Chapter 16

Detection of Peroxynitrite in Plants Exposed to Bacterial Infection

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Abstract

Peroxynitrite is a highly reactive derivative of nitric oxide (NO) which is gaining attention in the plant biology community because it may play a role in NO signaling during biotic stress. Peroxynitrite can react with many different biomolecules, but its ability to nitrate the tyrosine residues of proteins is particularly important because this may regulate defense signaling in response to pathogens. The analysis of peroxynitrite levels in the context of its proposed defense role requires an accurate and specific detection method. Here, we describe a photometric assay using the fluorescent dye Hong Kong Green 2 as a specific and quantitative probe for peroxynitrite in *Arabidopsis thaliana* plants challenged with an avirulent strain of *Pseudomonas syringae* pv. *tomato*. This protocol includes the preparation of plant samples, the assay procedure, the measurement of peroxynitrite-specific fluorescence, and data presentation.

Key words Nitric oxide, Peroxynitrite, *Pseudomonas syringae*, Hong Kong Green 2, Signaling, Superoxide, Nitrosative stress

1 Introduction

Peroxynitrite ($ONOO^-$) is a highly reactive molecule that forms in vivo during the diffusion-controlled reaction between nitric oxide (NO) and superoxide (O_2^-), i.e., the chemical reaction between both molecules is so quick that rate is only limited by the diffusion of NO and O_2^- until they encounter each other in the right stoichiometry, with a rate constant (k) of 6.7×10^9 M^{-1} s^{-1}. Because NO and O_2^- are produced simultaneously in plants during the hypersensitive response triggered by avirulent pathogens [1, 2], peroxynitrite may also form and accumulate in response to pathogen infection and may therefore play a physiologically relevant role during this process.

At physiological pH levels, $ONOO^-$ equilibrates with ONOOH (peroxynitrous acid) ($pKa = 6.8$). Peroxynitrate is a potent oxidizing agent attacking different biomolecules (proteins, lipids, DNA) in the plant cell. Accordingly, early studies in animals focused on its

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cytotoxicity [3]. However, peroxynitrite is also a strong nitrating agent, e.g., it can react with tyrosine residues in proteins to form nitrotyrosine. Proteins can be nitrated as a component of signal transduction [4, 5], thus supporting a potential role for peroxynitrite in the regulation of signaling in plants, particularly during defense responses [6]. Peroxynitrite breaks down rapidly (~10 ms) under physiological conditions into oxidizing intermediates derived from different reactions [3]. Therefore the production of peroxynitrite in plants cells undergoing the hypersensitive response is difficult to measure and until recently only limited research tools were available to monitor the accumulation of this molecule in vivo.

Nitrotyrosine-containing proteins were initially considered as markers of nitrosative stress and more specifically they were regarded as indicators of peroxynitrite accumulation. However, peroxynitrite is not the only trigger for tyrosine nitration. In animals, nitrotyrosine can also be produced via a mechanism based on heme peroxidase-NO_2-H_2O_2 [7]. Furthermore, three *Arabidopsis thaliana* hemoglobins with peroxidase activity have been shown to mediate nitrite-dependent tyrosine nitration [8]. This means that the potential role of peroxynitrite in NO signaling can only be investigated by measuring the levels of this molecule directly under physiological and pathological conditions known to induce the simultaneous production of both NO and superoxide.

As previously mentioned, given its high reactivity peroxynitrite breaks down rapidly under physiological conditions, so it cannot be quantified directly in processed biological samples. As an alternative, fluorescent probes can be introduced into living cells to report the presence of this highly reactive molecule. Among the various dyes already used to detect peroxynitrite in animal cells, aminophenyl fluorescein (APF) was the first to be used to investigate defense responses in plants, i.e., tobacco cells treated with the elicitor INF1 [9]. However, APF reacts also with the hydroxyl radical ($^{\bullet}OH$) and hypochlorite (OCl^-) with high sensitivity, so the activity it detects is not specific.

More recently, a new BODIPY-type fluorescent probe named Hong Kong Green 2 (HKGreen2) has been developed for the specific detection of peroxynitrite. The detection mechanism is based on photoinduced electron transfer (PET) and the probe has been used successfully to measure peroxynitrite generated in activated murine macrophages [10]. We therefore established a photometric assay using HKGreen2 that allowed us to monitor the dynamic levels of peroxynitrite in *Arabidopsis thaliana* plants challenged with an avirulent strain of *Pseudomonas syringae* pv. *tomato*. This assay finally demonstrated that peroxynitrite levels increase during the hypersensitive response, strongly supporting its proposed regulatory role during NO signaling [11].

2 Materials

2.1 Plants and Cultivation Equipment

1. The method described herein uses *Arabidopsis thaliana* Columbia 0 (Col-0) plants, the accession that is used most widely for research purposes.
2. Plastic pots (9 cm diameter).
3. Plastic flats (ARAFLATs, Arasystem, Gent, Belgium) and trays (ARATRAYs, Arasystem, Gent, Belgium).
4. Soil.
5. Growth chamber with controlled light, humidity and temperature.

2.2 Bacterial Strains and Cultivation Equipment

1. The *A. thaliana* hypersensitive response is induced here using the avirulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 carrying the avirulence gene *AvrB* (Pst *AvrB*). The protocol can be adapted for different pathogen strains depending on study requirements (*see* **Note 1**).
2. The following antibiotics should be prepared for selection. (a) (50 mg/mL) Kanamycin: dissolve powder in double distilled water (ddH_2O), filter-sterilize and store in 1-mL aliquots at −20 °C. (b) (50 mg/mL) Rifampicin: dissolve powder in dimethylsulfoxide (DMSO) and store in 1-mL aliquots at −20 °C.
3. The bacteria are cultivated in King's B broth, the following ingredients in ddH_2O and making topping up to 1 L: peptone20 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, K_2HPO_4 1.5 g, glycerol 10 mL. Agar15 g/L to prepare solid medium. The media should be autoclaved prior to use and antibiotics should be added when the media have cooled to below 65 °C.
4. Sterile 13-mL cell culture tubes with caps allowing gas exchange (Sarstedt, Verona, Italy).
5. 15-mL falcon tubes (Sarstedt, Verona, Italy).
6. Bench centrifuge for Falcon tubes (Eppendorf, Hamburg, Germany).
7. 1-mL syringe without needle (Soft-Ject, Henke Sass Wolf, Tuttlingen, Germany).
8. Incubator set at 28 °C with shaker.
9. Spectrophotometer set at fixed wavelength of 600 nm.

2.3 Peroxynitrite Detection

1. 10 mM stock solution in dimethylformamide (DMF): Hong Kong Green 2 (*see* **Note 2**). The solution can be aliquoted (*see* **Note 3**) and stored at −20 °C.
2. Cork borer.

3. 15-mL Falcon tubes (Sarstedt, Verona, Italy).
4. Vacuum source.
5. Sealed flask and connectors.
6. Flat-bottomed 96-well plates (Sarstedt, Verona, Italy).
7. Victor™ plate reader (Perkin Elmer, Waltham, MA, USA).

3 Methods

3.1 Cultivation of Arabidopsis thaliana Plants

It is important to grow healthy plants that will fully respond to pathogen infection. Most standard protocols will be effective and the following is given as an example.

1. Grow *Arabidopsis thaliana* plants in 9-cm pots filled with soil (*see* **Note 4**). Moisten the soil before sowing the seeds.
2. Sow approximately 50 seeds per pot, cover the pots with transparent film to maintain humidity and place pots in the dark for 1 day at 4 °C (*see* **Note 5**). Then transfer to the growth chamber for 10–12 days under the following conditions: 60 % relative humidity, 10-h photoperiod, light intensity 100 μmol $m^{-2}s^{-1}$ and day/night temperature 24/22 °C (*see* **Note 6**).
3. After 10–12 days, transfer plantlets individually into trays (*see* **Note 7**) and cultivate for 6–7 weeks. Cover the flats with a close-fitting clear plastic dome to maintain humidity for the first few days and then displace the dome slightly to allow air circulation and gradually reduce the humidity. After a few days of acclimation, remove the domes completely (*see* **Note 8**).
4. Water the pots and the flats by irrigating the base.

3.2 Preparation of the Bacterial Suspension

1. One day (16–20 h) before the assay, inoculate a single colony of Pst *AvrB* from a fresh King's B plate into 3 mL of King's B liquid medium supplemented with 50 μg/mL rifampicin and 50 μg/mL kanamycin (*see* **Note 9**). Incubate overnight (at least 16–20 h) at 28 °C with agitation at 200 rpm.
2. The following day transfer the liquid culture to a 15-mL tube.
3. Pellet the bacteria by centrifuging for 4000 × *g* at room temperature for 5 min.
4. Pour off the supernatant.
5. Resuspend the pelleted cells in an equal volume of autoclaved ddH_2O (*see* **Note 10**).
6. Repeat **steps 3–5**.
7. Measure the OD at 600 nm using a spectrophotometer (*see* **Note 11**) and dilute further with autoclaved ddH_2O if necessary to achieve a final $OD_{600} = 0.1$, corresponding to 10^8 colony forming units (cfu)/mL.

3.3 Infection of Plants

1. Infiltrate the diluted bacterial suspension through the abaxial surface of the leaves using a 1-mL syringe without a needle (*see* **Note 12**). A mock infiltration should be carried out with ddH_2O as a control.
2. Place infiltrated plants under constant illumination during the infection (*see* **Note 13**).

3.4 Sample Preparation and Assay Procedure

1. At different time points after infection (*see* **Note 14**), punch eight leaf disks (5 mm) from the infected and uninfected control leaves (*see* **Note 15**) with a cork borer.
2. Transfer the disks to a 15-mL tube containing 1 mL 20 μM HKGreen2 diluted with water and infiltrate the dye into the leaf disks under vacuum for 3 min (*see* **Note 16**). Prepare a second tube containing water only and use this as an autofluorescence control (*see* **Note 17**).
3. Incubate the leaf disks for 1 h at room temperature (*see* **Notes 18** and **19**).
4. When the incubation is complete, wash the leaf disks carefully with water to remove dye solution before measuring the fluorescence.

3.5 Photometric Measurement

1. Place leaf disks individually in the wells of a flat-bottomed 96-well plate containing 100 μL of water (*see* **Note 20**).
2. Read the fluorescence emission from leaf disks every 10 min for 1 h with a fluorimeter using excitation/emission wavelengths 485/530 nm. The plate should be shaken before each measurement.
3. Calculate the peroxynitrite-related fluorescence by subtracting the autofluorescence value (from disks that were not exposed to the dye, *see* Subheading 3.4) from the fluorescence emission values of the disks infiltrated with HKGreen2 (*Pst* treated or mock infiltration) at each time point. Some representative data from one of our assays is shown in Fig. 1 (*see* **Note 21**).

4 Notes

1. Pst *AvrB* is suitable for studying the hypersensitive response in *Arabidopsis thaliana* Col-0 which expresses the RPM1 resistance protein. Because the hypersensitive response is induced following a specific race/cultivar interaction depending on gene-for-gene recognition of the avirulent pathogen, it is necessary to define suitable pathogens that can induce the hypersensitive response if other plant species are used.
2. HKGreen2 was kindly provided by Prof. Dan Yang under the terms of a collaborative agreement. More recent generations of

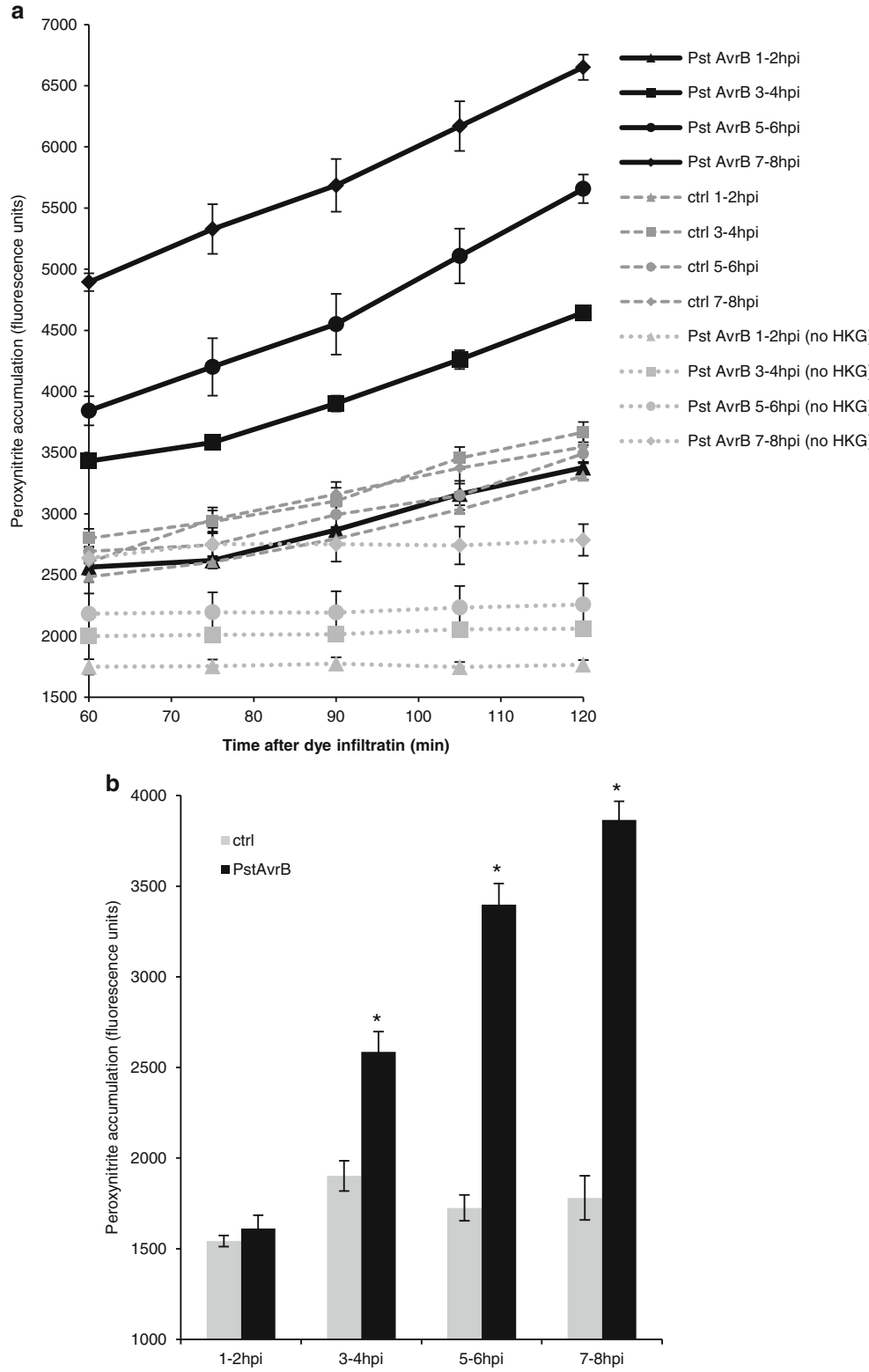

Fig. 1 Peroxynitrite formation in *A. thaliana* during the hypersensitive response induced by Pst *AvrB*. Peroxynitrite levels were estimated with HKGreen2 (20 μM) by monitoring the fluorescence intensity. Peroxynitrite

HKGreen dyes are now available such as HKGreen4, a small-molecule fluorescent probe developed for the exceptionally sensitive and selective detection of peroxynitrite in aqueous solutions, living cells and tissues [12]. These more recent dyes should be broadly compatible with the assay procedure we have described, but their chemical properties may differ slightly from HKGreen-2 and assay optimization may be necessary to find the best working solution concentrations and incubation times.

3. The dye should be divided into small aliquots to avoid freeze/thaw cycles.
4. Vermiculite and/or perlite can be added to the soil to improve aeration and inhibit fungal growth. The risk of fungal growth can also be reduced by autoclaving the soil before sowing seeds or transferring plants.
5. This step is necessary because *A. thaliana* seeds preferentially germinate after experiencing a period of cold temperatures.
6. The optimal day length and humidity depends on the species. When studying plant defense responses it is important to identify growing conditions that avoid early flowering. We recommend that *A. thaliana* plants are grown under short-day conditions.
7. Planting trays should be scrubbed to remove algae, fungi, old seeds and dead plants. Make sure the trays have drainage holes. Label the trays with the date the seeds were sown.
8. Particular care should be taken to avoid insect infestation because this may induce unanticipated defense responses that mask the response caused by the infiltrated pathogen. Pesticides cannot be used because many phytochemical products also contain molecules that induce defense responses. Therefore, infiltration should be carried out under clean-room conditions and older plants should be moved to a separate growth room for seed maturation and drying down.
9. The antibiotics used for selection depend on the choice of pathogen. *Pseudomonas syringae* pv. *tomato* DC3000 is inherently resistant to rifampicin and the *AvrB* gene confers kanamycin

Fig. 1 (continued) accumulation was (**a**) monitored in real time for 1 h or (**b**) estimated at different time intervals. Leaves were infected with an avirulent strain of *Pseudomonas syringae* pv. *tomato* carrying the *AvrB* gene ($OD_{600} = 0.1$) or with water (control). At different time points, leaf discs were vacuum infiltrated with 20 μM HKGreen2 and incubated with the dye for 1 h in darkness. Plant autofluorescence was estimated by measuring the fluorescence intensity of infected leaf discs that were not treated with HKGreen2 (no HKG). Fluorescence emission was monitored in a plate reader photometer for 1 h (2 h total including incubation with the dye) at room temperature. For peroxynitrite accumulation at different time points, the fluorescence values obtained at the end of the reading period (1 h) were subtracted from the autofluorescence background obtained with samples that had not been incubated with HKGreen2. In each panel, values shown are means of between six and eight biological replicates ± SE, where *asterisk* represents $p < 0.05$ vs. control. Abbreviations: *ctrl* control (H_2O), *hpi* hour postinfection

resistance. If other pathogens are used in this assay, the antibiotics should be chosen accordingly.

10. The dilution and pathogen infiltration steps do not require sterile conditions. Alternatively to water, the bacteria can be washed and resuspended in 10 mM $MgCl_2$. In this case, $MgCl_2$ is prepared as a 1 M stock solution which is autoclaved to avoid contamination. The 10 mM working solution is thus prepared freshly by diluting stock solution with autoclaved ddH_2O.
11. After growth for 16–20 h, the bacteria should reach an OD_{600} of 1.5–2. To keep within the linear absorbance range of the spectrophotometer, the bacterial suspension should be diluted 1:10 for OD measurement after washing. This should be included as a dilution factor in further calculations.
12. It is not necessary to punch holes in *A. thaliana* leaves to achieve bacterial infiltration. This step can be accomplished by exerting a weak pressure with a needless syringe while bracing the other side of the leaf against a finger. Special care should be taken to avoid causing tissue damage. Infiltration is complete when the leaf changes color to dark green. At least 20 μL of bacterial suspension is usually required for the complete infiltration of each leaf. Infiltrated leaves should be marked for identification because the color difference disappears soon after infiltration.
13. It is important to place infected plants under constant light because an appropriate light environment is required to establish a complete set of resistance responses in many plant–pathogen interactions.
14. Peroxynitrite is formed when NO and O_2^- come into contact. The selection of appropriate analytical time points is based on the kinetics for these two molecules, which in turn depends on the nature of the plant–pathogen interaction. In the case of incompatible interactions, it is necessary to consider how long it takes for the pathogen to introduce effectors that are recognized by plant receptors that trigger the hypersensitive response. This depends on the concentration of the bacterial suspension, with weaker suspensions inducing a slower and weaker response. A strong defense response can be ensured by using a concentrated bacterial suspension (e.g., 10^7–10^8 cfu/mL), which can be useful to study molecular mechanisms requiring a large number of infected cells for detection.
15. There may be some variation in the intensity of the fluorescent signal among plants so we recommend the analysis of several replicates for each condition and each time point. Eight disks sampled from at least three independent plants per condition provide statistically valid data, but this number can be modified depending on specific experimental conditions. For example, if there is substantial variability among replicates then the number of leaf disks per experiment should be increased.

16. Leaf disks representing one condition should be processed simultaneously in the same 15-mL tube containing the dye working solution (or water for the autofluorescence controls). Up to four tubes can be placed simultaneously in a sealed flask connected to vacuum for infiltration, which is carried out for 3 min with continuous manual shaking to remove air bubbles. The leaf disks should settle to the bottom of the tube by the end of the process if infiltration has been successful. If there are any floating disks then the vacuum infiltration must be repeated. As an alternative, the dye can be infiltrated using a 20-mL syringe. The leaf disks are placed into a syringe containing the dye solution, which is sealed with a cap or a tip. Pressure is then exerted with the syringe. The HKGreen2 working solution can be recovered, stored at 4 °C and reused several times for a few days. The solution should be discarded if the fluorescence becomes weak or the signal-to-noise ratio declines.
17. Leaves produce many autofluorescent compounds. Notably, secondary metabolites such as polyphenols are produced during plant–pathogen interactions and these emit strong fluorescence. Because the quantity of these compounds increases during infection, a control without the dye should be used to determine background autofluorescence and should be read at the same time points to allow for background correction. The fluorescence readings should be subtracted from the values obtained from the dye-infiltrated samples in order to determine the level of fluorescence specifically generated by HKGreen2, which represents the quantity of peroxynitrite. Similarly, certain plant species show high basal levels of autofluorescence that could interfere with the experiment so appropriate controls should be set up to ensure the assay values are corrected.
18. The incubation of samples in the dark before analysis is important. Indeed, a time course of different incubation periods with plant cell extracts in the presence of the dye showed that fluorescence intensity varies with time and that incubation for 1 h is necessary for an optimal fluorescence signal. We recommend that leaf disks should be incubated with HKGreen2 for at least 1 h and the same incubation period should be used for all experimental conditions to ensure the results are comparable.
19. The stability of the fluorescent form of the dye in different plant species should be determined to ensure that the compound is not recycled by cells and that the levels of peroxynitrite measured with the dye are representative of the actual amount in the cell. For this purpose, HKGreen2 can be added to plant extracts (or water as a control) in the presence or absence of exogenous peroxynitrite. HKGreen2 fluorescence caused by the presence of peroxynitrite can then be determined by comparing the absolute fluorescence values of the plant extracts and water.

20. Leaf disks should not be damaged when they are transferred to the 96-well plates, e.g., using a flat clamp or brush.
21. The sampling time point is distinct from the start of the fluorescence measurement. This time span should be exactly the same under all experimental conditions. It is also necessary to take into account the fluorescence reading time. Data can be presented showing all recorded fluorescence values during the reading period of 1 h (Fig. 1a) or as histograms obtained by subtracting autofluorescence background obtained from disks not exposed to the dye from the signal recorded at the end of the monitoring period (Fig. 1b). In the first case, the fluorescence reading represents peroxynitrite levels in real-time for 1 h with measurements beginning 1 h after the sampling/dye infiltration time. In the second case, each bar of the histogram represents the corrected amount of peroxynitrite produced in 2 h starting from dye infiltration.

References

1. Delledonne M, Xia RA, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature 394:585–588
2. Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci U S A 98:13454–13459
3. Szabó C, Ischiropoulos H, Radi R (2007) Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat Rev Drug Discov 6:662–680
4. Gow AJ, Duran D, Malcolmc S, Ischiropoulosa H (1996) Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. FEBS Lett 385:63–66
5. Liaudet L, Vassalli G, Pacher P (2009) Role of peroxynitrite in the redox regulation of cell signal transduction pathways. Front Biosci 14:4809–4814
6. Romero-Puertas MC, Laxa M, Mattè A, Zaninotto F, Finkemeier I, Jones AM, Perazzolli M, Vandelle E, Dietz KJ, Delledonne M (2007) S-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. Plant Cell 19:4120–4130
7. Oury TD, Tatro L, Ghio AJ, Piantadosi CA (1995) Nitration of tyrosine by hydrogen peroxide and nitrite. Free Radic Res 23:537–547
8. Sakamoto A, Sakurao SH, Fukunaga K, Matsubara T, Ueda-Hashimoto M, Tsukamoto S, Takahashi M, Morikawa H (2004) Three distinct Arabidopsis hemoglobins exhibit peroxidase-like activity and differentially mediate nitrite-dependent protein nitration. FEBS Lett 572:27–32
9. Saito S, Yamamoto-Katou A, Yoshioka H, Doke N, Kawakita K (2006) Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells. Plant Cell Physiol 47:689–697
10. Sun ZN, Wang HL, Liu FQ, Chen Y, Tam PK, Yang D (2009) BODIPY-based fluorescent probe for peroxynitrite detection and imaging in living cells. Org Lett 11:1887–1890
11. Gaupels F, Spiazzi-Vandelle E, Yang D, Delledonne M (2011) Detection of peroxynitrite accumulation in *Arabidopsis thaliana* during the hypersensitive defense response. Nitric Oxide 25:222–228
12. Peng T, Wong N-K, Chen X, Chan Y-K, Hoi-Hang Ho D, Sun Z, Hu JJ, Shen J, El-Nezami H, Yang D (2014) Molecular imaging of peroxynitrite with HKGreen-4 in live cells and tissues. J Am Chem Soc 136:11728–11734