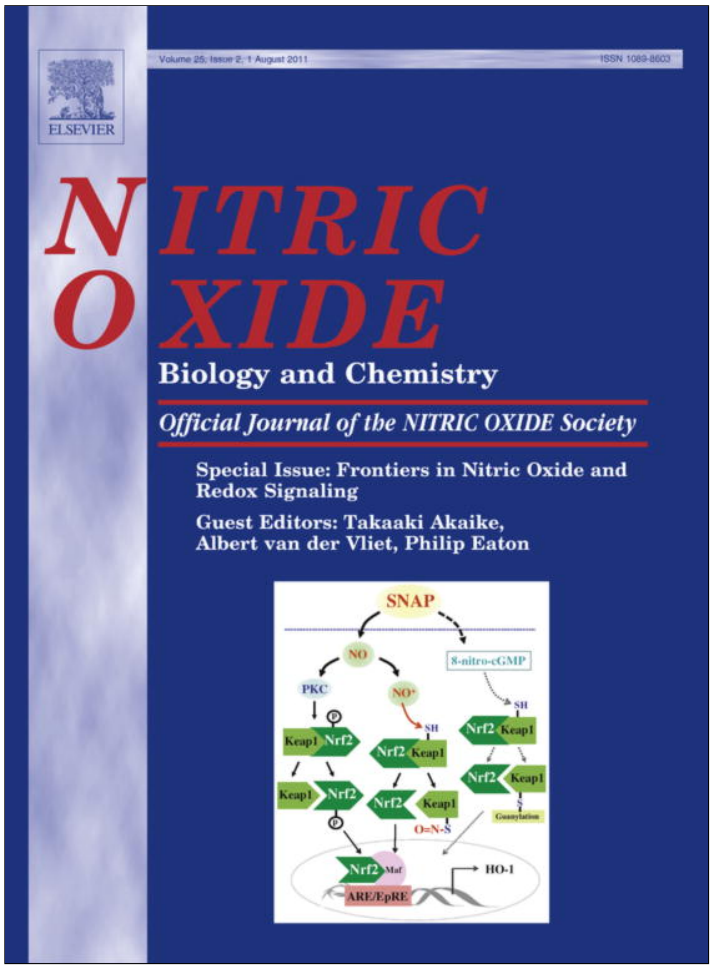

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Detection of peroxynitrite accumulation in *Arabidopsis thaliana* during the hypersensitive defense response

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ABSTRACT

Nitric oxide (NO) is synthesized in plants in response to stress, and its role in signaling is well-documented. In contrast, very little is known about the physiological role of its derivate peroxynitrite ($ONOO^-$), which forms when NO reacts with O_2^- and induces protein modification by tyrosine nitration. Infection with an avirulent pathogen triggers the simultaneous production of NO and reactive oxygen species, as well as an increase in tyrosine nitration, so peroxynitrite could be physiologically relevant during this process. To gain insight into the role of peroxynitrite in plants, we measured its accumulation during the hypersensitive response in *Arabidopsis thaliana* using the specific peroxynitrite-sensitive fluorescent dye HKGreen-2 in a leaf disc assay. The avirulent pathogen *Pseudomonas syringae* pv. *tomato*, carrying the *AvrB* gene (Pst AvrB), induced a strong increase in fluorescence 3–4 h post-infiltration (hpi) which peaked 7–8 hpi. The increase in HKGreen-2 fluorescence was inhibited by co-injecting the peroxynitrite-scavenger urate together with the pathogen, and was almost completely eliminated by co-infiltrating urate with HKGreen-2, confirming that HKGreen-2 fluorescence *in planta* is induced specifically by peroxynitrite. This establishes a link between peroxynitrite synthesis and tyrosine nitration, and we therefore propose that peroxynitrite transduces the NO signal by modifying protein functions.

Introduction

Nitric oxide (NO) is a gaseous signaling molecule that has multiple roles during plant development and stress adaptation [1–3], particularly in the hypersensitive response (HR) against pathogens [4,5]. The HR is triggered when plants recognize a pathogen, and is therefore typical of plant–pathogen interactions that follow the "gene-for-gene" model, in which an avirulent pathogen carrying an avirulence (*Avr*) gene induces defense mechanisms in a resistant plant carrying the corresponding resistance (*R*) gene. One of the main features of the HR is the formation of necrotic lesions at the infection site [6], a process that is precisely controlled by the synergistic action of NO and peroxide (H_2O_2) produced simultaneously at the onset of the HR [7,8].

NO signaling cascades ultimately trigger downstream effects in the cell by modifying target proteins at the post-translational level, i.e. NO reacts with particular amino acid side chains leading to a change in protein conformation and activity. The best-characterized protein modification mediated by NO in plants is *S*-nitrosylation, in which NO binds to the sulfhydryl groups of cysteine residues in target proteins [9]. Several targets of *S*-nitrosylation have been identified, in particular during the HR [10,11]. Of particular interest, *S*-nitrosylation inhibits the activity of peroxiredoxin IIE (Prx IIE), which can detoxify both H_2O_2 and peroxynitrite ($ONOO^-$) [12,13]. Peroxynitrite is a highly reactive molecule formed by the diffusion-limited reaction between NO and O_2^-. It has therefore been proposed that NO, via the inhibition of Prx IIE, could contribute to the accumulation of its own derivative [13].

Although peroxynitrite is highly reactive and toxic in animals, it is not involved in NO-mediated cell death in plants [8] and its physiological functions have yet to be determined. Peroxynitrite could play a role in NO signaling by mediating specific post-translational modifications, namely the nitration of tyrosine residues by the addition of a nitro group to the tyrosine aromatic ring [14]. Although the potential signaling role of peroxynitrite has not been investigated in detail, Tyr-nitrated proteins have been detected in various plant

Abbreviations: APF, aminophenyl fluorescein; HR, hypersensitive response; Prx IIE, peroxiredoxin IIE; Pst AvrB, *Pseudomonas syringae* pv. *tomato* carrying the *AvrB* avirulence gene; SIN-1, 3-(4-morpholinyl) sydnonimine hydrochloride; cPTIO, carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl.

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species including nitrite reductase-deficient transgenic tobacco [15], pea plants exposed to abiotic stress [16] and, of particular interest, *Arabidopsis thaliana* plants infected with an avirulent pathogen [13,17]. Tyr-nitrated proteins are considered to be markers of nitrosative stress and indicators of peroxynitrite accumulation. However, peroxynitrite is not the only cellular nitrating agent, e.g. Tyr-nitration can be achieved in animals using a mechanism based on heme peroxidase–NO_2–H_2O_2 [14,18], and three *A. thaliana* hemoglobins with peroxidase activity have been shown to mediate nitrite-dependent Tyr-nitration [19]. Because protein Tyr-nitration can occur in the absence of peroxynitrite, it is therefore necessary to measure peroxynitrite levels in plants under various physiological and pathological conditions to determine its potential role in NO signaling.

Thus far, only one study has established a direct link between peroxynitrite levels and Tyr-nitration in plants, following the treatment of tobacco cell suspensions with the pathogen-derived elicitor INF1 [20]. However, the authors detected peroxynitrite using the fluorescent dye aminophenyl fluorescein (APF), which is also known to detect reactive oxygen species (ROS) such as the hydroxyl radical [21]. Recently, a new fluorescent dye named HKGreen-1 was developed for the specific detection and imaging of peroxynitrite [22]. HKGreen-1 has a much higher reactivity for peroxynitrite than NO and ROS (including the hydroxyl radical) so has a greater specificity than APF, and a more sensitive version (HKGreen-2) allowed the detection of peroxynitrite in living animal cells [23]. Here, using a leaf disc assay, we show that HKGreen-2 can also be used for the specific detection of peroxynitrite in plant tissues, and we describe for the first time the accumulation of peroxynitrite in plants undergoing the HR.

Materials and methods

Biological material

A. thaliana ecotype Columbia 0 plants were grown in soil culture at 60% relative humidity, with a 10-h photoperiod (light intensity 100 μmol m^{-2} s^{-1}) and a day/night temperature of 24/22 °C. The avirulent *Pseudomonas syringae* pv. *tomato* (Pst) strain DC3000, carrying the *AvrB* avirulence gene, was grown overnight at 28 °C in King's B medium (2% w/v Proteose Peptone, 6.1 mM $MgSO_4$, 8.6 mM K_2HPO_4 and 1% v/v glycerol, pH 7.2) supplemented with 50 mg/ml kanamycin and 50 mg/ml rifampicin. The bacterial suspension was infiltrated at OD_{600} = 0.1 in water into the abaxial surface of *A. thaliana* leaves using a hypodermic syringe without a needle. As a control, leaves were infiltrated with water. After infection, plants were returned to the growth chamber until the start of the photometric measurements. *A. thaliana* leaves were then collected from plants 0, 2, 4 and 6 h post-infiltration (hpi) and processed for peroxynitrite analysis.

Photometric measurements of peroxynitrite in vitro

Peroxynitrite was detected using the fluorescent dyes APF (A36003, Invitrogen, Gaithersburg, MD, USA) and HKGreen-2 [23]. Both dyes were assessed using the peroxynitrite donor SIN-1 (3-(4-morpholinyl) sydnonimine hydrochloride; M5793, Sigma–Aldrich, St. Louis) prepared in 0.1 M phosphate buffer (pH 7.2). The fluorescence intensity of APF and HKGreen-2 was measured at room temperature on a Victor™ plate reader (Perkin–Elmer) at excitation/emission wavelengths of 485/535 nm.

Photometric measurements of peroxynitrite in planta

Peroxynitrite was detected in 5-mm leaf discs punched from infiltrated leaves and vacuum-infiltrated with 20 μM HKGreen-2 [23] for 3 min with continuous agitation. We also prepared a 1 mM solution of urate, a peroxynitrite scavenger, in 3.36 mM NaOH and this was either co-injected into leaves with the pathogen or co-infiltrated into leaf discs with HKGreen-2. Leaf discs were incubated in darkness for 1 h, washed carefully with water and individual discs were transferred to wells containing 100 μl of water in a flat-bottomed 96-well plate. HKGreen-2 fluorescence was measured at room temperature on a Victor™ plate reader at excitation/emission wavelengths of 485/535 nm. Fluorescence intensity was also measured in control leaf discs that were infected but not treated with HKGreen-2. The plate was shaken before each reading. Eight replicates (leaf discs) were prepared for each time point and condition.

Results

Comparison of APF and HKGreen-2 for the detection of peroxynitrite

In an initial set of experiments we carried out a comparative analysis of APF and HKGreen-2 under the same *in vitro* conditions to determine whether HKGreen-2 would be suitable for the specific measurement of peroxynitrite levels *in planta*. Fluorescence was measured in the presence of 1 mM SIN-1, a peroxynitrite donor that produces equal amounts of NO and O_2^- which then react rapidly to form peroxynitrite. The optimal dye concentration was 10 μM for both APF and HKGreen-2 (data not shown). Real-time monitoring of fluorescence emission for 2 h showed that the fluorescence readings reached a plateau after approximately 80 min for both dyes (Fig. 1). We then added different amounts of SIN-1 to fixed concentrations (10 μM) of APF and HKGreen-2 to investigate dose-dependency. Real-time monitoring of fluorescence emission for 2 h showed that the fluorescence readings increased linearly at the lowest SIN-1 concentrations but reached a plateau after ~80 min for the highest SIN-1 concentrations (Fig. 2). The slow increase in fluorescence observed with SIN-1 (Figs. 1 and 2) reflected the slow generation of peroxynitrite by the donor. In contrast, the addition of commercial peroxynitrite triggered a peak in fluorescence within a few seconds (data not shown). The sensitivity of each dye was then evaluated by determining fluorescence values after the 80-min period of linear intensification that occurred with all the test concentrations of SIN-1. As shown in Fig. 3, both dyes appeared equally sensitive becoming saturated at 0.6 mM SIN-1 (Fig. 3A and B). In both cases, fluorescence intensity increased in a linear manner from 0 to ~0.1 mM SIN-1 (Fig. 3, inset panels). We concluded from the above data that HKGreen-2 was a suitable probe for peroxynitrite detection *in planta*, providing sensitivity in the same range as that reported for APF in plant cell suspensions [20] but greater peroxynitrite specificity [21–23].

Detection of endogenous peroxynitrite in A. thaliana leaves during the HR

Although HKGreen-2 is sensitive enough to allow the detection of peroxynitrite in murine macrophages in response to various stimuli [23], it does not provide a sufficient signal-to-noise ratio for the detection of peroxynitrite in *A. thaliana* leaves by microscopy. We therefore developed a photometric assay using leaf discs. Leaves of the *A. thaliana* ecotype Col0, which contains the *RPM1* resistance gene, were infiltrated with an avirulent strain of *P. syringae* pv. *tomato* (Pst) carrying the *AvrB* avirulence gene (hereafter Pst AvrB), in order to induce the HR. Control leaves were infiltrated with water. For the analysis of peroxynitrite levels, leaf discs from infected and control plants were vacuum-infiltrated with HKGreen-2 at different time points post-infiltration, incubated in darkness for 1 h and then monitored for fluorescence emission

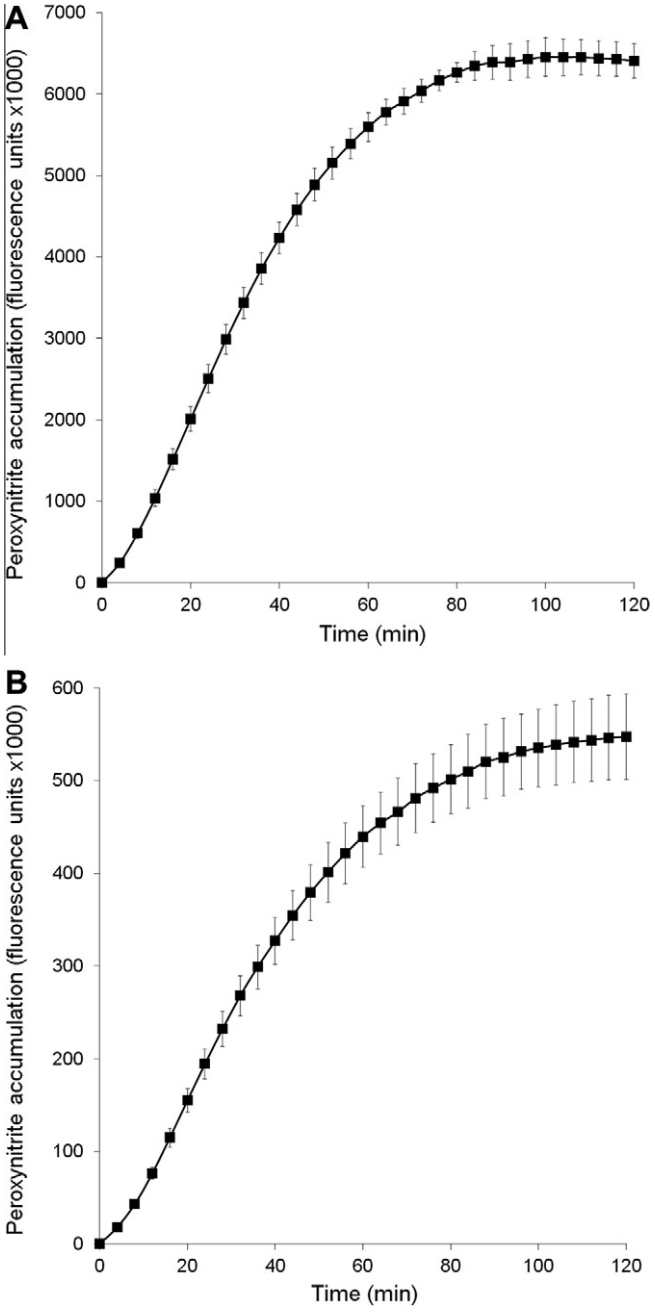

Fig. 1. Comparison of real-time detection of peroxynitrite by APF and HKGreen-2. Time course experiment for peroxynitrite detection by APF (A) and HKGreen-2 (B). Dye fluorescence emission was monitored in real-time using a plate reader photometer at room temperature for 2 h following the addition of APF or HKGreen-2, each prepared as a 10 μM solution, to 1 mM SIN-1 in 0.1 M phosphate buffer (pH 7.2). Values shown are means of four replicates ± SD. Fluorescence values have been divided by 1000 for clarity.

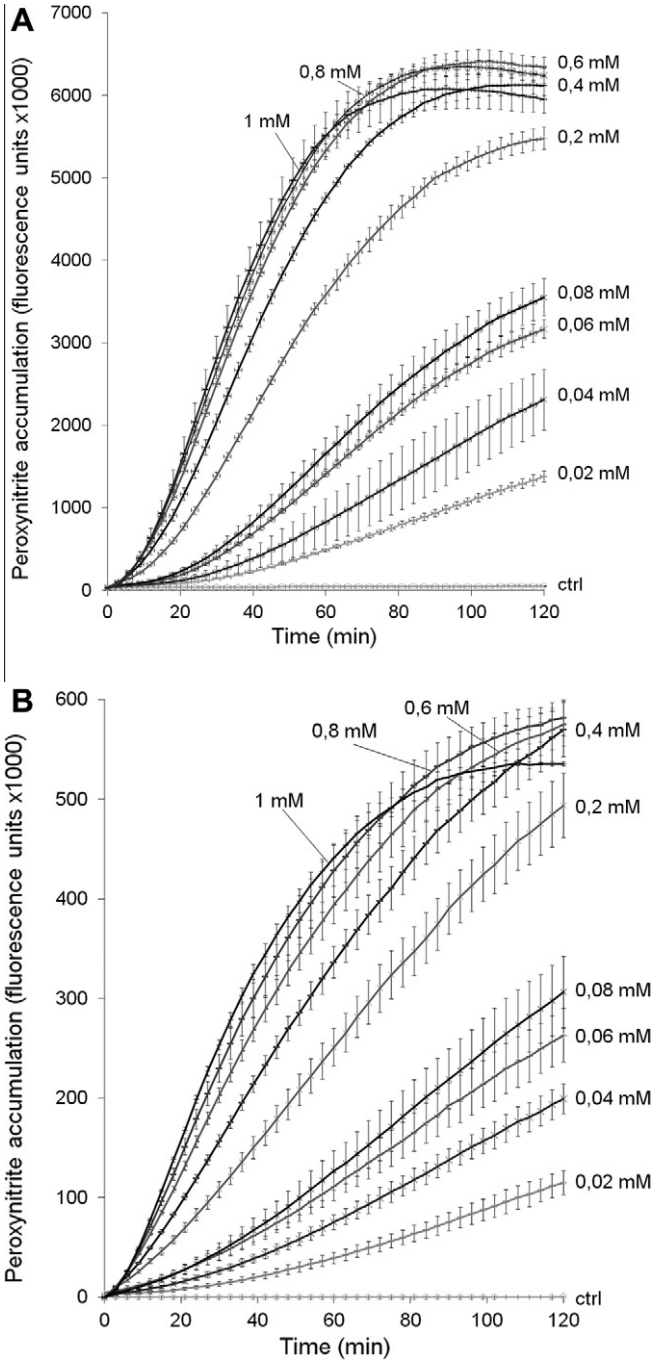

Fig. 2. SIN-1-dose dependency of APF and HKGreen-2 fluorescence intensity. APF or HKGreen-2, each prepared as a 10 μM solution, was added to different concentrations of SIN-1 (0.02–1 mM) prepared in 0.1 M phosphate buffer (pH 7.2). Fluorescence emission was measured for 2 h at room temperature in a plate reader photometer. Values shown are means of four replicates ± SD from a representative experiment (out of three). ctrl: Control (phosphate buffer). Fluorescence values have been divided by 1000 for clarity.

for 1 h. The evaluation of dye stability showed that HKGreen-2 is stable in cell extracts and that the 1-h incubation period appears to be necessary for the optimal detection of peroxynitrite because higher levels of fluorescence are observed in samples incubated for 1 h with the dye (data not shown). We found that dye-treated leaf discs from plants infiltrated with Pst AvrB were indistinguishable from controls 1–2 hpi, but thereafter displayed more intense fluorescence, indicating that peroxynitrite accumulation was induced by the avirulent pathogen (Fig. 4A). The increase in fluorescence induced by Pst AvrB is significantly higher in leaf discs treated with HKGreen-2 than in the absence of the dye, demonstrating that the increase in fluorescence observed during the HR is induced by peroxynitrite and is not caused by autofluorescence. It is noteworthy that the fluorescence intensity at the start of the monitoring period (1 h after infiltration with the dye) was already significantly higher in the Pst AvrB-infected discs compared to controls by 3 hpi, which shows there is a higher peroxynitrite content in infected leaves. Moreover, the fluorescence intensity increased during the HR, reaching a peak by 8 hpi, indicating that peroxynitrite continues to accumulate during this process (Fig. 4B). Accordingly, the

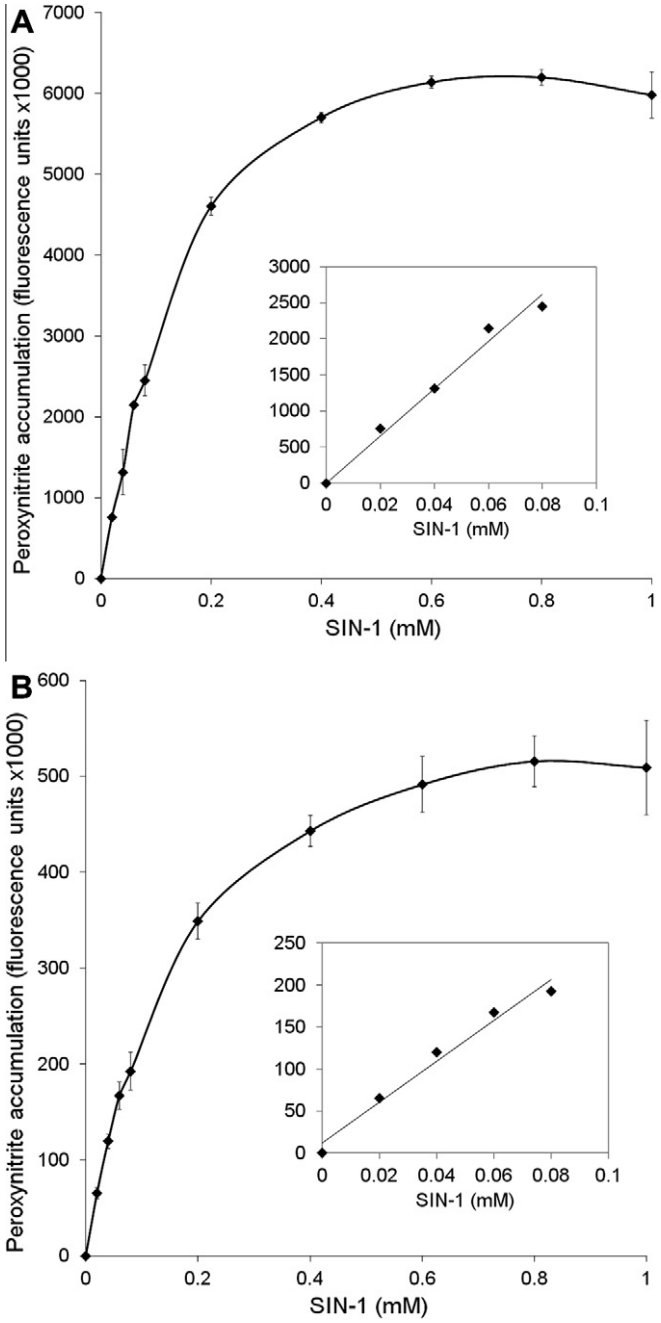

Fig. 3. Standard curve of peroxynitrite detected with APF or HKGreen-2. APF (A) or HKGreen-2 (B), each prepared as a 10 μM solution, was added to different concentrations of SIN-1 (0.02–1 mM) prepared in 0.1 M phosphate buffer (pH 7.2). Fluorescence emission was measured after 1 h in a plate reader photometer at room temperature. The inserts show fluorescence emission for the lowest concentrations of SIN1 (0.02–0.1 mM) in order to highlight the linear increase in fluorescence intensity within this range of peroxynitrite concentrations. Values shown are means of four replicates ± SD from a representative experiment (out of three). Fluorescence values have been divided by 1000 for clarity.

difference in fluorescence intensity observed during the monitoring period, corresponding to the real-time accumulation of peroxynitrite in response to infection, indicates that the rate of peroxynitrite production increases from 3 to 6 hpi and then remains stable until 8 hpi (Fig. 4C). In contrast, the fluorescence intensity increased much more slowly in control discs (Fig. 4A–C), the underlying accumulation of peroxynitrite presumably reflecting stress caused by vacuum infiltration of the dye. The comparison of infected and control samples leads to the conclusion that most of the fluorescence detected in the infected samples is due to peroxynitrite accumulation induced by the avirulent pathogen.

Effect of urate on HKGreen-2 fluorescence

In order to confirm that the observed increase in HKGreen-2 fluorescence was due to the presence of peroxynitrite, we introduced a peroxynitrite scavenger into the *in vitro* and *in planta* systems. Fig. 5A shows that, *in vitro*, 1 mM urate completely eliminated the increase in fluorescence normally induced by 1 mM SIN-1. Complete inhibition was also observed with 1 mM epicatechin (another peroxynitrite scavenger) and 500 μM carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (cPTIO, a scavenger of NO). This confirmed that the observed fluorescence signal was induced specifically by the release of peroxynitrite from SIN-1. Transferring this principle to the *in planta* system, we investigated two approaches, first introducing urate at the same time as the pathogen by co-injecting Pst AvrB and urate into intact leaves from which discs were later prepared, and second introducing urate at a later stage, by co-infiltrating leaf discs with urate and HKGreen-2. Fig. 5B shows that urate reduced HKGreen-2 fluorescence by ~25% when co-infiltrated with Pst AvrB at the beginning of the infection and by almost 80% when co-infiltrated with the dye 6 hpi, with both values monitored at 8 hpi. This difference could reflect the degradation of urate in plant cells in the 8 h that elapsed between infiltration and monitoring, so the effective concentration was lower by the time the dye was introduced into leaf discs. Notwithstanding the above, these data show clearly that HKGreen-2 fluorescence detected specifically during the HR is due to the accumulation of peroxynitrite.

Discussion

Peroxynitrite is a highly-reactive derivative of NO which is produced in plants under stress and causes specific post-translational modifications in proteins, namely tyrosine nitration. Little is known about the synthesis and potential physiological and pathological functions of peroxynitrite in plants, partly because the molecule has a short half life and is difficult to study directly. Its abundance is usually determined indirectly by the detection of reaction products such as nitrated lipids, amino acids and proteins using techniques such as chromatography, immunodetection and mass spectrometry [14,18,24].

Studies in animals have shown that Tyr-nitration is a widespread post-translational protein modification mediated by peroxynitrite [14], but only a few comparable investigations have been carried out in plants. These include reports showing that Tyr-nitrated proteins become more abundant in pea plants exposed to light and temperature stress, in elicitor-treated tobacco cell suspensions and in *A. thaliana* plants infected with the avirulent pathogen Pst AvrB [16,17,20]. The measurement of Tyr-nitration is difficult because only one in every 10,000 residues is modified, and its usefulness is debatable because although Tyr-nitration can be used as an indirect marker of peroxynitrite accumulation [25,26], the same modification can be caused by other mechanisms, e.g. peroxidase activity in the presence of reactive oxygen and nitrogen species (ROS/RNS) [19,25]. In order to gain insight into the precise function of peroxynitrite it plants, it is therefore necessary to establish assays that detect the molecule directly using fluorescent dyes that allow real-time monitoring of peroxynitrite levels. Current hypotheses dealing with the function of peroxynitrite rely on correlations between the production of NO and ROS and increases in Tyr-nitration, e.g. during the HR, and therefore lack conclusive data showing the production of peroxynitrite over time.

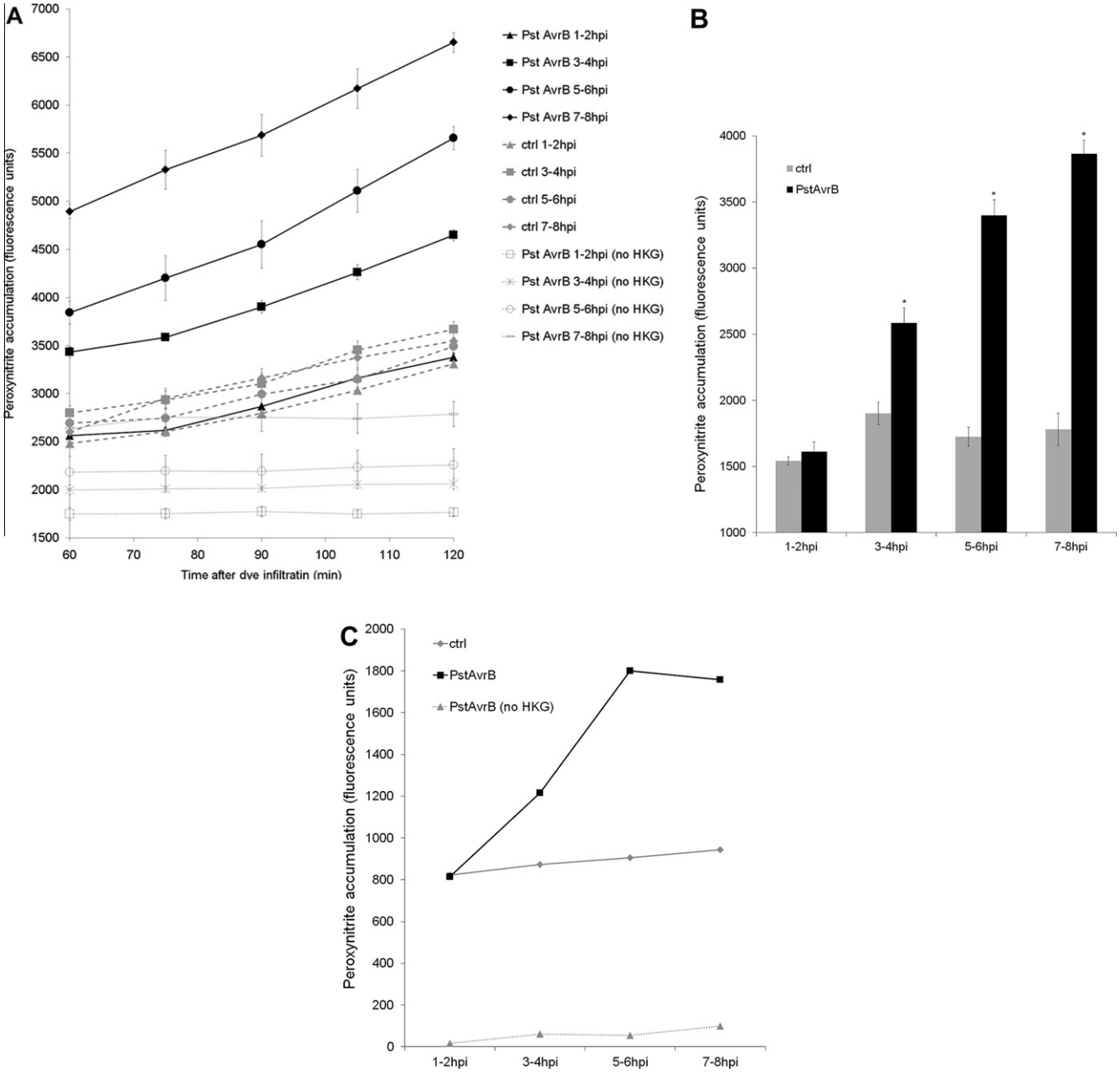

Fig. 4. Peroxynitrite formation in *A. thaliana* during the hypersensitive response. Peroxynitrite levels were estimated with HKGreen-2 (10 μM) by measuring fluorescence intensity. Fluorescence was monitored in real-time (A) or estimated at single time points (B) during the HR. Leaves were infected with 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 HKGreen-2 and incubated with the dye for 1 h in darkness. Fluorescence emission was monitored in a plate reader photometer for 1 h (2 h total after dye infiltration) at room temperature. The values shown in B were obtained by subtracting leaf disc autofluorescence values (from discs that were not treated with the dye) from the fluorescence signal in leaf discs infiltrated with HKGreen-2. The rate of peroxynitrite accumulation in real-time (C) in uninfected leaf discs and leaf discs infected with the pathogen was obtained by calculating the difference between fluorescence signal at the beginning and end of the monitoring period. As a control, the increase in fluorescence increase was measured in infected leaf discs that were not treated with the dye. Values shown are means of 6–8 biological replicates ± SE. ctrl, Control (H_2O); ∗, $p < 0.05$ vs. control.

Addressing the challenges listed above, we established a photometric leaf disc assay using the novel boron-dipyrromethene (BODIPY) dye HKGreen-2, which is highly specific for peroxynitrite. HKGreen-2 reacts only weakly with other ROS/RNS [23] and is therefore preferable to aminophenyl fluorescein (APF), which detects peroxynitrite but is also highly sensitive to hydroxyl radicals [21]. We carried out a comparative analysis of HKGreen-2 and APF using the peroxynitrite donor SIN-1, and found that both dyes displayed the same sensitivity towards peroxynitrite with the intensity of fluorescence increasing linearly with both dyes up to a SIN-1 concentration of 0.1 mM.

The main aim of the investigation was to study peroxynitrite accumulation during the HR in *A. thaliana* plants infected with Pst AvrB, as this pathogen has previously been shown to trigger protein Tyr-nitration when infiltrated at OD_{600} = 0.1 [17]. Leaf discs were punched from the infiltrated leaves 0, 2, 4 and 6 hpi and infiltrated with HKGreen-2 to detect peroxynitrite. There was no significant difference between infected plants and controls at 1–2 hpi, but a weak increase in fluorescence was observed in both cases, presumably reflecting the production of small amounts of peroxynitrite in response to the stress of wounding and vacuum infiltration. Fluorescence increased significantly at 3–4 hpi

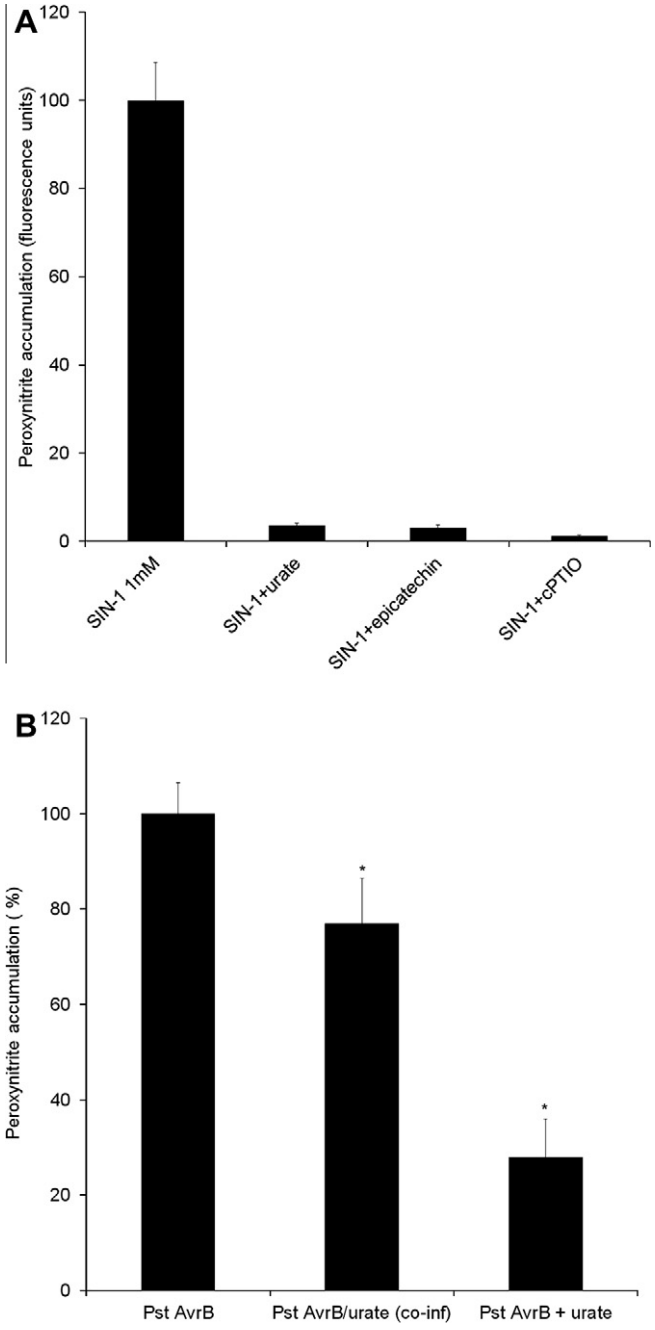

Fig. 5. Effect of urate on peroxynitrite accumulation in *A. thaliana* during the hypersensitive response. (A) Fluorescence emission of HKGreen-2 in the presence of SIN-1 (1 mM) ± urate (1 mM), epicatechin (1 mM), cPTIO (500 μM). A concentrated stock solution of urate was prepared in 1 M NaOH, whereas epicatechin and cPTIO were dissolved in water. The different scavengers were then added in a solution containing 1 mM SIN-1 prepared in 0.1 M phosphate buffer (pH 7.2). Fluorescence was measured after 80 min in a plate reader photometer at room temperature. (B) Urate (1 mM) was co-injected with the avirulent *Pseudomonas syringae* pv. *tomato* AvrB ($OD_{600} = 0.1$) or co-infiltrated with HKGreen-2 (10 μM). Fluorescence was measured in a plate reader photometer at room temperature after 2 h incubation with the dye, corresponding to 8 h after infection with the pathogen. Values shown are means of 6–8 biological replicates ± SE. ∗, $p < 0.05$ vs. Pst AvrB; co-inf, co-infiltration of Pst AvrB and urate.

specifically in the infected leaves, indicating that peroxynitrite was accumulating in response to the pathogen, and the intensity peaked at 7–8 hpi.

In order to determine that the increased HKGreen-2 fluorescence was specifically due to peroxynitrite and not other ROS/RNS, we introduced 1 mM urate into the system as a peroxynitrite scavenger. This resulted in complete suppression of HKGreen-2 fluorescence *in vitro* (confirmed with the additional scavengers epicatechin and cPTIO) and partial suppression *in planta*, specifically a ~25% reduction when urate was introduced into leaves along with the pathogen and an 80% reduction when urate was introduced into the leaf discs along with the HKGreen-2. These data indicated that the observed increases in HKGreen-2 fluorescence in infected leaf discs were predominantly, if not entirely, dependent on increasing peroxynitrite levels. The difference in the impact of urate in each experiment is likely to reflect the degradation of urate introduced into plant tissue several hours before fluorescence measurement, which would reduce its effective concentration.

In agreement with our data, the treatment of tobacco BY-2 cells with the fungal elicitor INF1 induced maximum peroxynitrite production (estimated using APF) and protein Tyr-nitration 6–12 h after elicitation, but no difference between treated cells and controls was apparent up to 2.5 hpi [20]. *A. thaliana* leaves infected with Pst AvrB therefore behave with remarkable similarity to suspension cells exposed to the HR-inducing elicitor INF1. It is likely that the dense population of bacteria in the intercellular spaces within the leaf following infiltration ensures that most plant cells are in contact with the avirulent pathogen, leading to the induction of a highly synchronized and uniform HR as seen in cell suspensions. However, INF1-induced APF fluorescence in tobacco cell suspensions could be almost completely suppressed by urate, whereas in our system HKGreen-2 fluorescence was suppressed by only 25%. This discrepancy indicates that urate may be less efficient in the complex environment of the leaf than in suspension cells, especially over an 8-h treatment period, perhaps because of degradation in plant, limited diffusion or a combination of the two.

Urate reduced fluorescence levels not only in the infected leaf discs but also in controls (data not shown), suggesting that HKGreen-2 is able to detect peroxynitrite induced both by pathogen/HR stress and wounding stress. This confirms the sensitivity of the assay *in planta*, and suggests HKGreen-2 could be useful for the detection of peroxynitrite in the context of many different forms of stress. The HKGreen-2 assay allowed us to show definitively that peroxynitrite accumulates during the HR in *A. thaliana* following infection with an avirulent Pst strain, beginning within 4 hpi and peaking 7–8 hpi. The profile of peroxynitrite accumulation reported here correlates well with the *S*-nitrosylation and inhibition of Prx IIE (which breaks down peroxynitrite) [13] and the accumulation of Tyr-nitrated proteins [17]. This suggests that the inhibition of Prx IIE by NO effectively contributes to peroxynitrite accumulation, which would in turn promote Tyr-nitration, further suggesting that peroxynitrite has an integral role in mediating NO signaling via protein Tyr-nitration. In two recent studies, a total of 21 Tyr-nitrated proteins were identified in untreated sunflower plants [27] and 12 nitro-proteins were identified in *A. thaliana* plants infected with Pst AvrB [17]. However, the physiological significance of peroxynitrite-mediated Tyr-nitration in plants remains unknown because none of the identified nitro-proteins has been characterized. These proteins must be identified and their functions must be established in order to fully elucidate the regulatory role of peroxynitrite in plant cell signaling.

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