for each line. Each replication well includes one or more separate leaf disks collected from the same line. Generally, the leaf discs are derived from at least two separate plants.

   A typical experimental setup can be found in Figure 1. For each line, two leaf discs are collected from two individual plants. These four discs are distributed in the plate as shown in Figure 1; one disc is assigned to a mock well and three disks are assigned to individual technical rep wells (TR1-3 in Figure 1). Four blank wells are also included. Using this protocol, 23 lines can be measured per 96-well plate. It is helpful to have a printed map of each 96-well plate to avoid confusion when matching lines and wells.

   Several plates can be run per day. The exact number will vary from lab to lab depending on facilities available, but the limiting factor is often that data collection from one plate takes one hour on the plate reader. With larger populations, experiments can be planned so appropriate multiples of 23 lines are planted 10 days (for maize) or 15 days (for sorghum) before they are to be assessed.

It is important to assess the whole population in as short a period as feasible. Due to the significant variability between measurements, at least two full (and preferably more) biological replications of the whole population should be assessed this way using a complete randomized block design.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Line1 Mock	Line2 Mock	Line3 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock
B	Blank	Line1 TR1	Line2 TR1	Line3 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1
C	Blank	Line1 TR2	Line2 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2
D	Blank	Line1 TR2	Line2 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2
E	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock	Line1 Mock
F	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1	Line1 TR1
G	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2
H	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2	Line1 TR2

Figure 1. Experimental set-up most commonly used by the Balint-Kurti lab. Four wells are used as blank wells. Four wells are used for each line. One mock and three technical replications (TR1, TR2, TR3). Twenty-three lines can be assayed per plate.

C. Sample preparation

1. Collect one to three leaf discs using a biopsy punch and place in a black 96-well polystyrene plate containing 50 µl of distilled water.
2. After tissue collection, seal the plate with an aluminum seal and place at room temperature overnight.

   Note: The leaf discs can be placed in either orientation in the well (abaxial or adaxial side up) but the orientation must be consistent within the experiment. To collect the leaf tissue, a rubber cork is placed on one side of the leaf and the biopsy tool is used to excise a leaf disc from the other side (Figure 2B).

The day before the assay

1. Put 50 µl H_2O water in each well of a 96-well black assay plate (Figure 2A).
2. For each line take two 3 mm diameter leaf discs from two 10-day-old maize or 15-day-old sorghum seedlings and float on 50 µl H_2O in the 96-well plate (Figures 2B and 2C). The discs are taken from the middle of the youngest fully-expanded leaf (fourth leaf). The two discs are taken from equivalent places either side of the mid-rib, equidistant between the edge of the leaf and the mid-rib.

   Note: Cover the plate with an aluminum seal to prevent evaporation and maintain darkness.
3. Keep the plate at room temperature in dark place overnight (we set up these plates in the

Bio-101: e3304.
DOI:10.21769/BioProtoc.3304

afternoon and perform the assays the following morning).

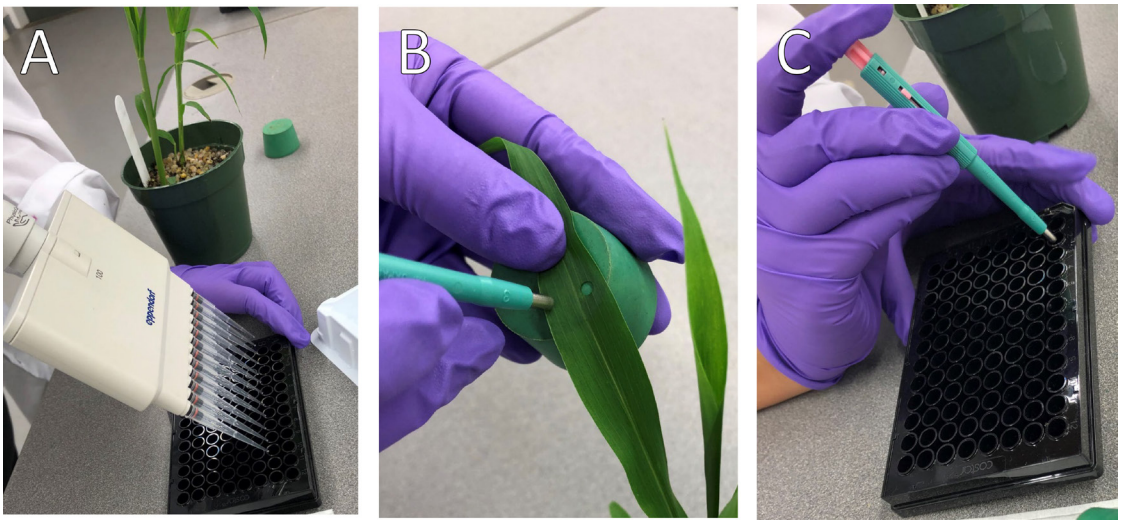

Figure 2. Leaf disc collection using punch with plunger in a 96-well black assay plate containing water on the day before the assay

The day of the assay

Setting up the Synergy 2 plate reader

Note: In order to minimize the time between adding the reaction solution and reading the plate, prepare the plate reader as step 5 below, then add the reaction solution to the plate (see the following section), then load it on the plate reader (step 6 below).

1. Switch on the Synergy 2 plate reader (Figure 3A).
2. Open the Gen5 software installed in the computer attached to the plate reader.
3. Set up the protocol by clicking procedure tab as shown in Figure 3B. This protocol measures luminescence every 2 min for the period of 1 hour-31 readings in all (Figure 3C).
4. Set up the plate based on how many samples and control you have as shown in Figure 3B.
5. Go to file export builder tab and select the parameters you want to export after the completion of the experiment (Figure 3D). Remember to select “all” as shown in Figure 3E to export all the data collected by the plate reader.
6. Once the program is set, insert the plate in the reader, start the reading.
7. The plate reader will generate a response curve for each sample as shown in Figure 3F.

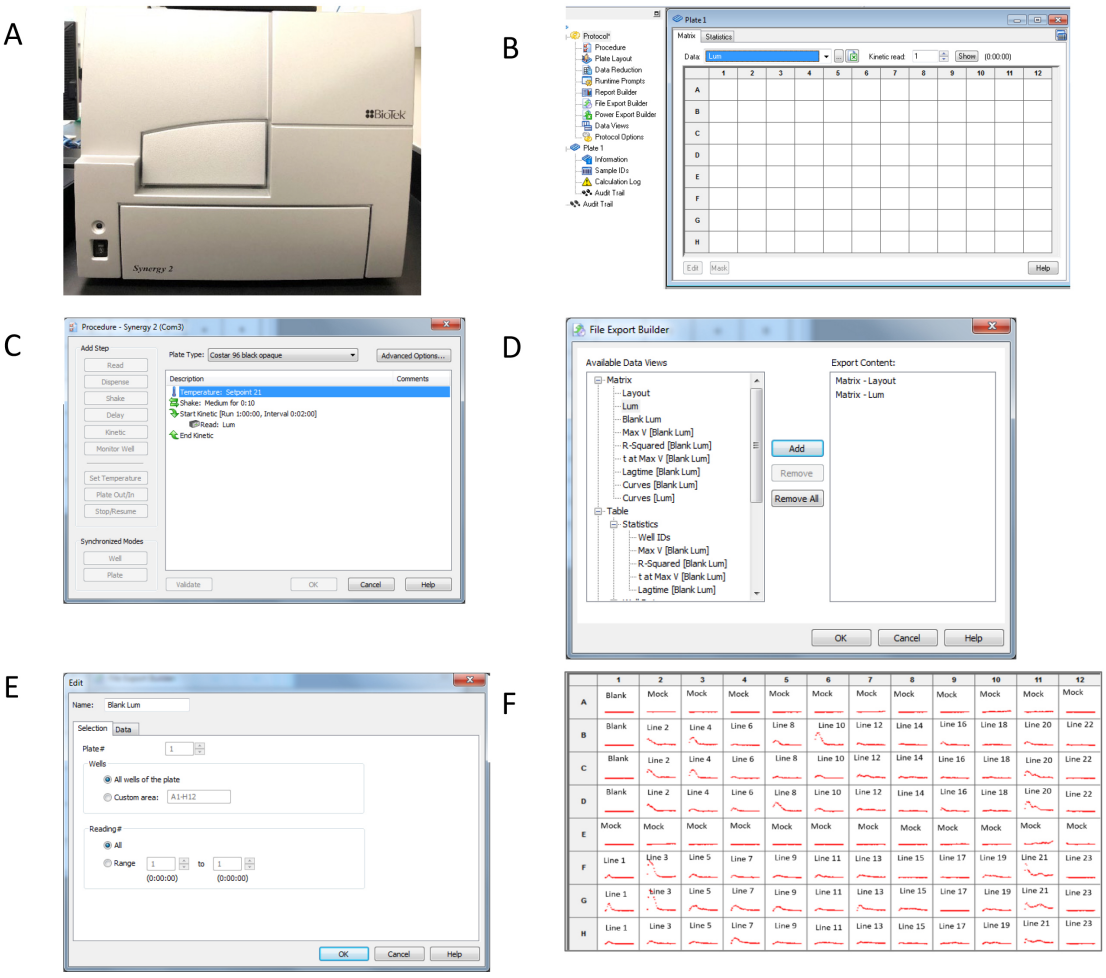

Figure 3. Setting up Synergy 2 multi detection plate reader. Using Gen5 software set up Synergy 2 multi detection plate reader (A) procedure (B), plate setup (C) and export builder (D) Configuring output (E) and final graphical output (F- also see figure 5).

Adding reaction solution to the plate

1. Add 50 µl of reaction solution using a multichannel pipette into each well just before measurement on the plate reader (we use a Synergy™ 2 multi-detection microplate reader made by BioTek). Maintain low-light conditions in the lab while adding chemicals to the leaf disc. Avoid touching leaf disc with pipette tips. Change tips when needed to avoid cross contamination among blank, mock and sample wells.

   For the 50 µl reaction, we use the following:

   For maize:

   1 µl 2 mg/ml L-012 in Dimethyl Sulphoxide (DMSO)

   1 µl of 2 mg/ml horseradish peroxidase

   48 µl 20 mg/ml chitin solution (see instructions for making the chitin solution below) or 2 µM of Flg22 solution

   Note: For the mock wells, omit the MAMP.

Bio-101: e3304.
DOI:10.21769/BioProtoc.3304

For sorghum:

0.5 µl of 2 mg/ml L-012 in water,

0.5 µl of 2 mg/ml horseradish peroxidase

49 µl of 100 mg/ml Chitin or 2 µM of Flg22 solution

Note: For the mock wells, omit the MAMP.

2. As soon as possible after adding the reaction and mock solution to each well, load the plate into the plate reader. The luminescence is recorded over a 60 min period 31 times at 2-min intervals and ROS production is calculated as the sum of 31 photon counts over this period.

D. Analysis and interpretation of results

A typical plot of luminescence over 60 min is shown in Figure 4. In a high-responding line, the signal generally peaks at about 10-20 min and then fades over the next 30 min.

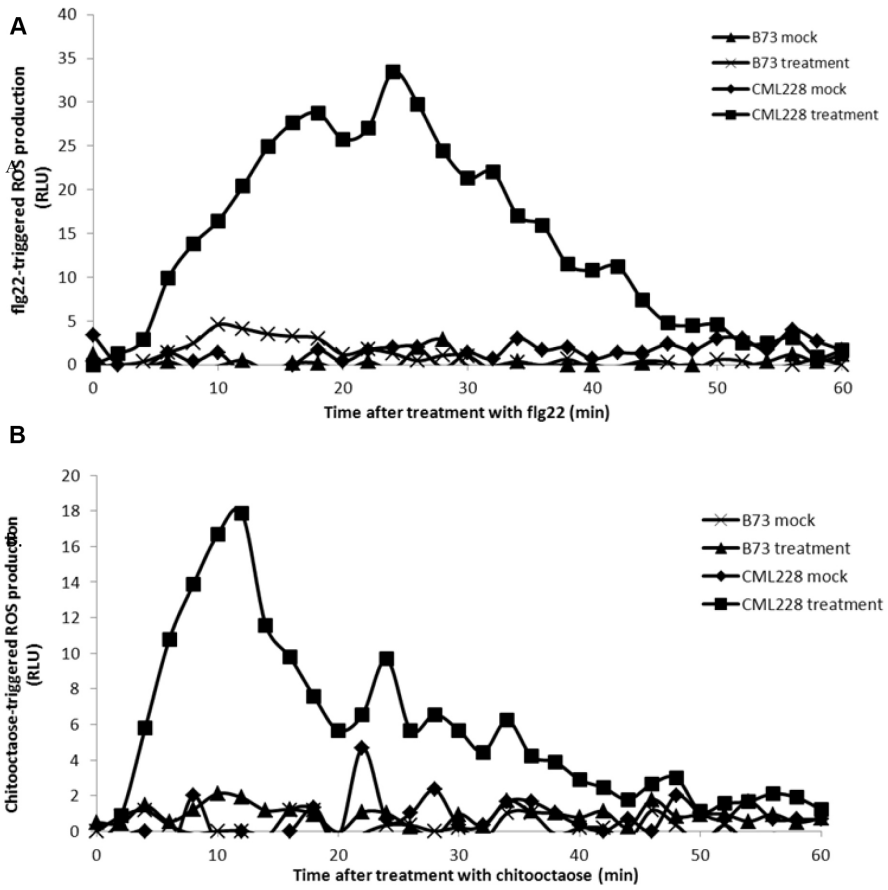

Figure 4. Time kinetics of (A) flg22-triggered and (B) chitin-triggered ROS production in maize. Results from mock and experimental treatments are shown for 2 maize lines: B73–a low responder and CML228–a high responder. Each data point represents the average of three biological replicates; ROS production measured in the relative light unit (RLU). This Figure is adapted from Zhang *et al.* (2017).

Bio-101: e3304.
DOI:10.21769/BioProtoc.3304

It is important that the mock reading is low. If the mock well shows a strong response, then the readings for that line should be ignored and redone. If all or most the mock wells in a plate show a response then the plate should be redone entirely. Note that to redo a line, the line should be planted afresh and the whole experiment should be performed. It is not acceptable to go back to the same sampled plants since at this point they are older and have been wounded and are therefore not equivalent to the rest of the sampled plants.

Plots from a typical successful plate are shown in Figure 5. Note that the mock plots are generally flat and that the three technical replications of each line show a generally similar response level.

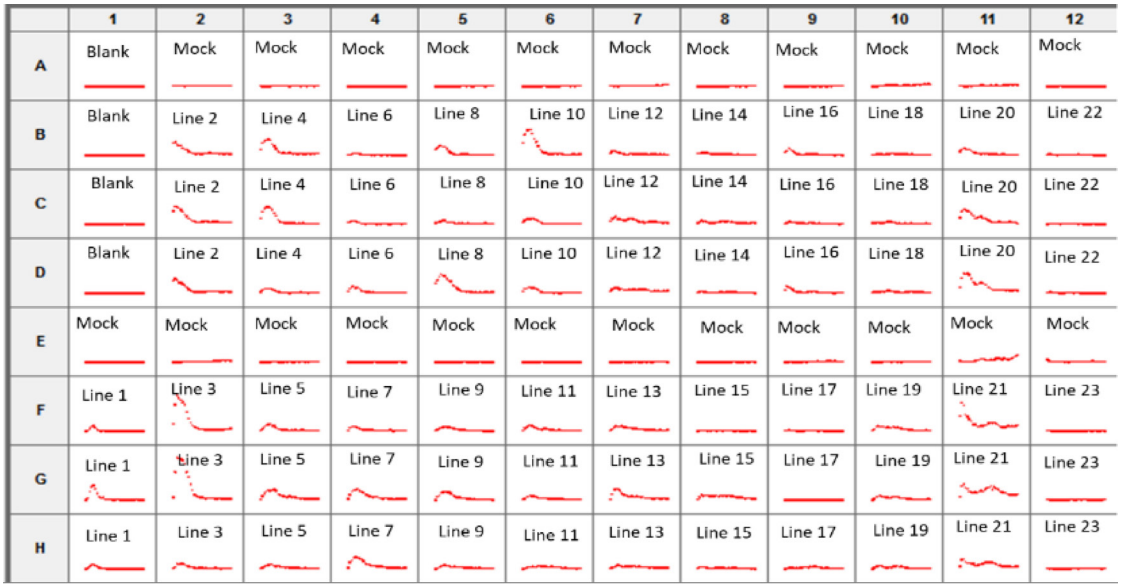

Figure 5. Plots showing levels of luminescence over an hour for each well of a typical 96-well plate assessed by the protocol described above. Note that this is an expanded view of Figure 3F.

Data analysis

1. Sum all 31 readings for each well. Subtract the blank reading. This is the value for the well.
2. For the experimental setup shown in Figure 1, the response of each line is assessed in three wells containing three separate leaf discs from the line (three technical replicates). In this case, we calculate the average of the values three experimental replicates and subtract the mock value to get the value for ROS response for each line.
3. Two or three biological replicates of the whole population should be performed. As long as the correlations between replications are significant, line values across replications can be averaged for further analyses. If correlations between replication are low, the data should be treated with caution.

Bio-101: e3304.
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Notes

1. The protocols used in the Balint-Kurti lab are described here. Almost every aspect of the protocols can be modified to suit the experiment being conducted, *e.g.*, the age of the plant, the number of replications and controls, the position of the sampled leaf. As with any experiment of this type, controlled, standardized growth conditions are very important. If possible, use growth chambers. If greenhouses are to be used, make every effort to standardize all growth conditions.
2. We have described the use of Synergy™ 2 multi-detection microplate reader but any standard plate reader that can measure luminescence can be used.
3. If reading more than two plates, make up fresh reaction solution for every two plates.
4. It is important to start measuring the luminescence as soon as possible once the reaction mixture is pipetted to the sample. Make sure the plate reader is set up before adding reaction mixture to the plate.
5. Maintain low light conditions during the entire experimental procedure on the second day due to the light-sensitive nature of the chemicals.
6. Chitin does not dissolve very well. Chitooctaose dissolves more easily, but it is more expensive. We were not able to detect a consistent response to chitooctaose in sorghum, though we did in maize and have used it in published studies (Zhang *et al.*, 2017). Both species respond to chitin.
7. It is best to collect leaf discs in the late afternoon in order to conduct ROS production experiments in the following morning.
8. The chemiluminescent probe Luminol can work well in these assays but researchers have found L-012 to produce more consistent results.
9. Levels of ROS production appear to depend on the species so researchers should test different concentrations of MAMPs for each new species they work with.
10. If low levels of ROS production are produced, increasing the concentration of the MAMP as well as the concentration of L-012 may improve results. We have also added multiple leaf discs to each well to increase the response.

Recipes

1. L-012 solution
   2 mg/ml in DMSO or water
   Make aliquots of 50 µl, wrap with a piece of aluminum foil and store in -20 °C freezer
   Note: This reagent is light sensitive.
2. Horseradish peroxidase solution (2 mg/ml)
   Prepare the stock solution in dH_2O
3. MAMP solutions
   a. Chitin stock solution (20 mg/ml for maize and 100 mg/ml for sorghum):
      Dissolve chitin in sterile water

This solution should be stored at 4 °C

Note: Chitin does not dissolve completely. So, vortex 2 times for 30 s and wait for 10 min after each vortex and then use.

b. Flg22 stock solution

Flg22 can be dissolved directly in water to the required concentration (20 µM)

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Competing interests

The authors declare no competing interests.

References

1. Cheng, C., Gao, X., Feng, B., Sheen, J., Shan, L. and He, P. (2013). Plant immune response to pathogens differs with changing temperatures. *Nat Commun* 4: 2530.
2. Lloyd, S. R., Ridout, C. J. and Schoonbeek, H. J. (2017). Methods to quantify PAMP-triggered oxidative burst, MAP kinase phosphorylation, gene expression, and lignification in Brassicas. *Methods Mol Biol* 1578: 325-335.
3. Lloyd, S. R., Schoonbeek, H. J., Trick, M., Zipfel, C. and Ridout, C. J. (2014). Methods to study PAMP-triggered immunity in Brassica species. *Mol Plant Microbe Interact* 27(3): 286-295.
4. Newman, M. A., Sundelin, T., Nielsen, J. T. and Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front Plant Sci* 4: 139.
5. Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G. and Boller, T. (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* 428(6984): 764-767.
6. Segonzac, C. and Zipfel, C. (2011). Activation of plant pattern-recognition receptors by bacteria. *Curr Opin Microbiol* 14(1): 54-61.
7. Singh, P., Yekondi, S., Chen, P. W., Tsai, C. H., Yu, C. W., Wu, K. and Zimmerli, L. (2014). Environmental history modulates *Arabidopsis* pattern-triggered immunity in a HISTONE ACETYLTRANSFERASE1-Dependent Manner. *Plant Cell* 26(6): 2676-2688.
8. Sun, Y., Li, L., Macho, A. P., Han, Z., Hu, Z., Zipfel, C., Zhou, J. M. and Chai, J. (2013). Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. *Science*

342(6158): 624-628.

9. Thomma, B. P., Nurnberger, T. and Joosten, M. H. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *Plant Cell* 23(1): 4-15.
10. Valdés-López, O., Khan, S. M., Schmitz R. J., Cui, S. Qiu, J., Joshi, T., Xu, D., Diers. B., Ecker, J. R. and Stacey, G., (2014). Genotypic variation of gene expression during the soybean innate immunity response. *Plant Genet Resour* 12(s1): S27-S30.
11. Valdés-López, O., Thibivilliers, S., Qiu, J., Xu, W. W., Nguyen, T. H., Libault, M., Le, B. H., Goldberg, R. B., Hill, C. B., Hartman, G. L., Diers, B. and Stacey, G. (2011). Identification of quantitative trait loci controlling gene expression during the innate immunity response of soybean. *Plant Physiol* 157(4): 1975-1986.
12. Veluchamy, S., Hind, S. R., Dunham, D. M., Martin, G. B. and Panthee, D. R. (2014). Natural variation for responsiveness to flg22, flgII-28, and csp22 and *Pseudomonas syringae* pv. tomato in heirloom tomatoes. *PLoS One* 9(9): e106119.
13. Vetter, M., Karasov, T. L. and Bergelson, J. (2016). Differentiation between MAMP triggered defenses in *Arabidopsis thaliana*. *PLoS Genet* 12(6): e1006068.
14. Vetter, M. M., Kronholm, I., He, F., Haweker, H., Reymond, M., Bergelson, J., Robatzek, S. and de Meaux, J. (2012). Flagellin perception varies quantitatively in *Arabidopsis thaliana* and its relatives. *Mol Biol Evol* 29(6): 1655-1667.
15. Zhang, X., Valdés-López, O., Arellano, C., Stacey, G. and Balint-Kurti, P. (2017). Genetic dissection of the maize (*Zea mays* L.) MAMP response. *Theor Appl Genet* 130(6): 1155-1168.
16. Zou, Y., Wang, S., Zhou, Y., Bai, J., Huang, G., Liu, X., Zhang, Y., Tang, D. and Lu, D. (2018). Transcriptional regulation of the immune receptor FLS2 controls the ontogeny of plant innate immunity. *Plant Cell* 30(11): 2779-2794.

