Measurement of Reactive Oxygen Species (ROS) in *Arabidopsis* and *N. benthamiana* plants

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- ROS measurement in *Arabidopsis* and *Nicotiana benthamiana* using a luminol reaction
- Usage of an IR cut off filter to eliminate signal of chlorophyll autofluorescence

Abstract

Production of reactive oxygen species (ROS) is a typical reaction of plants towards pathogen infections. Luminescence reactions can be used to measure these ROS and are therefore an easy way to analyze proteins involved in the early defense reaction against pathogens.

Introduction

Reactive oxygen species (ROS) are important molecules in plant cells. On the one hand, they occur as harmful by-products of aerobic metabolism, and have to be detoxified to prevent cell damage. On the other hand, they are actively produced by the plant. For example during root growth, ROS serve to loosen the cell wall. They also play a role in signaling during development and response to biotic and abiotic stress conditions[1].

Plants respond to pathogen attacks by mounting a multi-layered defense response[2]. The first recognition of pathogens occurs at the plasma membrane by specialized receptors that perceive conserved molecules, so called pathogen-associated molecular patterns (PAMPs), released by the pathogen. In *Arabidopsis thaliana*, two of these receptors detecting PAMPs from *Pseudomonas syringae* are well-characterized. FLS2 recognizes a conserved epitope of the bacterial motor protein flagellin flg22[3], whereas EFR recognizes elf18, an epitope of the prokaryotic elongation factor Tu[4]. Upon recognition of their respective PAMP, both receptors trigger a transient burst of ROS in the apoplast. This burst is mediated by the NADPH oxidase RbohD, which is localized in the plasma membrane. The rapid induction of this burst, within minutes upon PAMP perception and its transience, makes it an ideal response to study early events in plant immunity.

Here, we report on the measurement of transient production of ROS in response to pathogen attacks performing a luminescence coupled assay. This assay allows for a quantitative determination of ROS by performing a redox-reaction. Horseradish peroxidase reduces ROS while oxidizing luminol into an excited aminophtalate (AP*). Under the emission of blue (420 nm) light the excited AP* returns to its ground state. These measurements can serve as a robust output of pathogen induced signaling in *Arabidopsis* as well as in *N. benthamiana* leaves.

Experimental Procedures

1. Oxidative burst in *Arabidopsis*

Arabidopsis plants were grown for 4-6 week on soil under long-day conditions (16 h light, 8 h dark). 4 mm leaf disks were transferred to H_2O in a 96-well microtiter plate, and incubated overnight. H_2O was ex-changed 1 h prior to the measurement. To determine the background four cycles of luminescence measurement were performed. To induce PAMP-triggered ROS production, a 2x assay solution was injected leading to a final concentration of 10 ng/mL peroxidase, 20 µM luminol and 100 nM flg22 protein.

2. Oxidative burst in *N. benthamiana*

N. benthamiana were grown for 4-5 weeks before leaves were infiltrated with *Agrobacterium tumefaciens* carrying either an estradiol inducible EFR-GFP and/or p19 (silencing suppressor) plasmid as described before[5]. Three days after infiltration leaves were sprayed with 20 µM β-estradiol inducing EFR-GFP expression. 4 mm leaf discs of the induced

N. benthamiana leaves were cut out and floated abaxial site down in a petri dish filled with water over night. The next day the still floating leaf discs were carefully transferred abaxial site down into a white 96 well microtiter plate (Berthold) in 50 µL water each for 1 hour. ROS production was measured by injecting 50 µL of a 2x assay solution (containing 1 µM elf18 protein, 40 µM luminol and 20 ng/mL HRP) to each sample at time point 0.

Material

- Mithras LB 940 Multimode Reader with injectors (Berthold Technologies; figure 1)
- Filter 650 nm short pass built in position 8 in the emission wheel

Figure 1: Mithras LB941 Multimode Reader

Instrument Settings

Injectors were used to add assay solution to the microplate after 4 rounds of background measurements. An extra 650 nm SP filter was used to eliminate background signals caused by autofluorescence of chlorophyll. This autofluorescence – so called delayed fluorescence – is a weak light in the infra-red range emitted by pre-illuminated plants which lasts for minutes or even hours after illumination. Repeated luminescence measurements of the plate were done for 1 h with a counting time of 1 s and a cycle time of 129 s (*N. benthamiana*) respectively 150 s (*Arabidopsis*) resulting in 28 (*N. benthamiana*) respectively 21 (*Arabidopsis*) data points. Data analysis of kinetic was done with MikroWin 2000 and Excel software (figure 2). For each time point the average value of 8 replicates was used and standard deviations determined.

Results

We have measured ROS induced by flg22 in *Arabidopsis*. After 5 minutes upon treatment, ROS are produced by the plant. This burst is absent in the receptor mutant *fls2* (6), since flg22 is not perceived any more (Figure 3).

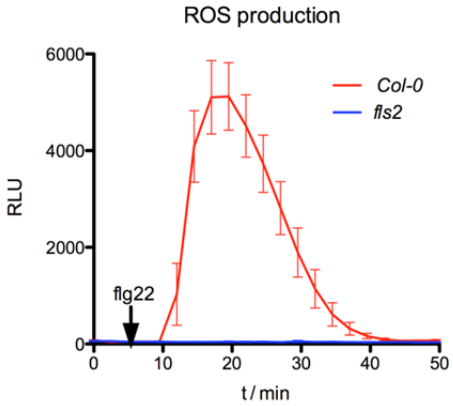

Figure 3: Measurement of reactive oxygen species (ROS) after flg22 addition in leaf discs of *Col-0* and *fls2* receptor mutant *Arabidopsis thaliana*. Shown are the average values ± standard deviations of relative luminescent units (RLU) (n=8 for each data point).

Elf18 induced ROS production in *N. benthamiana* leaves could also be measured (figure 4). As already reported EFR is not naturally expressed in *N. benthamiana* leaves and only when EFR is transiently expressed the expected ROS production can be triggered by elf18 addition (Zipfel, 2006). The p19 leaves serve as a negative control. The receptor like kinase EF-Tu (EFR) of Arabidopsis recognizes the bacterial oligopeptide elf18 of the elongation factor Tu (EF-Tu) (Zipfel et al. 2006). After binding of elf18 to EFR receptor a very fast response is an oxidative burst which can be measured by a luminol/HRP.

Conclusion

Measurement of ROS induced by PAMPs is a valuable tool to identify and characterize PAMPs, to analyze mutants with regard to their immune system and to transfer the knowledge from Arabidopsis to other plant species. To avoid background signals caused by autofluorescence of chlorophyll the usage of a 650nm SP filter proved to be a successful and easy way to eliminated chlorophyll emitted light.

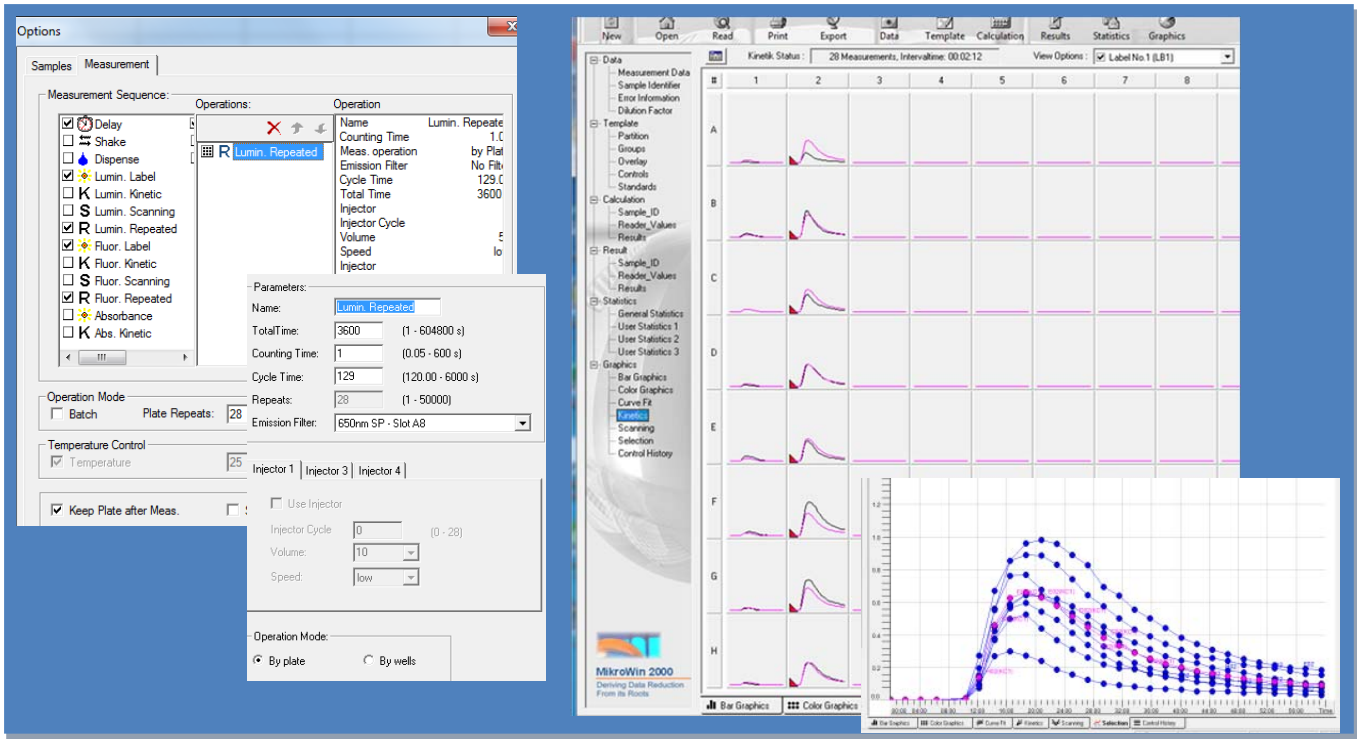

Figure 2: Software settings and data analysis done with MikroWin 2000. A. Settings, B. Kinetic Analysis: blue graphs show kinetic for single wells, pink graph based on average values.

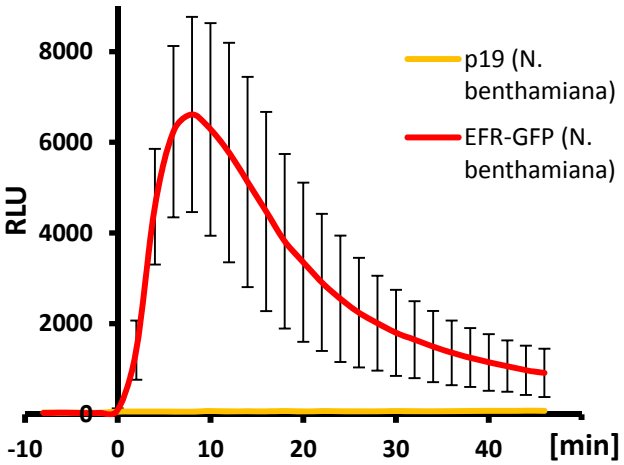

Figure 4: Measurement of ROS after elf18 addition in leaf discs of control (p19) and EFR-GFP expressing *Nicotiana benthamiana* plants. Shown are the average values ± standard deviations of relative luminescent units (RLU) (n=8 for each data point)

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