TECHNICAL ADVANCE

A High-Sensitivity, Microtiter-Based Plate Assay for Plant Pattern-Triggered Immunity

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The first step in the plant immune response to pathogen challenge involves the perception of conserved epitopes, called microbe-associated molecular patterns (MAMPs), by cell-surface pattern recognition receptors (PRRs). Given the key roles that MAMPs and PRRs play in plant innate immunity, great effort has been expended to identify these molecules. Current methods for assaying these immune responses are often limited in their resolution and throughput, and consequently, there is a need for medium- to high-throughput methodologies. Here, we describe the development of a 96-well microtiter plate-based assay for plant pattern-triggered immunity that measures the activity of plant peroxidase (POX) enzymes produced in response to treatment with bacterial MAMPs. The system has been optimized to minimize both the amount of plant tissue and MAMPs required and displays up to three orders of magnitude greater sensitivity than the traditional luminol-based reactive oxygen species assay when measuring the plant response to treatment with the bacterial MAMP *flg22*, reaching detection limits in the picomolar range. This high sensitivity opens the possibility of evaluating the immune-eliciting effects of weaker elicitors. The throughput and material requirements of the assay make it ideal for screens involving quantitative measurement of the plant innate immune response to MAMPs.

Plants rely on a multilevel innate immune system in order to protect themselves against pathogens (Dodds and Rathjen 2010; Mott et al. 2014). The initial step in the system involves the recognition of microbe-associated molecular patterns (MAMPs), which are highly conserved immune elicitors derived from invading microbes, and damage-associated molecular patterns (DAMPs), which are modified-self epitopes that result from pathogen activity. This perception event typically occurs through binding of the MAMP or DAMP to a plant cell-surface pattern-recognition receptor (PRR), which then activates tightly controlled intracellular signaling pathways to respond to invasion. The resulting innate immune reaction is termed pattern-triggered immunity (PTI) and is key to the control of many pathogens. One of the best studied MAMPs is *flg22*, a 22-amino acid peptide at the N-terminus of the bacterial flagellin

protein, which is recognized by the plant receptor FLS2 (Felix et al. 1999; Gómez-Gómez and Boller 2000). Due to the key roles that MAMPs, DAMPs, and PRRs play in plant innate immunity, great effort has been expended in order to identify these molecules. While much progress has been made, examples of cognate pairs, in particular, remain rare in the literature (Trdá et al. 2015).

The recent increase in the availability of genomic methodologies has allowed for both the identification of potential PRRs in many plant species (Lehti-Shiu et al. 2009; Liu et al. 2009; Magalhães et al. 2016; Sakamoto et al. 2012; Shiu et al. 2004) and the development of novel methods of elicitor identification in pathogens (Hou et al. 2014; McCann et al. 2012; Mott et al. 2016). These advances make possible the study of the plant innate immune system at a systems level. A more complete understanding of the interacting signaling events that underlie PTI could allow for the direct tuning of plant defense against pathogens as a strategy for crop protection. In contrast to existing techniques, engineering an increased PTI response promises to provide general and durable protection, which will be difficult for pathogens to overcome. Given the increasing desire for environmentally benign methods to achieve disease protection of crops and increase yields, this field is poised to continue to grow along with the desire for sensitive and high-throughput screening assays.

The current standard for the measurement of plant PTI is the luminol-based reactive oxygen species (ROS) burst assay (Felix et al. 1999; Keppler 1989). This assay gives excellent quantitative data, but its efficacy and ease of use varies greatly depending on the type of equipment available to the researcher. In addition, the assay sensitivity is such that reports from the literature generally require the use of purified *flg22* peptide at concentrations from 10 nM to 1 µM (Halter et al. 2014; Koller and Bent 2014; Yeh et al. 2016, 2015). While the ROS assay has played a central role in the study of highly active purified MAMPs and plant receptor knock-outs such as *flg22* and *fls2* plants, a complementary assay would be very useful for resolving some of the contradictory results that may be associated with differing growth conditions (Luna et al. 2011). Further, a more sensitive and high-throughput assay would enhance our ability to study weaker elicitors of innate immunity and more subtle plant phenotypes.

Here, we introduce a sensitive, high-throughput plant innate immunity assay that enables the rapid screening of large collections of MAMPs and candidate PRRs in a microplate-based assay that requires only small amounts of both MAMP and plant tissue. The assay measures the activity of plant peroxidase (POX) enzymes, which are produced in response to innate immune activation (Daudi et al. 2012; Felix et al. 1999;

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Mammarella et al. 2015; Minibayeva et al. 2015). The assay is able to detect innate immune activation by picomolar concentrations of flg22, demonstrating sensitivity greater than that displayed by other techniques. The combination of sensitivity

and minimal material requirements makes the POX assay an excellent additional screening tool for the field.

RESULTS

Workflow.

POX activity has been used previously to characterize flg22 activity in a petri dish format using cell culture suspension (Felix et al. 1999). While the assay showed excellent dose-dependent responses, it is laborious, hard to quantify, and unsuitable for high-throughput screening applications. We focused on developing a POX assay in a 96-well microtiter plate format using plant tissue rather than cell culture in order to increase the ease of use and throughput of the technique. The assay is conducted using a single 4-mm diameter leaf disk, which is accommodated in a microplate well. The assay can also be performed with larger leaf disks if increased activity is needed.

The small amount of plant tissue required for each assay permits high replication and allows for the study of tissue from a single leaf under a variety of compounds or conditions. This approach avoids the natural variation observed between plants and leaves and allows for a greater sensitivity and specificity, especially if the response is subtle, as is expected with weaker elicitors and incomplete mutant plant phenotypes.

The harvest of the leaf tissue induces physical damage, releasing POX enzymes into the media from the site of the cut. To remove this background activity, we washed the leaf disks for 1 h in fresh 1× Murashige Skoog (MS) media, after which the disks are arrayed into the 96-well plate with fresh media containing the desired treatments, after which only induced enzyme activity will accumulate. This procedure reduces the background of the assay, thereby increasing the observed sensitivity. As the assay is carried out in a minimal volume (50 µl), the amount of MAMPs required is also minimal. We seal the plate with Parafilm prior to the incubation step to minimize the effect of evaporation on the small volumes of the assay.

After an overnight incubation, the leaf tissue is removed from the wells and the assay substrate solution (50 µl of a 1 mg/ml solution of 5-aminosalicylic acid, pH 6.0, with 0.01% hydrogen peroxide) is then added to the remaining media in each well. This should be done with a multichannel pipette in a manner that will allow for minimal timing variability between treatments. At this point, the colored end-product is produced and the reaction is stopped by alkalization of the media through the addition of 20 µl of 2 N NaOH, followed by quantification at an optical density of 600 nm (OD₆₀₀).

Kinetics and enzyme dependence of the POX assay.

To characterize the kinetics of the plant POX response to flg22, we performed a time-course experiment with a fixed dose of flg22 (Fig. 1). Unlike the ROS burst observed using the luminol-based assay, which occurs over minutes, measurable POX activity is not observed until 16 h after treatment and increased levels are seen after 22 h. To ensure that the observed activity is, in fact, due to POX enzyme activity, we use the POX inhibitor salicylhydroxamic acid (SHAM) while treating with flg22 (Fig. 2). While the vehicle-treated samples show increased POX activity after flg22 treatment, the SHAM-treated samples show no response. In addition, we can observe a loss of background POX activity in these samples, resulting in a lower POX activity in SHAM-treated samples.

Quantifying PTI in *Arabidopsis*.

To test the sensitivity of the assay, we directly compared POX assay results to a traditional luminol-based ROS burst assay modeled after its adaptation for a 96-well microtiter plate (Albert et al. 2010). Using our optimized POX assay,

Fig. 1. Time course of peroxidase (POX) activity following flg22 application. Leaf disks from mature *Arabidopsis thaliana* ecotype Col-0 plants were treated with water or 100 nM flg22 peptide for the noted time period. Each timepoint had an associated control sample, with the exception of timepoints up to 2 h, which shared a common control. The graph shows data from a single representative experiment. Total POX activity was measured and data is presented as percentage of the appropriate water-treated control. The water control box is a compilation of all such samples and is shown for illustrative purposes only (mean ± standard error, $n = 6$, three asterisks (***) indicate $P < 0.001$, four (****) $P < 0.0001$, analysis of variance with Dunnett's multiple comparisons test or student's t test, as appropriate). Boxes show the lower quartile value, median value, and upper quartile value, while the whiskers extend to the lowest and highest values. The experiment was repeated twice with similar results.

Fig. 2. Treatment with peroxidase (POX) inhibitor eliminates response to flg22. Leaf disks from mature *Arabidopsis thaliana* ecotype Col-0 plants were treated with either 2 mM salicylhydroxamic acid (SHAM) or a vehicle control. At the same time, the noted dose of flg22 peptide or water was added to the wells. The graph shows data from a single representative experiment. Total POX activity was measured 20 h after treatment and data is presented as percentage of the water-treated control from the same SHAM or vehicle treatment (mean ± standard error, $n = 6$, one asterisk (*) indicates $P < 0.05$, two (**) $P < 0.01$, analysis of variance with Dunnett's multiple comparisons test). Boxes show the lower quartile value, median value, and upper quartile value, while the whiskers extend to the lowest and highest values. The experiment was repeated six times with similar results.

we performed a dose-response experiment using increasing concentrations of flg22 (Fig. 3A). The POX assay showed measurable activity with elicitor concentrations as low as 10 pM, although the activity only reached statistical significance at 100 pM. In contrast, ROS activity was only seen to increase once the elicitor was in the nanomolar range and reached statistical significance at 100 nM of flg22 (Fig. 3B). This observation is in line with reports in the literature, where commonly used doses for ROS burst assays range from the nanomolar to low micromolar range (Halter et al. 2014; Koller and Bent 2014; Yeh et al. 2016, 2015). This demonstrates that the improved POX assay performs better than the ROS assay at low MAMP concentrations, suggesting it would be preferable when studying weaker elicitors or subtle phenotypic changes.

Quantifying PTI in response to other MAMPs and DAMPs.

To confirm that the POX assay can detect a variety of innate immune activators, we tested the dose-dependent response to chitin and ATP. Treatment with increasing doses of the MAMP

chitin results in elevated POX activity, which reaches statistical significance at 100 mg/ml levels (Fig. 4A). In the literature, chitin is commonly used at dosages above 100 mg/ml to induce even small ROS bursts in *Arabidopsis* (Nekrasov et al. 2009). Thus, the POX assay responds at least as well as the ROS burst assay, though direct dosage comparisons may be difficult due to differences in chitin source, purity, and preparation. Increased activity was also observed following treatment with the DAMP ATP at doses above 100 μ M (Fig. 4B), demonstrating that the assay recognizes DAMPs in addition to MAMPs. An appropriate comparison in the literature using the traditional ROS burst technique is not available for ATP, but experiments using fluorescent probes to observe ATP-induced ROS accumulation in *Arabidopsis* root show marginal effects at 20 μ M, with robust activation with dosages of 100 to 200 μ M ATP (Clark et al. 2010; Demidchik et al. 2009).

Receptor dependence of the POX assay.

To ensure that the reaction we observe using the POX assay is specific to the flg22 treatment and not due to a nonspecific peptide response, we also tested flg22 in both *fls2* plants and the

Fig. 3. The peroxidase (POX) assay shows superior sensitivity at low flg22 doses. Leaf disks from mature *Arabidopsis thaliana* ecotype Col-0 plants were treated with water or the noted dose of flg22 peptide. Graphs show data from a single representative experiment. **A**, Total POX activity was measured 20 h after treatment and data is shown as percentage of the water treated control (mean $\pm$ standard error [SE], $n = 7$, one asterisk (*) indicates $P < 0.05$, four (****) $P < 0.0001$, analysis of variance [ANOVA] with Dunnett's multiple comparisons test). **B**, ROS production was measured for 2 s every 2 min for 60 min and total relative light units (RLU) were summed (mean $\pm$ SE, $n = 7$, four asterisks (****) indicate $P < 0.0001$, ANOVA with Dunnett's multiple comparisons test). Boxes show the lower quartile value, median value, and upper quartile value, while the whiskers extend to the lowest and highest values. Each experiment was repeated a minimum of five times with similar results.

Fig. 4. The peroxidase (POX) assay responds in a dose-dependent manner to chitin and ATP. **A**, Leaf disks from mature *Arabidopsis thaliana* ecotype Col-0 plants were treated with water or the noted dose of chitin or **B**, ATP. Graphs show data from a single representative experiment. Total POX activity was measured 20 h after treatment and data presented is shown as a percentage of the water-treated control (mean $\pm$ standard error, $n = 8$, one asterisk (*) indicates $P < 0.05$, four (****) $P < 0.0001$, analysis of variance with Dunnett's multiple comparisons test). Boxes show the lower quartile value, median value, and upper quartile value, while the whiskers extend to the lowest and highest values. Each experiment was repeated a minimum of seven times with similar results.

Wassilewskija (Ws) ecotype. Both *fls2* and Ws lack the ability to respond to flg22, due to mutation of the FLS2 receptor (Gómez-Gómez and Boller 2000; Gómez-Gómez et al. 1999). Even at the highest tested dose of 1 μ M flg22, neither *fls2* nor Ws show any increased POX activity, while Col-0 plants show a robust response (Fig. 5), indicating that the POX assay performs as expected and accurately reflects the innate immune reaction to flg22 treatment.

Quantifying PTI in other plant species.

Reliable immune assays in crop plant species are also needed to quickly and effectively translate research findings into marketable products. To test whether the POX assay is suitable for the study of PTI in crop species, we tested its efficacy in both tomato and bell pepper, which have previously been shown to respond to flg22 (Clarke et al. 2013). We treated tomato cultivar Glamour and bell pepper cultivar Fascinato with increasing doses of flg22 and measured POX activity. Both species showed excellent dose response, with significantly increased POX activity again observed in the picomolar range (Fig. 6). Other groups have also adopted the assay and optimized it for use in other species, demonstrating its utility even in nonmodel organisms, such as the invasive weed yellow starthistle (Kaczowka et al. 2017).

DISCUSSION

We have developed a sensitive, medium to high-throughput method to measure plant PTI activation via the activity of extracellular POX enzymes. The plant-specific class III POXs are encoded by large numbers of genes in terrestrial plants, including 73 sequences in *Arabidopsis* (Tognolli et al. 2002). The enzymes in this class have a signal sequence targeting them for the secretory pathway, and, once outside the cell, they play important roles in a variety of processes, including defense, growth, and development (Shigeto and Tsutsumi 2016). In relation to plant defense, these enzymes are known to act in the initial ROS burst upon MAMP treatment (O'Brien 2012) in addition to the canonical role they play in cell-wall fortification

Fig. 5. Response to flg22 treatment is FLS2-dependent. Leaf disks from mature *Arabidopsis thaliana* plants were treated with water or 1 μ M of flg22 peptide. The graph shows data from a single representative experiment. Total peroxidase (POX) activity was measured 20 h after treatment of Col-0, *fls2*, and ecotype Wassilewskija (Ws) plants and data is shown as percentage of the water-treated control (mean $\pm$ standard error, $n = 7$, three asterisks (***) indicate $P < 0.001$, Student's t test). Boxes show the lower quartile value, median value, and upper quartile value, while the whiskers extend to the lowest and highest values. Each experiment was repeated a minimum of three times with similar results.

via lignification and other processes (Shigeto and Tsutsumi 2016). While there is considerable interest in assigning biological function to individual enzymes, such characterization is limited by several factors. First, the large expansion of the family is thought to have led to functional redundancy, complicating traditional reverse-genetic approaches. Second, POXs can act both as generators of ROS and as catalytic enzymes while switching between these functions based on several factors, including substrate availability and other reaction conditions. Thus, determination of in-vitro enzyme function does not necessarily correlate to in-vivo biological activity. Given these limitations, we are unable to identify the enzyme responsible for the observed POX activity, but it is likely to be involved in cell-wall reinforcement rather than ROS burst, as any burst-associated activity would be lost during the initial wash step.

While this new method provides advantages over other PTI assays, there are important technical considerations relevant to its use. The first is the choice of an appropriate endpoint, to prevent any treatment condition from causing saturation and a resulting loss of resolution. The assay also requires the reaction

Fig. 6. Peroxidase (POX) assay functions in both tomato and pepper. Leaf disks were treated with water or the noted dose of flg22 peptide. Total POX activity was measured 20 h after treatment and data is shown as percentage of the water-treated control. Graphs are data from a single representative experiment. **A**, Leaf disks from tomato cultivar Glamour (mean $\pm$ standard error [SE], $n = 8$, one asterisk (*) indicates $P < 0.05$, four (****) $P < 0.0001$, analysis of variance [ANOVA] with Dunnett's multiple comparisons test). **B**, Leaf disks from sweet pepper cultivar Fascinato. (mean $\pm$ SE, $n = 8$, one asterisk (*) indicates $P < 0.05$, two (**) $P < 0.01$, and four (****) $P < 0.0001$, ANOVA with Dunnett's multiple comparisons test). Boxes show the lower quartile value, median value, and upper quartile value, while the whiskers extend to the lowest and highest values. Each experiment was repeated four times with similar results.

substrate be prepared fresh for each experiment, and the resulting variation in the substrate, in addition to minor changes in environmental conditions and reaction timing between experiments, means that direct comparisons between readings from different experiments must be approached with caution. The use of relative measures, using internal controls for normalization or the adoption of a fold-increase framework, makes such comparisons more robust. Finally, users should be cognizant of the risk of increased background from induced tissue damage. This risk is compounded by the number of POX enzymes in plant tissue and their promiscuous activity in a given condition, providing many potential sources of background activity. The leaf-disk harvest induces edge damage, which is removed during the wash step, however, the user must be careful not to damage the disks during the transfer steps in order to minimize background release.

With these caveats noted, the POX assay should be a valuable new tool in the PTI research space. While no single assay should be depended upon to function in all contexts, the method shows greater sensitivity than traditional ROS burst assays, making it a candidate for screening weaker elicitors and subtle phenotypic changes (Mott et al. 2016). This will allow for screening of novel MAMPs, and the identification not only of immune receptors but of proteins that modulate the immune response in nonbinary fashions. The same assay can also be used in crop species and nonmodel organisms, allowing for translational studies to be conducted and findings from *Arabidopsis* to be quickly tested in other species.

MATERIALS AND METHODS

Plant materials and growth conditions.

All plants were grown, with a 12-h photoperiod (100 to 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 21°C followed by a 12-h dark period at 20°C, in a Conviron growth room (Conviron).

POX assay.

Single leaves were excised from *Arabidopsis*, tomato, or sweet pepper plants. Leaf disks (diameter 4 mm) were taken from each leaf and were washed for 1 h in 1 ml of 1× MS solution, with agitation. After washing, disks were carefully transferred to individual wells of a clear 96-well assay plate, using forceps while minimizing damage. Each well received 50 μl of 1× MS buffer with vehicle control (H_2O) or was supplemented with the noted dose of flg22 peptide, chitin, or ATP, unless otherwise noted. Plates were sealed with Parafilm and were incubated for 20 h at room temperature with agitation, unless otherwise noted. The leaf disks were removed and each well received 50 μl of a 1-mg/ml solution of 5-aminosalicylic acid, pH 6.0, with 0.01% hydrogen peroxide, using a multichannel pipette to minimize timing differences. The reaction was allowed to proceed for 1 to 3 min and was stopped by the addition of 20 μM 2 N NaOH, using a multichannel pipette in the same order as above, to ensure equal time for enzymatic reaction in each well, prior to reading the OD₆₀₀ on an Infinite M1000Pro microplate reader (Tecan Group Ltd.).

Luminol-based ROS generation assay.

Measurement of ROS production was performed using a modified version of the luminol-based assay as described (Felix et al. 1999). Leaves were excised from mature Col-0 *A. thaliana* plants and were vacuum-infiltrated with sterile water. The leaves were then washed in sterile water for 5 h, with agitation. Single leaf disks (diameter 4 mm) were then taken and were placed in individual wells of a 96-well assay plate with 150 μl of sterile water, until the commencement of the assay. The water was removed and was replaced with sterile water containing 20 μg of horseradish POX per milliliter and 34 μg of

luminol per milliliter. Wells were treated with vehicle control (H_2O) or the noted dose of flg22 peptide. The luminescence was summed over a 2-s interval every 2 min for a period of 30 min, on an Infinite M1000Pro microplate reader (Tecan Group Ltd.).

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