Video Article

Catalytic Scavenging of Plant Reactive Oxygen Species *In Vivo* by Anionic Cerium Oxide Nanoparticles

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Abstract

Reactive oxygen species (ROS) accumulation is a hallmark of plant abiotic stress response. ROS play a dual role in plants by acting as signaling molecules at low levels and damaging molecules at high levels. Accumulation of ROS in stressed plants can damage metabolites, enzymes, lipids, and DNA, causing a reduction of plant growth and yield. The ability of cerium oxide nanoparticles (nanoceria) to catalytically scavenge ROS *in vivo* provides a unique tool to understand and bioengineer plant abiotic stress tolerance. Here, we present a protocol to synthesize and characterize poly (acrylic) acid coated nanoceria (PNC), interface the nanoparticles with plants via leaf lamina infiltration, and monitor their distribution and ROS scavenging *in vivo* using confocal microscopy. Current molecular tools for manipulating ROS accumulation in plants are limited to model species and require laborious transformation methods. This protocol for *in vivo* ROS scavenging has the potential to be applied to wild type plants with broad leaves and leaf structure like *Arabidopsis thaliana*.

Video Link

The video component of this article can be found at https://www.jove.com/video/58373/

Introduction

Cerium oxide nanoparticles (nanoceria) are widely used in living organisms, from basic research to bioengineering, due to their distinct catalytic reactive oxygen species (ROS) scavenging ability[1,2,3]. Nanoceria have ROS scavenging abilities due to a large number of surface oxygen vacancies that alternate between two oxidation states (Ce^{3+} and Ce^{4+}) [4,5,6]. The Ce^{3+} dangling bonds effectively scavenge ROS while the lattice strains at the nanoscale promote the regeneration of these defect sites via redox cycling reactions[7]. Nanoceria have also been recently used for studying and engineering plant function[8,9]. Plants under abiotic stress experience accumulation of ROS, causing oxidative damage to lipids, proteins, and DNA[10]. In *A. thaliana* plants, nanoceria catalytic scavenging of ROS *in vivo* leads to improved plant photosynthesis under high light, heat, and chilling stresses[8]. Applying nanoceria to soil also increases shoot biomass and grain yield of wheat (*Triticum aestivum*)[11]; canola (*Brassica napus*) plants treated with nanoceria have higher plant biomass under salt stress[12].

Nanoceria offer bioengineers and plant biologists a nanotechnology-based tool to understand abiotic stress responses and enhance plant abiotic stress tolerance. Nanoceria's *in vivo* ROS scavenging capabilities are independent of plant species, and the facile delivery into plant tissues has the potential to enable broad application outside of model organisms. Unlike other genetically-based methods, nanoceria do not require generating plant lines with the overexpression of antioxidant enzymes for higher ROS scavenging ability[13]. Leaf lamina infiltration of nanoceria to plants is a practical approach for lab-based research.

The overall goal of this protocol is to describe 1) the synthesis and characterization of negatively charged poly (acrylic) acid nanoceria (PNC), 2) the delivery and tracking of PNC throughout leaf cells, and 3) the monitoring of PNC-enabled ROS scavenging *in vivo*. In this protocol, negatively charged poly (acrylic) acid nanoceria (PNC) are synthesized and characterized by their absorption spectrum, hydrodynamic diameter, and zeta potential. We describe a simple leaf lamina infiltration method to deliver PNC into plant leaf tissues. For *in vivo* imaging of nanoparticle distribution within mesophyll cells, a fluorescent dye (DiI) was used to label PNC (DiI-PNC) and observe the nanoparticles via confocal fluorescence microscopy. Finally, we explain how to monitor *in vivo* PNC ROS scavenging through confocal microscopy.

Protocol

1. Growing *A. thaliana* Plants

1. Sow *A. thaliana* seeds in 5 cm x 5 cm disposable pots filled with standard soil mix. Put 32 of these pots into a plastic tray filled with water (~ 0.5 cm depth) and transfer the plastic tray with the plants into a plant growth chamber.
    1. Set the growth chamber settings as follows: 200 µmol/ms photosynthetic active radiation (PAR), 24 ± 1 °C day and 21 ± 1 °C night, 60% humidity, and 14/10 h day/night light regime, respectively.
2. Thin each pot to leave only one individual plant after one week of germination. Take note to keep the seedlings with similar size in each pot.
3. Water the pots by pouring tap water directly on the plastic tray once every two days. Grow the plants for four weeks. *A. thaliana* plants are ready for further use.

2. Synthesis and Characterization of PNC

1. Weigh 1.08 g of cerium (III) nitrate and dissolve it in 2.5 mL of molecular biology grade water in a 50 mL conical tube.
2. Weigh 4.5 g of poly (acrylic) acid and dissolve it in 5 mL of molecular biology grade water in a 50 mL conical tube.
3. Mix these two solutions thoroughly at 2,000 rpm for 15 min using a digital vortex mixer.
4. Transfer 15 mL of ammonium hydroxide solution (7.2 M) to a 50 mL glass beaker.
5. While stirring at 500 rpm, add the mixture from Step 2.3 dropwise to the ammonium hydroxide solution and stir at 500 rpm at room temperature for 24 hr in a fume hood.
6. Cover the beaker with a piece of paper to avoid the substantial loss of solution during the overnight reaction.
7. After 24 h, transfer the resulting solution to a 50 mL conical tube and centrifuge it at 3,900 x g for 1 h to remove any possible debris and large agglomerates.
8. Transfer this 22.5 mL of supernatant solution into three 15 mL 10 kDa filters and fill the remainder of the filter with molecular grade water to make a total dilution of 45 mL.
9. Purify the supernatant solution from free polymers and other reagents with a benchtop centrifuge by adding the supernatant to a 15 mL 10 kDa filter and centrifuge at 3,900 x g for 15 min. Repeat this step at least six times.
10. Measure the absorbance of the eluent in each cycle with a UV-VIS spectrophotometer from 220-700 nm to ensure no free polymers and other reagents are present in the final PNC solution.
11. Take the collected PNC solution into the 5 mL syringe and filter it against a 20 nm pore size syringe filter. Collect the filtered PNC solution in a 50 mL conical tube.
12. Take a diluted final PNC solution in a plastic cuvette and measure its absorbance with the UV-VIS spectrophotometer from 220-700 nm. PNC absorbance peak is at 271 nm.
13. Calculate its concentration by using Beer-Lambert's law: $A = \varepsilon CL$. A is the absorbance of the peak value for a given sample, ε is the molar absorption coefficient of PNC (cm^{-1} M^{-1}), L is the optical path length (cuvette width, 1 cm in this method), and C is the molar concentration of measured nanoparticles.
14. Measure the hydrodynamic diameter and zeta potential of the synthesized PNC using a particle size and zeta potential analyzer (**Figure 1**).
15. Store the final PNC solution in a refrigerator (4 °C) until further use.
NOTE: Please refer to Wu *et al.*[8] for more protocol details about PNC characterization.

3. Labeling PNC with DiI Fluorescent Dye

1. Mix 0.4 mL of 5 mM (58 mg/L) PNC with 3.6 mL of molecular biology grade water in a 20 mL glass vial and stir at 500 rpm.
2. Add 24 µL 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate dye solution (DiI, 2.5 mg/mL; dilute in DMSO) into 176 µL of DMSO (dimethyl sulfoxide) to make the DiI dye solution.
3. Add the DiI dye dropwise to the PNC solution, stirring at 1,000 rpm for 1 min at ambient temperature.
4. Transfer this resulting mixture into a 15 mL 10 kDa filter and fill the tube to the top with molecular biology grade water to make the total dilution 15 mL.
5. Purify the DiI labeled PNC (DiI-PNC) solution from DMSO and any possible free DiI dye by a benchtop centrifugation with the 15 mL 10 kDa filter at 3,900 x g for 5 min.
    1. Repeat Step 3.5 at least five times.
6. Filter the final DiI-PNC solutions through a 20 nm pore size syringe filter.
7. Measure the absorbance of final DiI-PNC by UV-VIS spectrophotometry and calculate its concentration according to Beer-Lambert's law (**Figure 2**). See Step 2.13 for more details.
8. Store it in a refrigerator at 4 °C for further use.

4. Infiltration of Plant Leaves with PNC

1. Add 0.1 mL of infiltration buffer (100 mM TES, 100 mM $MgCl_2$, pH 7.5, adjusted by HCl) into 0.9 mL of 0.5 mM PNC or DiI-PNC solution and vortex it. Use a solution of 10 mM TES infiltration buffer as a negative control.
2. Transfer 0.2 mL of the PNC or DiI-PNC infiltration solution to a 1 mL sterile needleless syringe. Tap to remove any possible air bubbles.
3. Retrieve the plant from the growth chamber just before infiltration with nanoparticles to avoid possible stomata closure under room light conditions.

4. Before infiltration, measure the chlorophyll content from *A. thaliana* leaves with similar size using a chlorophyll meter. Measure each leaf with three replicates (each replicate consisting of at least three measurements)[14]. Choose the *A. thaliana* leaves with similar chlorophyll content for the infiltration experiment.
5. Infiltrate the leaves slowly with the recently prepared PNC or DiI-PNC solution by gently pressing the tip of the needleless syringe against the bottom of the leaf lamina (abaxial side) and depress the plunger (**Figure 3A**).
6. Gently wipe off the excess solution that remains on the surface of leaf lamina (**Figure 3B**) using a delicate task wiper (**Figure 3C**) and label the plant. Use new delicate task wipes for each group of leaves.
7. Keep the infiltrated *A. thaliana* plants on the bench for leaf adaptation and incubation with PNC or DiI-PNC for 3 h.
NOTE: Infiltrated *A. thaliana* plants are then ready for further use (**Figure 3D**).

5. Preparation of Leaf Samples for Confocal Microscopy

1. Roll a pea-size amount of observation gel to about a 1 cm radius (**Figure 4A**) and then spread it out until it is 1 mm thin on a glass slide (**Figure 4B**).
2. Use a cork borer (diameter 0.3 cm) to cut out a circular section at the center of the observation gel on the glass slide (**Figure 4C**).
3. Fill the cut well entirely with perfluorodecalin (PFD) for deeper and better confocal imaging resolution in leaf tissues.
4. Use a cork borer (diameter 0.2 cm) to collect leaf discs from the adapted DiI-PNC infiltrated *A. thaliana* plants (**Figure 4D**).
5. Mount the leaf disc in the PFD filled well; face the infiltrated (abaxial) side of the leaf up.
6. Put a square coverslip on top of the leaf disc and gently press on the slide coverslip evenly to seal it with the well of observation gel and ensure no air bubbles remain trapped (**Figure 4E**).

6. Imaging DiI-PNC in Leaf Tissues by Confocal Microscopy

1. Use a 40X objective lens in an inverted laser scanning confocal microscope.
2. Drop two to three drops of ddH_2O on the top of the 40X objective lens.
3. Place the prepared DiI-PNC infiltrated leaf sample slide on top of the inverted 40X objective lens.
    1. Make sure the coverslip side but not the glass slide contact directly with the ddH_2O on the lens.
4. Find a region of interest in the sample under the microscope with either laser light or bright field.
5. Start the microscope software and turn on the Argon laser (set at 20%).
6. Set the pinhole to collect an optical slice less than 2 µm and a line average of 4.
7. Image the sample with confocal microscope settings: 514 nm laser excitation (30 %); Z-Stack section thickness: 2 µm; PMT1: 550-615 nm (for DiI-PNC imaging); PMT2: 700-800 nm (for chloroplast imaging).
8. Take representative confocal images of leaf samples from different individuals, a minimum of three biological replicates.

7. Imaging PNC *in vivo* ROS Scavenging by Confocal Microscopy

1. Prepare 25 µM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, a dye for indicating a general ROS) and 10 µM dihydroethidium (DHE, a dye for indicating superoxide anion) dyes in TES infiltration buffer (pH 7.5) in 1.5 mL microcentrifuge tubes, separately.
2. Use a cork borer (diameter 0.2 cm) to collect leaf discs from the adapted PNC infiltrated *A. thaliana* plants.
    1. Use the sharp tip of the forceps to make three to four holes on the leaf discs to accelerate dye loading process.
3. Transfer the leaf discs to microcentrifuge tubes with H_2DCFDA and DHE separately and incubate for 30 min under darkness.
4. After incubation, rinse the leaf discs with ddH_2O three times and mount it into the glass slide with observation gel (see Protocol Section 5).
5. Put the slide on the confocal microscope and manually focus to a region of leaf mesophyll cells. See Protocol Section 6 for details.
6. Expose the leaf discs to the UV-A (405 nm) laser for 3 min to generate ROS and record the ROS signal intensity change in time-series ("xyt") per leaf disc.
7. Image the leaf disc with confocal microscope settings: 40X water objective; 496 nm laser excitation; PMT1: 500-600 nm (for DHE and DCFDA dye detection); PMT2: 700-800 nm (for chloroplasts detection). Use a plant infiltrated with only infiltration buffer solution as the negative control.

8. PNC Scavenging of H_2O_2 *in vitro*

1. Conduct the CAT (catalase) mimetic activity of the synthesized PNC *in vitro* by following the methods in previous publications[3,8,15]
2. Add 45.4 µL of 1x TES infiltration buffer (10 mM TES, 10 mM $MgCl_2$, pH 7.5, adjusted by HCl), PNC (60 nM, 3 µL), and H_2O_2 (2 µM, 1 µL) into a well (white round bottom 96 well plate), and gently mix it by pipetting.
3. Add 10-acetyl-3,7-dihydroxyphenoxazine (working concentration 100 µM, 0.5 µL) and horseradish peroxidase (HRP; working concentration 0.2 U/mL, 0.1 µL) into the well, gently mix it by pipetting, and incubate it for 30 min. 10-acetyl-3,7-dihydroxyphenoxazine reacts with H_2O_2 and is converted into resorufin in the presence of HRP.
    1. Wrap the plate with aluminum foil to avoid light during the incubation.
    2. Prepare a negative control by using reaction buffer or water to replace H_2O_2.
    3. Except for the stock solution, prepare all other solutions at ambient temperature.
4. After the incubation, with a plate reader, monitor the absorbance at 560 nm to use resorufin for indicating the level of H_2O_2. Set time regime at 0, 2, 5, 10, 20, and 30 min.

Representative Results

PNC synthesis and characterization.
PNC were synthesized, purified and characterized following the method described in Protocol Section 2. **Figure 1A** shows the coloration of the solutions of cerium nitrate, PAA, the mixture of cerium nitrate and PAA, and PNC. A color change from white to light yellow is seen after PNC is synthesized. After purification with a 10 kDa filter, PNC were characterized with a UV-VIS spectrophotometer. A peak of absorbance for PNC was observed at 271 nm (**Figure 1B**). The final eluent was also measured with UV-VIS to confirm that the non-reacted chemicals were washed during the purification. The hydrodynamic diameter and zeta potential of the synthesized PNC were measured with a particle sizer and zeta potential analyzer (**Figure 1C**).

PNC labeling with DiI dye.
To determine the distribution of the nanoparticles *in vivo*, PNC were labeled with a fluorescent DiI dye following the method described in Protocol Section 3. DiI dye embeds within the PNC coating spontaneously since it can encapsulate into the hydrophobic domains inside the polymer coatings of nanoceria[16]. After adding DiI dye to the PNC solution, a rapid color change to pink was observed (**Figure 2A**). The DiI labeled PNC were then purified with a 10 kDa filter and characterized by a UV-VIS spectrophotometry. Three clear peaks of absorbance for the DiI labeled PNC were observed (**Figure 2B**). The final eluent was measured by UV-VIS spectrophotometry to confirm that the non-reacted chemicals were washed out during the purification.

Leaf Lamina infiltration.
PNC or DiI-PNC were delivered into *A. thaliana* leaf via leaf lamina infiltration method as described in Protocol Section 4. Leaf was infiltrated at four different spots to ensure the full leaf area was perfused with PNC solution (**Figure 3A**). Any remaining solution was removed from the leaf surface (**Figure 3B** and **3C**). The leaf color changed during infiltration from green to darker green (**Figure 3D**). The syringe was gently pressed against the leaf to avoid any physical damage.

Leaf sample preparation for fluorescence microscopy.
Leaf samples were mounted on glass slides within an observation gel made well filled with PFD. After rolling the pea size observation gel on the slide (**Figure 4A** and **4B**), a well was made in the middle of the flat gel (**Figure 4C**). Then, the freshly prepared leaf disc was transferred to the well previously filled with PFD solution (**Figure 4D**). A coverslip was used to immobilize the leaf sample on the slide (**Figure 4E**).

Confocal imaging of DiI-PNC in vivo.
DiI-PNC infiltrated *A. thaliana* leaves were used for determining the distribution of DiI-PNC in leaf mesophyll cells via confocal imaging (**Figure 5A** and **5B**). To visualize colocalization between the DiI-PNC and chloroplasts, DiI-PNC infiltrated leaf samples were excited with a 514 nm laser. The emission of DiI-PNC was set at 550-615 nm to avoid the possible interference of chloroplast pigments signals after ~650 nm[17]. The chlorophyll auto-fluorescence from chloroplasts were detected from 700-800 nm. The confocal imaging settings were set (laser power and gain) to make sure no DiI dye signals were detected in the control leaf sample (infiltrated with only buffer) (**Figure 5C**). The colocalization of DiI-PNC with chloroplasts in leaf mesophyll cells can be observed by the overlay image of detected DiI-PNC and chloroplast pigment autofluorescence (**Figure 5D**).

Confocal imaging of PNC ROS scavenging *in vivo*.
PNC in 10 mM TES buffer solution were delivered into *A. thaliana* leaves via the leaf lamina infiltration method as described in Protocol Section 7. DHE (dihydroethidium) and H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) fluorescent dyes are used to visualize ROS in plant tissues[8,18]. H_2DCFDA is known to be converted to fluorescent DCF (2′,7′-dichlorofluorescein, an indicator of the degree of general oxidative stress) due to the cleavage of the acetate groups by ROS[19]. DHE is a more specific dye for superoxide anion having its fluorescent product (2-hydroxyethidium) increase upon reaction with a superoxide anion[20]. *In vivo* ROS scavenging enabled by PNC was monitored in leaf discs measuring DHE and DCF dye fluorescence intensity changes (**Figures 6A and 6B**). PNC infiltrated leaf samples were excited with 496 nm laser. The emission of DHE and DCF dye was set at 500-600 nm to avoid the possible interference with chloroplast auto-fluorescence signals. Pigment auto-fluorescence from chloroplasts were detected from 700-800 nm. After 3 minutes of UV stress, the ROS dye signals in PNC and buffer- infiltrated leaf samples were monitored separately. Compared to the no-nanoparticle buffer control (NNP), PNC infiltrated leaves showed significantly less ROS-activated fluorescent DCF dye signal (**Figure 6A**). Similar results were also found with the superoxide anion-activated DHE dye, where PNC infiltrated leaves had significantly less DHE dye intensity than buffer infiltrated control leaves (**Figure 6B**).

PNC CAT mimetic activity assay. Assay of PNC scavenging of H_2O_2 was described in Protocol Section 8. A decrease of resorufin which indicates H_2O_2 level in the reaction mixture containing PNC was observed (**Figure 7**), confirming the CAT mimetic activity of the synthesized PNC.

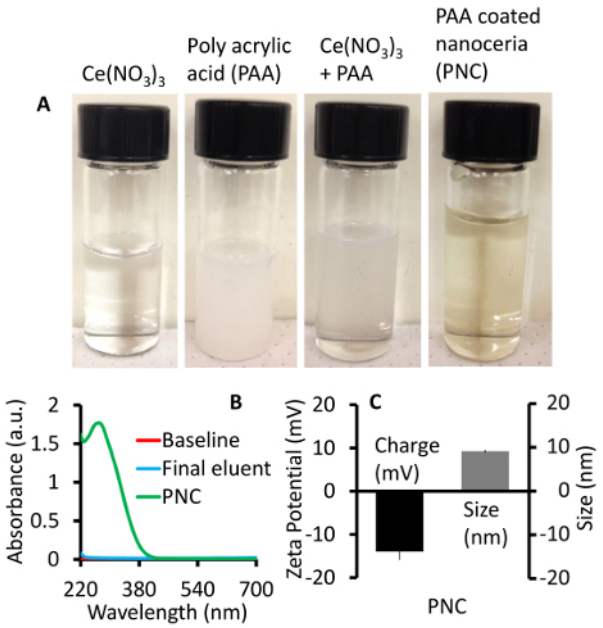

Figure 1: Synthesis and characterization of PNC. A. Coloration of cerium nitrate, poly acrylic acid (PAA), mixture of cerium nitrate and PAA, and the synthesized PNC (PAA coated cerium oxide nanoparticles, light yellow). B. Absorbance spectrum of PNC measured by UV-VIS spectrophotometry. C. Hydrodynamic diameter and zeta potential of the synthesized PNC. Mean ± standard error (n = 4). Please click here to view a larger version of this figure.

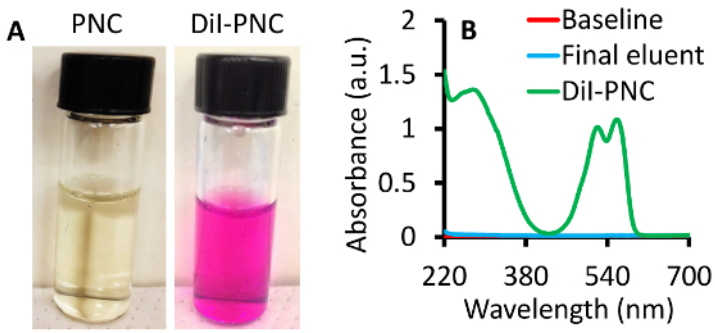

Figure 2: PNC labelling with DiI fluorescent dye. A. PNC (light yellow) and DiI dye labelled PNC solution (DiI-PNC, pink). B. Absorbance spectrum of DiI-PNC solution. Please click here to view a larger version of this figure.

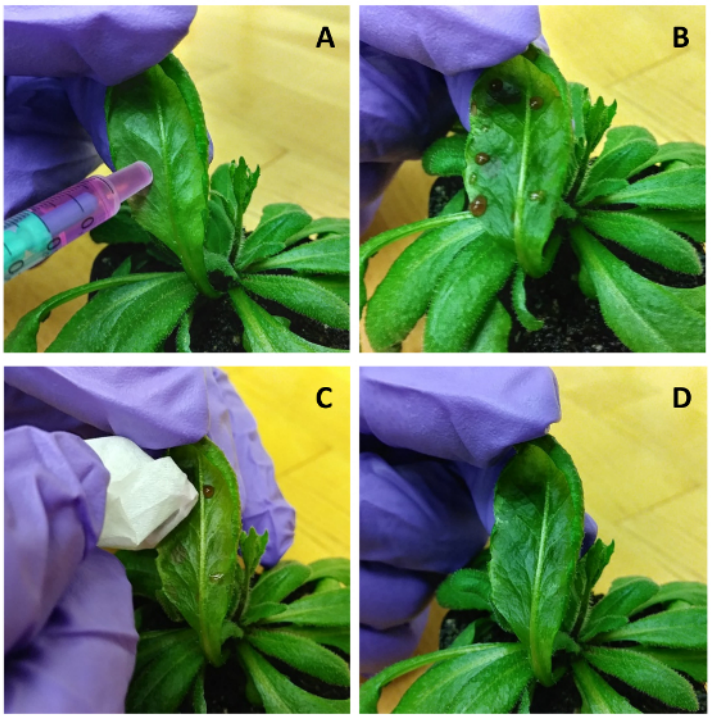

Figure 3: Leaf lamina infiltration of PNC or DiI-PNC. A. *A. thaliana* leaf before infiltration. The solution inside the syringe is DiI-PNC. B. Leaf infiltrated with DiI-PNC. C. Cleaning the remaining solution from the leaf surface with delicate task wipes. D. Cleaned *A. thaliana* leaf infiltrated with DiI-PNC. Please click here to view a larger version of this figure.

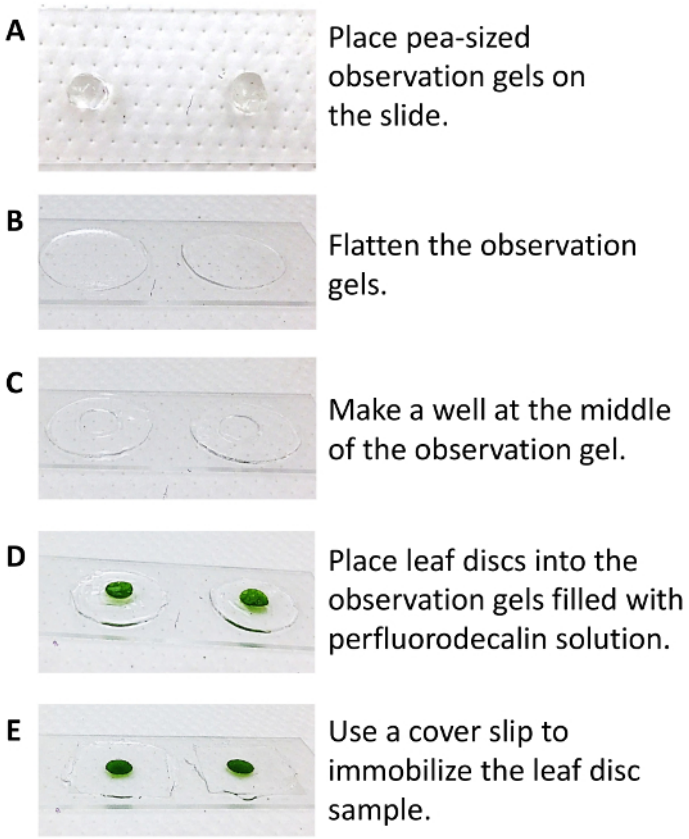

Figure 4: Preparation of leaf sample slides. A. Microscopy glass slide with pea size observation gels. B. Slide with flat observation gels. C. Slide with flat observation gel having a well at the middle. D. Slide with leaf discs in the observation gel well filled with perfluorodecalin (PFD) solution. E. Slide with leaf discs immobilized by cover slip. Please click here to view a larger version of this figure.

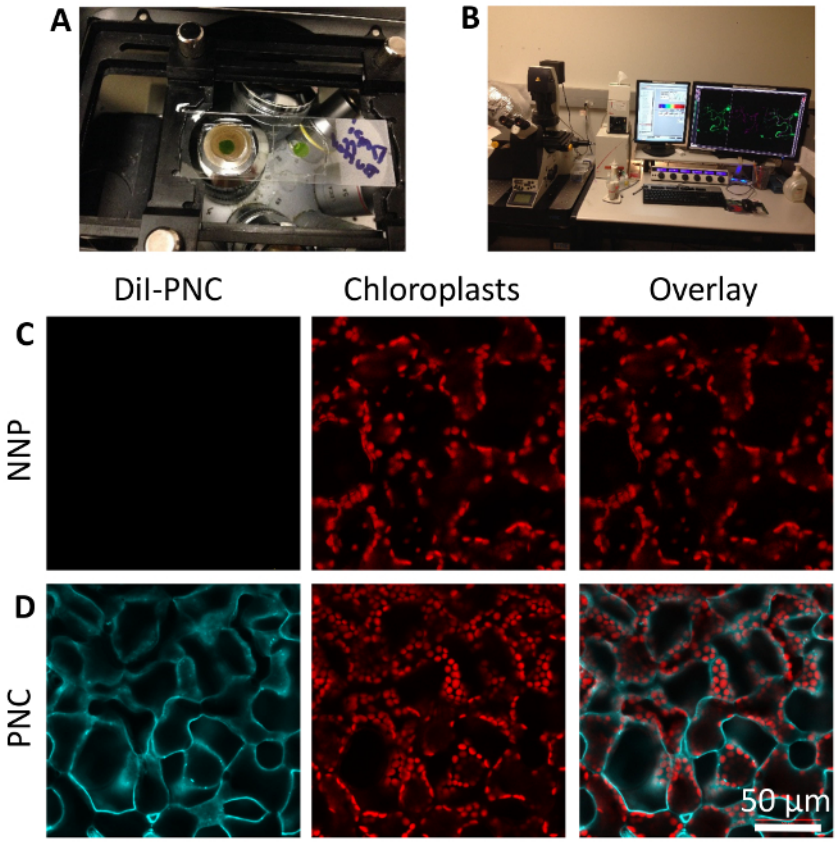

Figure 5: Imaging DiI-PNC in leaf mesophyll cells via confocal microscopy. A. Leaf sample is mounted on an inverted confocal microscope having a 40X water immersion lens. B. A confocal microscope is used for imaging DiI-PNC and chloroplasts. C. Chloroplast auto-fluorescence is recorded in buffer infiltrated leaf samples without nanoparticles (NNP). D. DiI-PNC signal and chloroplast auto-fluorescence is imaged in DiI-PNC infiltrated leaf samples. Please click here to view a larger version of this figure.

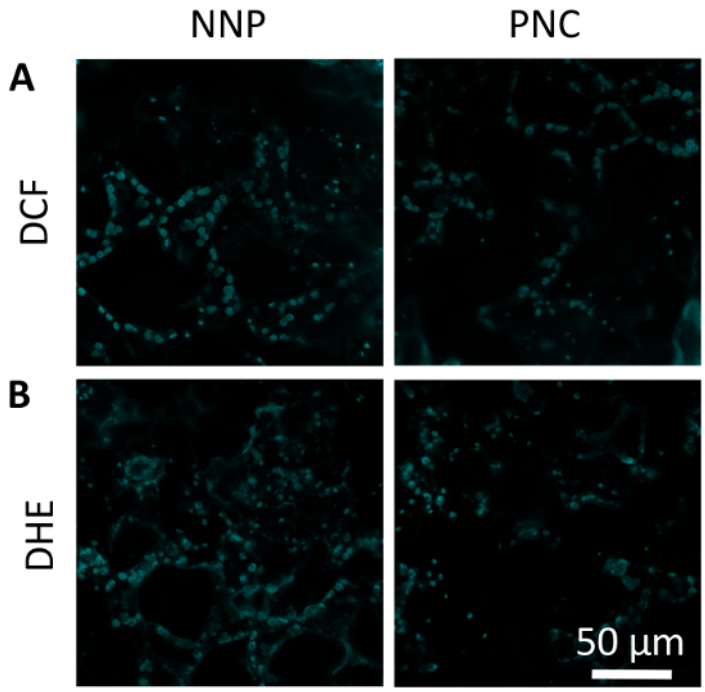

Figure 6: Monitoring PNC ROS scavenging *in planta* via confocal microscopy. A. DCF fluorescent dye (for monitoring general ROS signal) is significantly lower in mesophyll cells of PNC infiltrated plants than buffer control (no nanoparticles, NNP). B. Reduced DHE fluorescent dye (for monitoring superoxide anion) in mesophyll cells of PNC infiltrated plants relative to that of buffer control (NNP). Please click here to view a larger version of this figure.

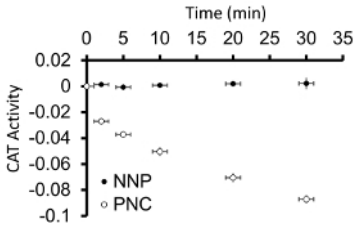

Figure 7: Catalase (CAT) mimetic activity of PNC. In the presence of horseradish peroxidase, the fluorescent probe reacts with hydrogen peroxide and is converted to resorufin (absorbance 560 nm). Absorbance of resorufin, which is indicative of hydrogen peroxide levels, was monitored at 560 nm. PNC showed a CAT mimetic activity. Mean ± SE (standard error) (n = 4). Please click here to view a larger version of this figure.

Discussion

In this protocol, we describe PNC synthesis, characterization, fluorescent dye labeling, and confocal imaging of the nanoparticles within plant mesophyll cells to exhibit their *in vivo* ROS scavenging activity. PNC are synthesized from a mixture of cerium nitrate and PAA solution in ammonium hydroxide. PNC are characterized by absorption spectrophotomery and the concentration determined using Beer-Lamberts law. Zeta potential measurements confirmed the negatively charged surface of PNC for enhancing delivery to chloroplasts[8]. Labeling of PNC with a fluorescent DiI dye enables *in vivo* imaging by confocal microscopy within leaf mesophyll cells where the nanoparticles show high levels of colocalization with chloroplasts. Using DHE and DCF fluorescent dyes, we confirmed that PNC act as a potent scavenger of superoxide anion and ROS *in vivo*.

The method for synthesizing PNC is a simple step-wise procedure that generates cerium oxide nanoparticles with controlled size, negative charge, and ROS scavenging capabilities[16]. Other methods, such as thermal hydrolysis, require high temperatures and expensive chemistry equipment[21,22]. The synthesis and characterization of PNC is a low-cost method performed with common laboratory equipment. It does not require a steep learning curve compared with molecular methods in plants based on overexpression of an antioxidant enzyme, *e.g.*, *SOD*, *APX*, and *CAT*, for scavenging ROS species in model systems[23]. PNC is a robust, water-soluble ROS catalytic scavenger that will not require laborious cloning and transformation methods that are dependent on the plant's genetic tractability and available molecular toolkit.

A critical step in this protocol is the syringe-based infiltration of leaf mesophyll cells with PNC. Infiltration of nanoparticles into live plants should be done gently to avoid physical damage to the leaf[24]. Thus, as in Protocol Step 4 of the methods, gently push against the leaf surface with the syringe to avoid tearing or puncturing the abaxial leaf surface. It is better to infiltrate from the abaxial surface of *A. thaliana* leaf since it has

higher stomatal density than the adaxial surface[25,26]. Furthermore, a buffered solution of PNC or DiI-PNC within the physiological pH range (~ pH 7.5) should be used during leaf lamina infiltration. Another critical step in this protocol is to apply the appropriate concentration of PNC to the studied plant tissue. In this protocol, 50 mg/L of PNC was not toxic to *A. thaliana* leaves while enabling catalytic PNC ROS scavenging. Some limitations of the current nanoparticle delivery method are 1) not applicable to plant species having thick and waxy cuticles or low stomatal densities, 2) not scalable for applications in the field, 3) the high cost of a confocal microscopy system to monitor *in vivo* nanoparticle distribution and ROS scavenging.

This protocol demonstrates the application of ROS scavenging PNC for studying and improving abiotic stress tolerance in plants through a facile method of leaf lamina infiltration. ROS accumulation is accompanied by abiotic stresses in plants, which in turn reduces plant photosynthesis, growth, and yield[8,27,28]. Plant genetic modifications methods for ROS manipulation *in vivo* are often limited to plant model species while PNC ROS-scavenging has the potential to be applied to diverse wild type plant species. Leaf lamina infiltration is a practical research method to increase ROS scavenging in leaf tissues for understanding and engineering plant abiotic stress tolerance.

Disclosures

The authors have nothing to disclose.

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