Chapter 26

Analysis of the Immunity-Related Oxidative Bursts by a Luminol-Based Assay

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Abstract

The rapid production of reactive oxygen species (ROS) in response to biotic and abiotic cues is a conserved hallmark of plant responses. The detection and quantification of ROS generation during immune responses is an excellent readout to analyze signaling triggered by the perception of pathogens. The assay described here is easy to employ and versatile, allowing its use in a multitude of variations. For example, ROS production can be analyzed using different tissues including whole seedlings, roots, leaves, protoplasts, and cultured cells, which can originate from different ecotypes or mutants. Samples can be tested in combination with any ROS-inducing elicitors, such as the FLS2-activating peptide flg22, but also lipids or even abiotic stresses. Furthermore, early (PAMP-triggered) and late (effector-triggered) ROS production induced by virulent and avirulent bacteria, respectively, can also be assayed.

Key words Reactive oxygen species (ROS), NADPH oxidase, Immunity, flg22, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*)

1 Introduction

The enhanced production of ROS is a hallmark response of plants to diverse stress stimuli. The production of ROS in response to pathogen attack, also known as the oxidative burst, was first described by Doke [1]. Doke described the production of superoxide ($O_2^{\bullet-}$) in potato tubers triggered by the infection of the oomycete *Phytophthora infestans* and also in potato tuber protoplasts in response to hyphal cell wall components [1, 2]. The generation of ROS has since been proposed to have various functions, such as antimicrobial activity, cross-linking of cell wall polymers at sites of attempted penetration by fungi, as a local signal to mediate stomatal closure, or as systemic secondary messengers to activate immune responses in distal tissues [3–5].

Early studies showed that the ROS production during an infection can be biphasic (Fig. 1). Virulent as well as avirulent pathogens trigger a general but relatively weak first oxidative burst that

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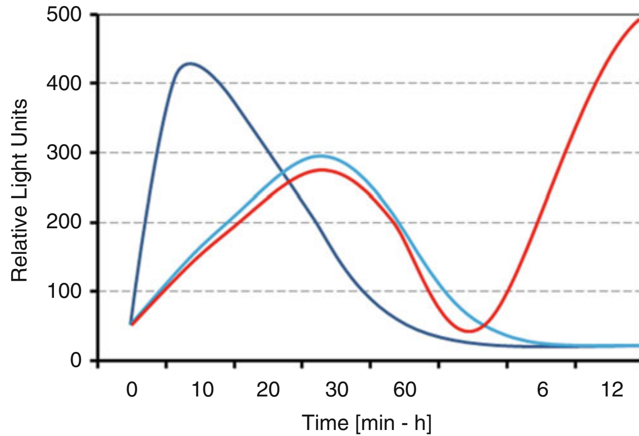

Fig. 1 ROS burst patterns during immune responses. The ROS production pattern differs depending on whether it is triggered by a purified minimal elicitor, such as flg22, or by pathogens. Leaf discs treated with flg22 react with a fast production of ROS which peaks at approximately 10 min after elicitation (*dark blue*). The response to virulent bacteria results in a weaker and delayed ROS generation, which peaks at approximately 30 min and is characteristic for the initiation PTI (*light blue*). The initial ROS burst of plants inoculated with avirulent bacteria is similar to that of virulent bacteria. By contrast, only avirulent bacteria activate a second massive production of ROS (*red*), which is characteristic for ETI

takes place within the first hour after infection. However, only avirulent pathogens trigger a second, massive, and prolonged oxidative burst, which is restricted to specific host cultivars and pathogen race combinations (Fig. 1) [4]. The early oxidative burst was first described as being a "general unspecific reaction." However, it has become clear that this reaction is the result of the perception of pathogen-associated molecular patterns (PAMPs) by plasma membrane located receptor-like kinases (RLKs). The best described example is the flagellin-sensitive 2 (FLS2) receptor of the bacterial flagellin [6, 7]. Activation of RLKs by the perception of PAMPs is the initial step in the activation of ROS production and PAMP-triggered immunity (PTI) [3, 5].

The second oxidative burst, on the other hand, is dependent on the recognition of pathogen virulence factors, so-called effectors, which are perceived directly or indirectly, by a resistance (R)-gene product, generally a nucleotide-binding leucine-rich repeat (NB-LRR) protein [8]. These intracellular sensors mediate the activation of effector-triggered immunity (ETI). The second stronger ROS production is a hallmark of the hypersensitive response (HR) which includes cell death [8].

Several sources for ROS production have been proposed or identified. However, it is important to differentiate between extracellular (apoplastic) and the intracellular generation of ROS [3]. In *Arabidopsis thaliana* (hereafter *Arabidopsis*), the apoplastic ROS

production triggered by RLKs such as FLS2 is completely dependent on the NADPH oxidase respiratory burst homologue (RBOH)D [9], but may also rely after NADPH oxidase activation on the cell wall peroxidases (PRX)33 and 34 [10]. NADPH oxidases catalyze the generation of superoxide, which can either spontaneously or enzymatically be converted into hydrogen peroxide (H_2O_2) by superoxide dismutases or peroxidases. By contrast, the second ROS burst triggered during ETI is likely to include sources such as chloroplasts, peroxidases, and mitochondria [3].

ROS production, or to be precise, hydrogen peroxide generation, can be quantified using luminol. Luminescence from luminol requires its activation by an oxidant which in this case is the indirect NADPH oxidase-dependent generation hydrogen peroxide and hydroxide ions. In the presence of the horseradish peroxidase, which acts as a catalyst, the hydrogen peroxide is decomposed to form oxygen and water. The reaction of luminol with the hydroxide ion results in the formation of a dianion. The oxygen released from the hydrogen peroxide subsequently reacts with the luminol dianion. As a result, an unstable organic peroxide is generated, electrons change from the excited to the ground state, and finally energy is emitted in the form of a photon which can be quantified by a luminescence detector.

2 Materials

2.1 Media for Plants and Bacteria

1. Medium for *Arabidopsis* seedlings: Half-strength Murashige and Skoog (MS) salts with vitamins, sucrose 0.5 % [w/v], MES 500 mg/l pH 5.6.
2. King's B medium for *Pseudomonas*: Add to 1 L distilled water, 10 g proteose peptone no. 2 (Difco), 1.5 g anhydrous K_2HPO_4, 15 g glycerol, 5 ml $MgSO_4$ (1 M stock, sterile), antibiotics (as needed). Add water to first three ingredients, adjust the pH to 7.0 with HCl, and bring volume to 1 L. Autoclave and then add 5 ml of sterile 1 M $MgSO_4$ and antibiotics (as needed).

2.2 Solutions and Chemicals

1. Reaction solution: Prepare a 500× horseradish peroxidase stock solution of 10 mg/ml in water. Make 50 μl aliquots and store at −20 °C. Prepare a 500× stock solution of 15 mg/ml luminol in DMSO and store at −20 °C. Example: To prepare 10 ml of a 20 μg/ml peroxidase and 30 μg/ml luminol reaction solution mix 20 μl of each of the stock solutions with 10 ml of water.
2. Peptide elicitors, such as flg22, are diluted from a 10 mM stock solution in water.

2.3 Equipment

1. Microtiter plate reader, e.g., Tecan Infinite F200 PRO fitted with a luminometer.

3 Methods

3.1 Plant Material

1. Stratify *Arabidopsis* seeds for 3 days at 4 °C and subsequently grow under short day conditions (8-h light/16-h dark), 65 % humidity, 21 °C, and 120–150 μmol/m^2 s.
2. To analyze leaves use adult plant material, sow *Arabidopsis* seeds directly onto soil and grow plants for 5–7 weeks. Use fully expanded leaves of the same age (*see* **Note 1**).
3. To analyze seedlings, sterilize approximately 100 *Arabidopsis* seeds with chlorine gas using 100 ml of commercially available bleach and add carefully 5 ml of 37 % HCl. Close the desiccator and sterilize for 3–4 h. Subsequently, ventilate seeds for 30 min and submerge in half-strength MS in an appropriate vessel such as a petri-dish or multiwall plate. After 10 days, pass two to three seedlings into a well from a 96-well plate, also containing half-strength MS such that they stay submerged, and grow for another 2 days before measurement.
4. To analyze roots, sterilize seeds as described above and grow seedlings vertically on solid half-strength MS medium for approximately 14 days.

3.2 Bacteria

1. Streak out the desired strain of bacteria, e.g., *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*, empty vector with Kan resistance) [11] from a glycerol stocks (stored at −80 °C) onto King's B medium agar plates containing 50 μg/ml kanamycin and 30 μg/ml rifampicin 1 day before the assay.
2. Incubate inoculated KMB plates for 24 h at 28 °C.
3. For elicitation, scrape bacteria from plates and wash twice in sterile water by centrifugating at 5000 × *g* for 10 min and resuspending the pellet.
4. Measure the OD_{600} employing spectrophotometer to quantify bacteria of a 1:10 dilution, to ensure measurement within the linear range.
5. The final bacterial solution can be adjusted to an OD_{600} of 0.1 (approx. 1×10^6 colony-forming units/ml). Ideal concentration of the bacterial suspension may need to be determined individually for different strains.

3.3 Sample Preparation

1. Leaves: Prepare leaf discs from fully expanded leaves (*see* **Note 2**) using a sharp biopsy puncher of 5 mm and swiftly place them with the abaxial side in contact with the water to avoid desiccation (critical, *see* **Note 3**). Leaf discs can additionally be sliced in half to increase the reaction area. Well plates of all sorts are suitable but 96-well plates allow more replicates.

2. Seedlings: 10 days after germination, pass two to three seedlings into a well from a 96-well plate, also containing half-strength MS such that they stay submerged. Allow to grow for another 2 days before measurement.
3. Roots: Prepare root tissue by cutting into in 0.5 cm long pieces using a new scalpel blade and place two to three pieces per well in a 96-well plate. Use the lower half of the roots and exclude the meristematic tissue area at the tip.

3.4 Measurement

1. Prepare samples as described above 1 day before assaying ROS production.
2. Float samples overnight at room temperature in 150 μl of sterilized tap water.
3. Immediately before starting the assay, prepare the reaction solution containing 20 μg/ml peroxidase and 30 μg/ml luminol.
4. Carefully remove the water in which the samples were incubated overnight without damaging sample tissue (critical).
5. Swiftly add 120 μl of the aqueous reaction solution using a multichannel pipet to avoid desiccation (critical) and incubate for 20 min at room temperature. Be careful not to stress the samples mechanically.
6. Add 120 μl of reaction solution containing double concentration of elicitor (e.g., peptide or bacteria) using a multichannel pipet and mix carefully (*see* **Note 4**).
7. Place samples in the microtiter plate reader or photon counting camera and wait for 1 min before starting to allow autofluorescence to cease.
8. Start monitoring luminescence (*see* **Note 5**).

4 Notes

1. The main drawback of the ROS assay is its variability. However, there are several steps that can be taken which will help to considerably increase reproducibility. Most important are the growth conditions; these need to be identical, especially light conditions. Use seeds harvested from plants grown simultaneously. It is imperative that plants do not have any sort of infection or wounding. Also, infestation with dark-winged fungus gnats, which produce larvae that will feed on leaves and roots, will result in considerable variability between samples.
2. We always obtained the most consistent results with plants older than 6 weeks and grown under short day. However,

attention must be paid to that there are no senescent leaves present on the plants.

3. The measured luminescence that is produced by the reaction of luminol with hydrogen peroxide in leaf discs originates from the edges resulting from cutting. Two points are pivotal: first, avoid desiccation of the edges and second, always use very sharp biopsy punchers which will reduce wounding responses.
4. Duration of the measurement is dependent on the elicitor, the bacteria strain, and the ROS burst type. Most elicitors which are perceived by RLKs, including flg22, elf18, but also chitin and oligogalacturonides, induce the production of ROS very quickly. Within 5–10 min, the ROS burst peaks and ebbs off in the next 50 min. However, when using virulent bacteria such as *Pst*, PAMP-dependent ROS production is triggered after 30–60 min and ebbs off within the next 30 min (Fig. 1) [12, 13]. By contrast, when measuring the oxidative burst which is triggered by the recognition of effectors by R-proteins such as NB-LRRs, ROS production starts 6–8 h after inoculation.
5. Luminol-based measurement of ROS production can be carried out with a variety of equipment that is able to detect luminescence. However, the bottleneck is always the sensitivity. Two settings may help to improve sensitivity: (1) increased binning when using a camera, although at the cost of a reduced resolution, and (2) increasing the integration time, which results in an increased dwelling time on each sample.

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