Chapter 6

Improved ROS Measurement in Root Hair Cells

Silvina Paola Denita Juárez*, Silvina Mangano*, and José M. Estevez

Abstract

Reactive oxygen species (ROS) are recognized as important signaling components in various processes in plants. ROS are produced for NADPH oxidase in different subcellular compartments and they are involved for a wide range of stimuli, such as cell cycle, growth, plant defenses, abiotic stress responses, and abscisic acid signaling in guard cells. In Arabidopsis, root hairs ROS also play a key role in root hair growth and they control the activity of calcium channels required for polar growth (Takeda et al. Science 319:1241–1244, 2008). The production of reactive oxygen species is under a specific molecular control in order to avoid detrimental side effects. Here we describe a protocol to detect ROS by oxidation of a derivative of fluorescein: 2′,7-dihidro dicloro fluorescein (H2DCFDA).

Key words ROS, Root hair, 2′,7-Dihidro dicloro fluorescein

1 Introduction

Reactive oxygen species (ROS) play a key signal transduction role in cells. They are involved in different physiological process like growing, development, homeostasis, and survival of plants [1]. The level of ROS in cells is determined by interplay between ROS producing pathways and ROS scavenging mechanisms, part of the ROS gene network of plants. Key players are NADPH oxidases (NADPHox). These catalyze the production of superoxides, a type of reactive oxygen species (ROS) which is rapidly converted to hydrogen peroxide [2].

In plant NADPH oxidase regulates developmental programs such as polarized cell expansion in root hair formation [3] and pollen tip growth [4]. In Arabidopsis, the Root Hair Defective 2 (RHD2) or AtrbohC protein is required for root elongation. The roots of plants homozygous for loss-of-function rhd2 mutations have decreased levels of ROS and are shorter than the wild type, indicating that cell expansion is defective in these plants [3, 5] and the ROS production is crucial for tip growth of root hair.

*Both the authors contributed equally for this chapter.

José M. Estevez (ed.), *Plant Cell Expansion: Methods and Protocols*, Methods in Molecular Biology, vol. 1242,
DOI 10.1007/978-1-4939-1902-4_6,

Fluorophores typically change their emission properties due to the interaction with ROS [5]. The addition of exogenous fluorescence probes, which penetrate into the cell and change their fluorescence properties due to reaction with ROS. In principle, the permeability across membranes and the ROS specificity are necessary for the applicability of the exogenous ROS-sensing fluorophores. However, for a quantitative analysis, it is necessary to know the reaction mechanism in detail, as well as possible interfering side effects and the cellular localization of these dyes. Some of these ROS probes can be turned regarding their properties inside the cell by enzymatic reactions. For example, 2′,7′-Dichlorofluorescin diacetate is a nonfluorescent probe in the reduced state and diffuses passively through the cellular membrane. Into the cytosol it is rapidly cleaved by unspecific esterases. The resulting molecule emits green fluorescence when it is oxidized and it is excited with blue light [6]. It could be oxidized by different ROS like anion superoxide, hydrogen peroxide, or nitrogen monoxide. This technique does not differ between the different ROS. We describe here an optimized technique by which to measure ROS specifically in the root hair.

2 Materials

1. *Arabidopsis thaliana* seeds.
   Arabidopsis thaliana Col-0 ecotype and mutants seeds can be obtained from the Arabidopsis Biological Resource Center. All mutants in this work are T-DNA insertions lines.
2. Agar 1 %.
3. H2DCFDA 50 mM (Sigma).
4. Sterilized water.
5. Slide and coverslip.

2.1 Equipment

1. Biological Control Camera (Bio-Control).
2. Confocal Microscopy (Olympus FV300/BX61).

3 Method

3.1 Plant Growth

Plants were grown in Agar 1 % (Chemit) at 22 °C under continuous light conditions for 10 days.

3.2 ROS Measurements

1. Grow sterilized Arabidopsis seed in plate with agar 1 % sterile for 8 days in chamber at 25 °C with a continuous light.
2. Remove seedlings from sterile culture and incubate in darkness in a slide for 10 min with the H2DCFDA 50 μM at room temperature (*see* **Note 1**).
3. Wash the seedling with Milli Q water.

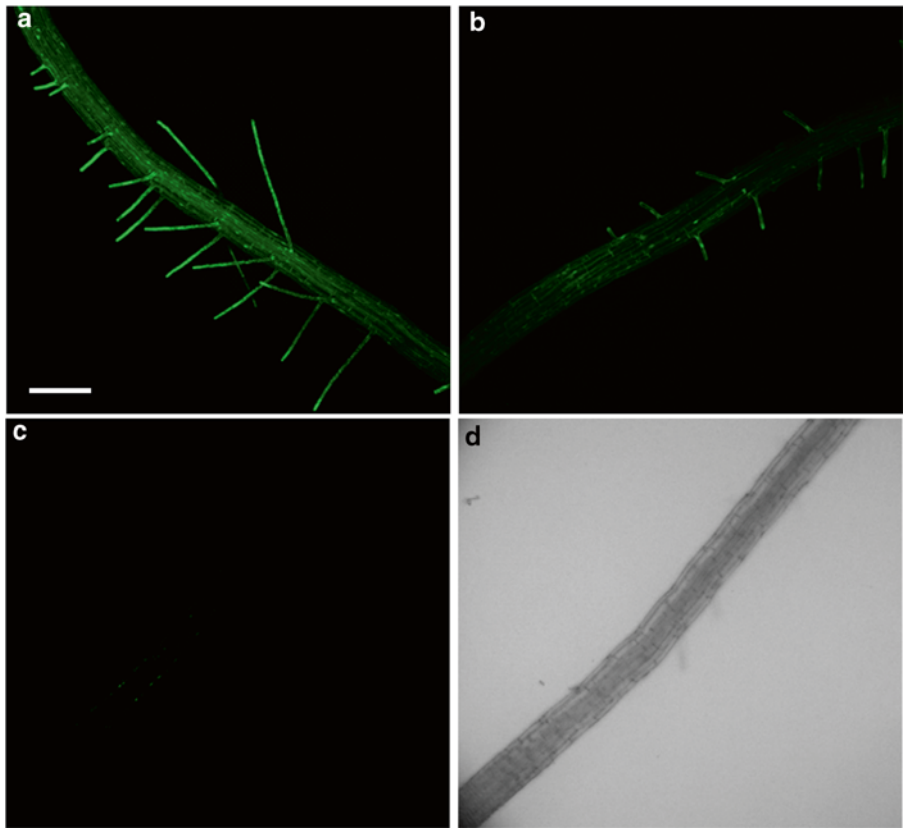

Fig. 1 Images that obtained with this technique. (**a**) ROS in a wild type root hair. (**b**) ROS in a rbohc, a insertional mutant. This mutant has been described with short root hair, which is a product of decrease in ROS levels [3]. (**c**) Wild type root with 140 nM of DPI. DPI is an inhibitor of NADPH oxidases [1]. (**d**) Wild type root with Bright field view

4. Mount seedlings in water and cover the sample with a cover slip for direct microscopic observation.
5. Examine the samples (*see* **Note 2**) using a confocal microscope equipped with the 488 nm argon laser and BA510IF filter sets. Use 10× objective and 0.30 NA (*see* **Note 3**).
6. Take the image scanning XZY with a 2 μm between the focal planes. Take all the optical sections necessary to get a clearly image of the root (Fig. 1).
7. Transfer the large data files to a computer (Fig. 2). Images were taken in different root hair developmental stages in order to measure ROS levels during cell expansion process.

3.3 Analyze the Images with ImageJ

1. Open the *Stack* in ImageJ.
2. Integrate the slices. Click on *Image—Stacks—Z project*: Projection type: Max Intensity.
3. Select an area including the root hair (*see* **Note 4**).

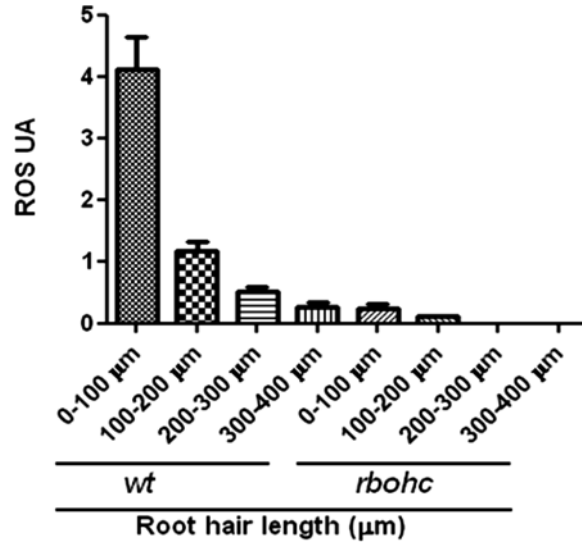

Fig. 2 Example of the experience. We examine the root hairs in different states of development. Measure ROS in different stages of root hair is important because levels can oscillate during the development

4. Quantify the intensity. Click on *Analyze—Set Measurement*, select Mean gray value. Click on *Analyze—Measure* (*see* **Note 5**).
5. Measure length.
6. Create a table to upload data.

4 Notes

1. H2DCFDA is highly sensitive to light.
2. As a negative control it was used a WT seedling incubated in a slide for 10 minutes in water (without the H2DCFDA) at room temperature in darkness. Another negative control was root hairs treated with 140 nM DPI (Fig. 1).
3. Parameters used were laser: 4.7, PMT: 503, gain: 1.1, off set: 3 %, exposure time: fast. This condition was set so as not to detect autofluorescence from root.
4. For quantification with Image J, only include the area of the root hair, be careful you do not include background.
5. Another option is to click on the letter M in the keyboard.

References

1. Suzuki N, Miller G, Morales J, Shulaev V, Torres M, Mittler R (2011) Respiratory burst oxidases: the engines of ROS signaling. Plant Biol 14:691–699
2. Roppolo D, De Rybel B, Tendon V, Pfister A, Alassimone J, Vermeer J, Yamazaki M, Stierhof Y, Beeckman T, Geldner N (2011) A novel protein

family mediates Casparian strip formation in the endodermis. Nature 473:380–384

3. Takeda S, Gapper C, Kaya H, Bell E, Kuchitsu K, Dolan L (2008) Local positive feedback regulation determines cell shape in root hair cells. Science 319:1241–1244
4. Potocký M, Jones M, Bezvoda R, Smirnoff N, Zársky V (2007) Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. New Phytol 174:742–751
5. Foreman J, Demidchik V, Bothwell J, Mylona P, Miedema H, Torres M, Linstead P, Costa S, Brownlee C, Jones J (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422: 442–446
6. Karlsson M, Kurz T, Brunk U, Nilsson S, Frennesson C (2010) What does the commonly used DCF test for oxidative stress really show? Biochem J 428:183–190