Chapter 18

In Planta Analysis of Leaf Mitochondrial Superoxide and Nitric Oxide

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Abstract

Superoxide (O_2^-) and nitric oxide (NO) are produced within plant mitochondria and may have signaling functions within the cell. Here we describe semiquantitative fluorescence imaging-based approaches to estimate the mitochondrial amount of these reactive and short-lived species within intact leaf tissue. We also outline a biochemical method using oxyhemoglobin to measure NO within a whole leaf tissue extract. This quantitative method, while not specifically evaluating mitochondrial localized NO, does nonetheless provide another independent measure of NO that can be useful.

Key words DAF-FM, Fluorescence confocal microscopy, Leaf tissue, Mitochondria, MitoSOX, Nitric oxide (NO), Oxyhemoglobin, Superoxide (O_2^-)

1 Introduction

A secondary consequence of plant mitochondrial electron transport chain (ETC) activity is the leakage of single electrons to O_2, producing the superoxide radical anion (O_2^-). This reactive oxygen species (ROS) may then be converted to other ROS such as H_2O_2 and the hydroxyl radical [1]. There is accumulating evidence that plant mitochondria are also a source of reactive nitrogen species (RNS) [2]. For example, electron leakage from the ETC to nitrite can produce nitric oxide (NO). NO may then react with O_2^-, producing another RNS, peroxynitrite.

It seems that plant cells can use mitochondrial ROS and RNS as signaling molecules to control diverse plant processes [3–5]. However, there are major challenges to studying these signaling functions. Methods are required to evaluate the concentration and subcellular localization of these short-lived and reactive species. Further, methods are required that better distinguish between the different specific species of ROS and RNS within the cell. Further, the investigator must strive to maintain physiological conditions as much as possible during such measurements. For example, while

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isolated mitochondria have been instrumental in determining the general mitochondrial metabolic conditions that promote ROS generation by the ETC [1], they would not be an appropriate system to elucidate a broader signaling function of these ROS within the intact cell or tissue and in response to particular internal or external (environmental) conditions.

In the case of mitochondria, O_2^- and NO are species of particular interest since these are the ROS and RNS that are immediate by-products of ETC activity. Since NO is volatile, methods for its detection and quantification can be divided into those that attempt to directly measure the dissolved *in planta* tissue concentration of NO and those that attempt to indirectly estimate tissue concentration by measuring the NO being released to the atmosphere [6]. Classic early experiments that demonstrated the presence and biological significance of NO in plant tissues made use of an oxyhemoglobin-based biochemical assay to measure the NO [7, 8].

The concentrations of NO and O_2^- in intact cells and tissues can be evaluated using cell-permeable small-molecule probes, combined with live cell imaging by fluorescence microscopy [9, 10]. Compartment-specific O_2^- probes such as the mitochondrion-localizing fluorescent probe MitoSOX (Invitrogen), provide a means to directly establish the local concentration of this ROS [11]. Such an analysis is aided by the inability of O_2^- to readily cross membranes, hence minimizing its potential to relocate elsewhere following generation within the mitochondrion. We provide below a protocol to assess mitochondrial O_2^- in the mesophyll cells of near-intact tobacco leaf (lower epidermis removed) using MitoSOX. Unlike O_2^-, NO readily crosses biological membranes, complicating efforts to understand its localized concentration, as well as its site(s) of synthesis [2]. We provide below two methods to examine NO in tobacco leaf. An oxyhemoglobin-based biochemical assay provides a direct *in planta* measure of total NO in a leaf homogenate. Meanwhile, the cell-permeable small-molecule probe DAF-FM (Invitrogen) [12] combined with fluorescence confocal microscopy provides an independent measure of NO concentration in the near-intact leaf. In this case, double-labeling experiments utilizing DAF-FM and a mitochondrion-localizing fluorescent probe such as MitoTracker (Invitrogen), followed by colocalization analyses of their fluorescent signals, provide one means to evaluate the local (mitochondrial) concentration of NO.

2 Materials

2.1 Reagents and Equipment for Fluorescence Imaging of O_2^- and NO

1. MitoSOX Red mitochondrial superoxide indicator (Life Technologies, Invitrogen #M36008) is stored at –20 °C as a desiccated stock and protected from light. A 5 mM stock solution is made by dissolving the reagent in dimethyl sulfoxide

(DMSO) (*see* **Note 1**). A working solution (3 μM in distilled water) is prepared fresh on the day of use and kept in the dark.

2. 4-Amino-5-methylamino-2′7′-difluorofluorescein diacetate (DAF-FM diacetate, Invitrogen #D23844) is stored at –20 °C as a desiccated stock and protected from light. A 5 mM stock solution is made by dissolving the reagent in DMSO (*see* **Note 1**). A working solution [10 μM in a DAF-FM loading and wash buffer (10 mM KH_2PO_4, pH 7.4)] is prepared fresh on the day of use and kept in the dark.
3. MitoTracker Red (Invitrogen #M22425) and MitoTracker Green (Invitrogen #M7514) are stored at –20 °C as desiccated stocks and protected from light. A 1 mM stock of each is prepared by adding DMSO (*see* **Note 1**). When using MitoTracker Green in double-labeling experiments with MitoSOX Red, a working solution of MitoTracker Green (0.35 μM in distilled water) is prepared fresh on the day of use and kept in the dark. When using MitoTracker Red in double-labeling experiments with DAF-FM diacetate, a working solution of MitoTracker Red (0.35 μM in 10 mM KH_2PO_4, pH 7.4) is prepared fresh on the day of use and kept in the dark.
4. Standard microscope slides and coverslips.
5. Flat-end tweezers and scalpel blades.
6. Sealing grease: We use Dow Corning high-vacuum silicone grease (Sigma-Aldrich, St. Louis, MO, USA).
7. Confocal laser scanning microscope with appropriate filter combinations: We use an LSM510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany).
8. Software for image processing and analysis: We use ImageJ (open source) with the JACoP plug-in for colocalization analyses.
9. The cell-permeable O_2^- scavenger superoxide dismutase-polyethylene glycol (SOD-PEG) (Sigma-Aldrich #S9549), the cell-permeating NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (Sigma-Aldrich #C221), the NO donor sodium nitroprusside (SNP) (Sigma-Aldrich #S0501), and the mitochondrial Complex III inhibitor antimycin A (Sigma-Aldrich #A8674).

2.2 Reagents and Equipment for NO Quantification Using Oxyhemoglobin

1. Hemoglobin: We use lyophilized hemoglobin from bovine blood (Sigma-Aldrich #H2500).
2. Tris buffer (50 mM Tris, pH 7.0).
3. Sodium hydrosulfite ($Na_2S_2O_4$).
4. Sephadex G-25 column: We use prepackaged disposable PD-10 desalting columns (GE Healthcare, Cooksville, ON, Canada).

5. Source of pure O_2.
6. Extraction buffer: 100 mM KH_2PO_4 pH 7.0, 0.6 % (w/v) insoluble PVP (added after autoclaving).
7. Powdered activated carbon.
8. Catalase: We use catalase from bovine liver supplied as an aqueous solution, >30,000 U/mg protein (Sigma-Aldrich #C3155).
9. Superoxide dismutase (SOD): We use SOD from bovine erythrocytes, supplied as a lyophilized powder, 2,500–7,000 U/mg protein (Sigma-Aldrich #S2515).
10. Spectrophotometer to measure absorbance at 401 and 421 nm.

3 Methods

All of the methods have been optimized for use with tobacco plants (*Nicotiana tabacum* L. cv Petit Havana SR1). The plants are grown in controlled-environment growth chambers (Model PGR-15, Conviron, Winnipeg, Canada) with a 16-h photoperiod, a temperature of 28/22 °C (light/dark), a relative humidity of 60 %, and at an irradiance of 130–150 μmol/m^2/s. Examples of published work utilizing the methods can be found elsewhere [13–16].

3.1 Detection of O_2^- and NO Using Fluorescence Confocal Microscopy

1. Obtain fresh leaf tissue and use flat-end tweezers to gently peel off the lower epidermis.
2. Using a scalpel blade, cut a section of leaf (~5-mm square) from the tissue lacking lower epidermis and place it in one of the working solutions of probe (as described in Subheading 2.1, **items 1–3**). Ensure that the side of the leaf lacking lower epidermis is in contact with the solution (*see* **Note 2**). For small samples, it is convenient to use 0.5 mL of solution in a 2-mL microcentrifuge tube, while for larger samples, a 5 mL solution in a small dish works fine also.
3. Incubate the samples for 30 min in the dark at room temperature (RT) to allow for loading of the probe into the tissue.
4. Transfer the samples from the working solutions to the same solution but without probe (distilled water or 10 mM KH_2PO_4, pH 7.4) to wash the sample. Several washes may be necessary to remove probe that has not been loaded into cells (*see* **Note 3**). Protect the sample from light during these washes.
5. Apply a uniformly thin layer of sealing grease to the edges of a coverslip and add several drops of the washing solution directly on the coverslip. Place the leaf sections in this solution, ensuring that the side without epidermis is facing down against the coverslip. Carefully place a microscope slide atop the coverslip while ensuring that no air bubbles become trapped in the sample.

6. The prepared slides should be protected from light and viewed under the microscope as soon as possible.
7. The microscope should be adjusted to the appropriate excitation/emission settings (MitoSOX Red, 488/585–615 nm; MitoTracker Green, 488/500–530 nm; MitoTracker Red, 543/585–615 nm; DAF-FM diacetate, 488/500–530 nm) (*see* **Note 4**). A recent study suggests that the excitation/emission settings for MitoSOX may be further optimized [17]. However, regardless of the settings used, it is critical to verify their suitability through a series of control experiments (*see* **steps 8** and **9** below).
8. It is critical to perform a series of controls prior to starting a new experiment in order to confirm the validity of the images being obtained. To ensure no confounding effects due to sample autofluorescence, obtain images of unlabeled plant tissue (prepared as above but without probe) using the same acquisition settings and longest exposure times being used for labeled samples. Chlorophyll autofluorescence should be detected at higher wavelengths (>630 nm) but not in the 585–615 and 500–530 nm range. Further, examine the fluorescence of a cell-free mixture of probe and loading/washing solution over an extended period of time to confirm no rise in fluorescence signal resulting from auto-oxidation of probe. Finally, in cases where the tissue is being double-labeled with two probes, establish that there is no significant bleed-through and cross talk between channels using a single-labeled experiment.
9. It is critical that additional positive and negative controls are used to ensure that the signals resulting from MitoSOX Red and DAF-FM labeling are due to the presence of O_2^- and NO, respectively, in the sample tissue. This is most effectively confirmed by preincubating leaf segments (with lower epidermis removed) with various donors, inducers, and scavengers of O_2^- and NO. In our experiments, we treated leaf segments with antimycin A (10 μM, 1 h, RT), which is known to increase the production of mitochondrial O_2^-, prior to loading with MitoSOX Red. This treatment dramatically elevated MitoSOX Red fluorescence. Further, a co-treatment (1 h, RT) with both antimycin A and SOD-PEG (100 U/mL) strongly reduced the subsequent MitoSOX Red signal. Alternatively, pretreatment with SNP (2 mM, 1 h, RT, 130 μmol/m^2/s) dramatically increased subsequent DAF-FM fluorescence, while a co-treatment (1 h, RT, 130 μmol/m^2/s) with both SNP and cPTIO (200 μM) could strongly reduce the subsequent DAF-FM signal.
10. Establish clear criteria for selecting cells for image acquisition and subsequent analysis. Special care should be taken to follow these criteria throughout the experiment as the

mesophyll cells are irregular in shape. Confirm that the sample has similar fluorescence signal throughout by observing many fields of view.

11. All images within an experiment should be acquired using the same microscope settings. It is necessary to take separate images in all channels in double-labeling experiments.
12. Using a choice of image analysis software, overlay images taken in different channels. For example, overlay MitoTracker Red fluorescence images collected in the red channel with DAF-FM fluorescence images collected in the green channel (*see* **Note 5**).
13. Threshold each channel to differentiate between the presence of the fluorophore and background fluorescence. Image quality is critical to successfully establish the spatial relationship between two fluorophores in double-labeling experiments, so it is important to eliminate noise (background fluorescence, reflection from cell walls, low-level autofluorescence, etc.) from the images.
14. Colocalization between two fluorophores (for example, colocalization of MitoTracker Red and DAF-FM) can be evaluated using a variety of methods and coefficients, depending on the software used. We have used the Pearson's coefficient, where a value of 1 corresponds to absolute colocalization of the two fluorophores, a value of 0 indicates random localization, and a value of −1 indicates an absolute mutual exclusion of the two signals. We have also used the Manders' coefficient, which represents the fraction of red pixels in the one fluorescence channel that overlap with green pixels in the second fluorescence channel. This coefficient will range from 0 (random localization) to 1 (complete colocalization). We recommend using at least two methods of colocalization analysis when examining the relationship between two fluorophores.

3.2 Detection of NO Using the Oxyhemoglobin Method

1. The method was adapted from that described to measure NO in animal tissues [18].
2. Condition the Sephadex G-25 column by filling the column with Tris buffer and allowing the solution to penetrate the packing bed fully. The conditioning procedure should be repeated at least four times and the flow-through discarded. The column should be used immediately.
3. On the day of use, gently dissolve 25 mg of hemoglobin in 2 mL of Tris buffer in a 4 mL glass vial. When fully dissolved, the solution should be dark red or brown.
4. Add ~4 mg of sodium hydrosulfite and swirl gently. The solution should turn dark purple.

5. Cover the open vial with Parafilm and blow a gentle stream of O_2 into the vial for 5–10 min with gentle shaking. The color of the solution should change to bright red indicating a change from methemoglobin to oxyhemoglobin (*see* **Note 6**).
6. Add ~1.5 mL of the oxyhemoglobin solution directly to the Sephadex G-25 column and wait until the solution penetrates the packing bed completely. Then add ~1 mL of Tris buffer.
7. Elute the sample with ~4 mL of Tris buffer. Collect the eluate in a glass vial and then close the vial with a cap and intact septum. Care should be taken to avoid the leading and trailing ends of the eluate (*see* **Note 7**). The eluate (oxyhemoglobin stock solution) should now be kept on ice and in the dark. A syringe through the septum should be used to sample from the stock.
8. Determine the concentration of the stock oxyhemoglobin by diluting an aliquot 100× in Tris buffer and measuring its absorbance at 415 nm. Calculate the concentration using the extinction coefficient of 131/mM/cm. For best results, the solution should be used as soon as possible.
9. Grind leaf tissue under liquid nitrogen.
10. Transfer 150 mg of leaf powder to a 1.5-mL microcentrifuge tube. Add 1 mL of extraction buffer and vortex thoroughly (30 s).
11. Add powdered activated carbon (~100 mg) and vortex again (30 s).
12. Centrifuge the sample (11,000 × *g*, 10 min, 4 °C).
13. Remove the supernatant to a fresh tube and repeat **steps 10** and **11** until the sample is clear (i.e., no green).
14. Transfer an aliquot of the clear supernatant (~700 uL) to a fresh tube. To each sample add 100 U of catalase and 50 U of SOD. Briefly mix the sample and incubate at RT for 5 min (*see* **Note 8**).
15. Add 10 μM of the freshly prepared oxyhemoglobin to the sample, briefly vortex the sample, and incubate at RT for 5 min.
16. Measure the absorbance of the sample at 401 nm and 421 nm. Calculate the NO concentration by subtracting the absorbance at 421 nm from the absorbance at 401 nm, and using the extinction coefficient of 77/mM/cm (*see* **Note 9**).

4 Notes

1. MitoSOX Red and DAF-FM stock solutions in DMSO can be stored at −20 °C in the dark for up to 1 month. MitoTracker stock solutions in DMSO are stable for up to 3 months when stored at −20 °C in the dark.

2. For good image quality, it is critical not to damage the mesophyll layer during these steps. Be careful not to touch this layer with tweezers or a scalpel blade at any time.
3. The number and duration of the washes should be determined empirically. The goal of these washes is to obtain a sample that shows clear and defined fluorescence signals, by limiting the amount of nonspecific fluorescence arising due to the presence of excess probe. The majority of our experiments did not require extensive washing.
4. The testing and use of appropriate excitation/emission combinations is critical due to the autofluorescence inherent in plant tissues. The detection range for each probe should be as narrow as possible in order to avoid collecting fluorescence signal that is not due to the probe.
5. If the experiment involves the acquisition of z-stacks, make sure that you are analyzing a single image layer at a time. This is as opposed to analyzing a maximum intensity projection of the entire stack which might not reflect spatial relationships between the two fluorophores.
6. The conversion from methemoglobin to oxyhemoglobin can also be achieved with a stream of air (rather than pure O_2) but this procedure takes a longer time and does not give as high oxyhemoglobin yields.
7. The hemoglobin solution should be desalted quickly to avoid conversion of oxyhemoglobin to methemoglobin. After passing through the Sephadex G-25 column, the color of the solution should remain bright red. A stream of O_2 can be used if the solution begins to turn brown.
8. Catalase and SOD are added to scavenge H_2O_2 and O_2^-, which could otherwise react with the oxyhemoglobin.
9. A test can be performed to check the sensitivity of the assay. SNP can be dissolved in 10 mM KH_2PO_4 (pH 7.4) to different final concentrations (0.5, 1, 2.5, 5, and 10 mM). These samples are incubated under light (400 μmol/m^2/s) for 1 h at RT to facilitate NO release. Under these conditions, 1 mM SNP releases ~5 μM NO [19]. The samples are then treated with catalase and SOD prior to the addition of 10 μM oxyhemoglobin and the measurement of NO concentration (as described above). This test is useful to estimate the range of plant NO concentrations that the assay might be expected to measure. If the NO concentrations in plant samples do not fall within this measurable range, then a possible solution is to empirically adjust the oxyhemoglobin concentration being used.

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