Biophoton Imaging: A Nondestructive Method for Assaying *R* Gene Responses

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Plant disease resistance (*R*) proteins of the nucleotide binding-leucine rich repeat class are responsible for pathogen recognition and activation of defense signaling networks leading to the hypersensitive response (HR). Genetically, *R*-protein signaling appears to be integrated through a limited set of common downstream components. However, the timing of development of visible HR is unique to individual *R* proteins. By utilizing the phenomena of ultraweak photon emission from leaves undergoing an incompatible interaction, a powerful nondestructive and facile assay is described to compare timing of defense responses elicited by different *R* proteins. We demonstrate that ultraweak photon emission, or “biophoton generation,” is demonstrated to be associated with hypersensitive cell death. Biophoton emission requires an intact *R* signaling network and increases in cytosolic calcium and nitric oxide, but elevated reactive oxygen species are not necessary. Importantly, the assay is robust and applicable to a range of incompatible interactions in various plant species. The ability to assay *R* responses nondestructively in real time and a chosen genetic background makes this technique amenable to subtle genetic dissection of plant defense responses.

Additional keywords: RPM1, *Pseudomonas syringae*

Ultraweak photon emission in living systems was initially described by A. Gurwitsch during the early 1920s (Lipkin 1996). Gurwitsch’s morphogenetic field theory correlated “mitogenetic radiation” (photon emissions at approximately 260 nm) with cell division rates in yeast and onion cells. A half century later, the term “biophotons” was introduced to describe permanent light emission from all biological systems in terms of single photons emitted within the spectral range of approximately 300 to 800 nm with an intensity of up to a few hundred photons per second per centimeter (Popp 1978). Although originally coined to describe single quanta continuously emitted by living systems and thus distinguish it from bioluminescence (enzymatic photon generation, e.g., luciferase-mediated), the term biophotons has become used increasingly as a general term to describe significant spatial-temporal emissions from biological systems subjected to biotic or abiotic stress.

The molecular mechanisms underlying biophoton emissions are unknown. Biophotons are thought to represent spontaneous

photon emissions generated by way of chemical excitation arising from cellular processes, usually oxidative metabolism. The decay of transition state complexes of excited species such as carbonyls and singlet oxygen generated by radical chain reactions are the likely source of conventional biophoton emissions. Therefore, under conditions of stress, particularly oxidative or nitrosative stress, significant amounts of biophotons are likely to be generated locally, following lipid peroxidation. In mammalian systems, biophotons are emitted in the range of visible up to UV. Changes in biophoton emission have been used to describe the functional state of a living cell. Cancer cells can be discriminated from normal cells based upon reproducible differences in their respective biophoton signatures (Kalikin et al. 2003). In contrast, reports on biophoton emission in plants are rare and incomplete. Biophoton generation (BG) during germination of soybean seeds comprise two spectral components, a UV component, correlated to the original “mitogenetic radiation,” and emission in the red and far-red spectra (Kobayashi et al. 1997). Biophoton emission has also been detected during *Fusarium* infection of cut potato tubers (Makino et al. 1996).

The majority of plant disease-resistance genes encode structurally similar proteins comprising an amino terminal coiled-coil or TIR (toll interleukin 1) effector domain, a central nucleotide binding (NB) region with homology to AAA-ATPases, and a carboxyl terminal region of a variable number of leucine rich repeats (LRR) (Dangl and Jones 2001). The sequence of the model plant *Arabidopsis thaliana* predicts approximately 150 such NB-LRR *R*-like gene sequences potentially capable of specifying resistance to a diverse range of pathogens (Meyers et al. 2003). Activation of a NB-LRR protein is commonly associated with a plethora of biochemical changes (including increases in reactive oxygen and nitrogen species) and concomitant transcriptional reprogramming that usually lead to activation of a hypersensitive cell death program and pathogen restriction. Surprisingly, the majority of characterized *R* genes signal through a few defined genetic loci, given the disparate modes of pathogen presentation (Parker 2003). In spite of this apparent communality of response, actual individual *R*-gene recognition occurs with a specific temporal framework. Currently, a robust nondestructive assay for measuring and comparing activated resistance processes is lacking.

In this study, the utility of biophotons to report activation of plant resistance responses is investigated. We describe a facile, noninvasive assay to measure BG, a key molecular event associated with gene-for-gene interactions. BG is dependent upon increases in intracellular calcium and reactive nitrogen species, but not reactive oxygen species (ROS). The bioluminescence provides both temporal and spatial information on *R*-protein elicitation leading to hypersensitive cell death.

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RESULTS

Arabidopsis thaliana plants carrying the *RPM1* disease resistance gene (Grant et al. 1995) were challenged with *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) with or without the cognate avirulence gene *avrRpm1*. The *RPM1* gene product recognizes, directly or indirectly, Type III mediated cellular delivery of the AvrRpm1 effector protein and orchestrates a rapid defense response. Measurable parameters associated with this defense response include rapid changes in ion fluxes,

followed by an oxidative burst (Grant et al. 2000), NO generation (Delledonne et al. 1998), and induction of a specific set of genes (de Torres et al. 2003), culminating in restriction of pathogen growth and macroscopic leaf collapse approximately five hours following challenge.

Biophotons are generated during an incompatible interaction.

Arabidopsis plants, accession Col-5, were infiltrated at a bacterial concentration of 5×10^7 CFU/ml, and were placed immediately into a dark box. Figure 1 shows real-time integration of photons arising from either an incompatible (DC3000(*avrRpm1*)) (Fig. 1B) or compatible (DC3000 challenge) (Fig. 1C) interaction. The avirulent bacteria generate a rapid and sustained emission of biophotons in challenged leaves, beginning around 2 to 2.5 h postinoculation (hpi). Bioluminescence lasted for approximately 2 to 3 h, with maximal intensity recorded between 2.5 to 3.5 hpi. No photon emission was visible from leaves infiltrated with the virulent pathogen until approximately 16 to 18 hpi (Fig. 1C, integration 17 to 19 hpi). Photon emissions associated with the compatible interaction were weaker than the signal generated by the avirulent pathogen and correlated with the collapse of DC3000-inoculated leaves at approximately 24 hpi. No biophoton emission was detected in mock-challenged (MgCl_2) leaves, indicating that bioluminescence was not generated through perturbation of the apoplastic osmoticum nor wounding associated with the inoculation process (Fig. 1B and C).

The effect of inoculum concentrations on the intensity of photon emission was examined by challenging leaves with bacterial suspensions of 0.5×10^8 , 1×10^8 , 2×10^8 , and 4×10^8 CFU/ml. No significant difference in BG could be detected over this inoculum range (data not shown), indicating the bacteria concentration was saturating, and increasing levels of bacteria did not quench BG.

To demonstrate the specificity of BG for incompatible interactions, we counted photons following challenge with DC3000(*avrRpm1*) in the *RPM1*-signaling mutants, *rpm1* (*rpm1*-3; Grant et al. 1995) (Fig. 1D and E), and *Atrarl* (*Atrarl*-28; Tornero et al. 2002), or in the *NahG* transgenic line overexpressing salicylate hydroxylase (Delaney et al. 1994; Fig. 1F and G). No BG was observed in either mutant (Fig. 1D and E), confirming the dependency on a functional *RPM1* signal transduction pathway. In contrast, reduced salicylic acid (SA) levels had no effect on BG (Fig. 1G), suggesting SA is not a requirement for BG.

Spatial specificity of BG.

We examined the spatial distribution of BG during an incompatible interaction by integrating counts every 15 min. Figure 2

Fig. 1. Biophotons are generated during the incompatible interaction following *RPM1* elicitation. **A**, Natural bioluminescence during the first 15 min, resulting from stored photons. Mock-challenged leaves (MgCl_2) or leaves challenged with either DC3000(*avrRpm1*) or DC3000 are marked. **B**, Integration of photon emissions (2.5 to 3.5 h postinoculation [hpi]) resulting from the incompatible interaction through recognition of the *RPM1* disease resistance protein and AvrRpm1 delivered by DC3000(*avrRpm1*). **C**, Biophoton generation is not evident in DC3000-challenged leaves until 16 to 18 hpi, just prior to visible leaf collapse caused by this highly virulent pathogen. **D** and **E**, No biophotons were generated in the *rpm1* mutant following challenge with DC3000(*avrRpm1*) compared with Col-5 control (integration time 2.5 to 3.5 hpi). **F** and **G**, DC3000(*avrRpm1*) inoculation of the *RPM1* signaling mutant *rar1* fails to elicit biophoton emission, demonstrating the requirement for an intact *RPM1* signal transduction pathway. However, the *NahG* lines with reduced salicylic acid (SA) still undergo a wild-type response (**G**, integration of 3 to 4 hpi), consistent with the SA-independent hypersensitive response elicited by *RPM1*.

traces the spatial-temporal patterns of BG in a single leaf inoculated with DC3000(*avrRpm1*). Biophotons are first generated within the vicinity of the major leaf veins beginning 2.25 hpi. As the reaction develops, a wave of photons is propagated, moving radially across the leaf towards the edge of the blade (Fig. 2B to F). Quantitation of pixels emitted during the incompatible interaction clearly reflects an increased photon emission spreading from the central midvein, toward the leaf margins (Fig. 2G to K).

The robustness of using biophotons to report gene-for-gene interactions was investigated using other *R*-gene combinations. Both the *AvrRpt2*/RPS2 (another coiled-coil (CC)-NBS-LRR R protein; Mindrinos et al. 1994) and *AvrRps4*/RPS4 (a TIR-NBS-LRR protein, Gassmann et al. 1999) interactions elicited a temporally distinct biophoton signature (Fig. 3A to D). Biophotons generated by RPM1 recognition occurred at approximately 2.5 hpi (Fig. 3B), while RPS2-mediated bioluminescence occurred approximately 7 to 8 hpi (Fig. 3C) and RPS4-mediated bioluminescence was first detected approximately 11 to 12 hpi (Fig. 3D). We also examined the ability to generate biophotons in bean pods and tomato leaves undergoing recognition of the respective gene-for-gene pairs, *avrPphE*/R2 (Stevens et al. 1998) (Fig. 3E and F) and *avrPtoB*/*Pto* (Kim et al. 2002) (Fig. 3G and H). Bean pods were inoculated using a syringe and needle. The *avrPphE*/R2 incompatible interaction to race 7 strain 1449B led to strong photon emission (beginning approximately 5 hpi), whereas no photons were detected following inoculation with the virulent race 6 strain 1448A of *P. syringae* pv. *phaseolicola* (Fig. 3E and F). Figure 3G and H shows BG resulting from the *avrPtoB*/*Pto* interaction in tomato beginning approximately 3 hpi. Plants carrying *Pto* were challenged with *P. syringae* pv. *phaseolicola* RW60 containing *avrPtoB*. Like DC3000, in the absence of *avrPtoB*, RW60 also caused hypersensitive response (HR) collapse after 24 h, but the reaction was initiated much more rapidly by RW60 plus *avrPtoB*. In all incompatible interactions investigated, photon emission significantly preceded the onset of macroscopic HR.

Biophotons are generated by a plant-specific process.

To demonstrate BG was strictly dependent upon host *R* gene function and not an indirect consequence of a pathogen-derived

signal, we examined the plant response to conditional in planta expression of *avrRpm1* or *avrRpt2* driven off a dexamethasone (DEX)-inducible promoter (Ayoma and Chua 1997). In planta expression of *avrRpm1* resulted in BG approximately 2 h after DEX application (15 μ M). This timing is nearly coincident with BG initiated through *AvrRpm1* delivery by DC3000 (Fig. 4A and B). Interestingly, by 16 h post-DEX application, a low level of photon emission was detected throughout the *avrRpm1*-expressing plants, indicative of systemic induction of *AvrRpm1* and consistent with a whole-plant HR evident in all three independent lines examined (data not shown). Conditional expression of *avrRpt2* also resulted in BG (Fig. 4C and D). Intriguingly, although DEX induction of *avrRpt2* and *avrRpm1* transcripts occurs within a similar time frame, RPS2-mediated bioluminescence following conditional expression of *avrRpt2* occurs within approximately 30 min of BG measured following DC3000(*avrRpt2*) challenge. Assuming both transcripts are translated with an equivalent efficiency, these data are consistent with a requirement for *AvrRpt2* posttranscriptional processing prior to RPS2 activation, as recently demonstrated by Axtell and associates (2003).

BG requires increases in cytosolic Ca^{2+} but not an oxidative burst.

Since increases in intracellular calcium and generation of ROS are integral to active defense responses (Grant and Loake 2000; Grant et al. 2000), we investigated the role of selected pharmacological inhibitors on BG. Plants were coinfiltrated with the avirulent pathogen DC3000(*avrRpm1*) and either the calcium channel blocker, lanthanum chloride (LaCl_3 , 1.5 mM) or the NADPH oxidase inhibitor diphenyl iodonium (DPI, 9 μ M). LaCl_3 but not DPI was capable of preventing BG (Fig. 5A and B), suggesting increases in cytosolic calcium are necessary for BG.

The ability to generate biophotons in the presence of DPI was unexpected. Biophoton emission in mammals has historically been associated with oxidative stresses (Devaraj et al. 1991, 1997). To address the question whether BG was caused by an alternative source of reactive oxygen, we coinfiltrated DC3000(*avrRpm1*) with catalase (25 μ g/ml). Under these conditions, very little H_2O_2 accumulates (Bestwick et

Fig. 2. The spatial and temporal dynamics of biophoton generation (BG) were measured during the *avrRpm1*/RPM1 interaction. **A**, Biophotons generated in the challenged leaf spread radially from **B**, the central midvein beginning 2.25 h postinoculation (hpi) toward **C** through **F**, the laminar margins. **G** through **K**, Quantitation of BG clearly shows a wave of photons originating along the midvein and extending outward as the interaction develops.

al. 1998; Grant et al. 2000), yet BG was undiminished (Fig. 5C and D).

We next examined a possible role for ROS in BG by generating superoxide or hydrogen peroxide in planta, using, respectively, xanthine oxidase/xanthine or glucose oxidase/glucose reaction systems (Jabs et al. 1996; Overmyer et al. 2000). Neither ROS generating system elicited any detectable photon emission (Fig. 5E and F), although biophotons were generated on adjacent leaves following DC3000(*avrRpm1*) inoculation with or without DPI.

Finally, we examined the role of ROS in BG, using one of the *Arabidopsis* NADPH oxidase respiratory-burst homologs, *AtrbohD*. DC3000(*avrRpm1*)-challenged *AtrbohD* mutant plants fail to accumulate H_2O_2 but still undergo a typical RPM1 macroscopic HR and concomitant bacterial restriction (Torres et al. 2002). BG was undiminished in the *AtrbohD* mutant compared with that in Col-5 (Fig. 5G). Our data indicate *AtrbohD*-generated H_2O_2 is not necessary for BG but support the hypothesis that BG is correlated with hypersensitive cell death (Fig. 5G). Further genetic evidence for this

hypothesis was derived from studying BG in *ndr1* plants. *ndr1* plants still undergo an *RPM1*-mediated HR but fail to fully restrict bacterial growth (Century et al. 1995). It has previously been reported, based upon measurements of H_2O_2

Fig. 3. Biophoton generation (BG) is common to gene-for-gene interactions and defines a unique signature for each *R* gene response. **A**, Leaves of *Arabidopsis thaliana* Col-5 were challenged with DC3000 alone or DC3000 containing either *avrRpm1*, *avrRpt2*, or *avrRps4*, as indicated. BG was measured over 20 h postinoculation (hpi). **B** through **D**, Each *R* gene elicited a unique biophoton signature that was strongly correlated with the timing of leaf collapse. Photon emissions were integrated over the following times (hpi); **B**, *avrRpm1* = 2.75 to 3.5 hpi; **C**, *avrRpt2* = 7.5 to 8.25 hpi; **D**, *avrRps4* = 11.5 to 13.5 hpi. **E** and **F**, Rapid BG (within 5 hpi) was also measured in bean pods containing the R2 resistance gene following inoculation with *Pseudomonas syringae* pv. *phaseolicola* race 7 strain 1449B containing *avrPphE* but not the virulent race 6 strain 1448A. **G** and **H**, In tomato leaves containing Pto, BG was rapid, occurring approximately 3 h after challenge with *P. syringae* pv. *phaseolicola* RW60 carrying *avrPtoB*.

Fig. 4. In planta expression of *avr* genes generates biophoton emissions with a similar temporal signature to that generated by bacterial delivery. **A**, In planta expression of *avrRpm1* under the dexamethasone (DEX) promoter results in biophoton generation within 2 h postinoculation (hpi) compared with approximately 2.5 hpi after *AvrRpm1* delivery by DC3000. **B**, Photon integration for 1 h from 2.75 hpi. **C**, The timing of biophoton emission following in planta expression of *avrRpt2* under control of the DEX promoter (right hand side) recapitulates that seen by DC3000 delivery of *AvrRpt2*. **D**, Biophoton emission integrated between 7 to 8 hpi.

production (Shapiro and Zhang 2001) and NO generation (Zhang et al. 2003), that RPM1 elicitation in *ndr1* is delayed relative to wild-type Col-0. We also observed biophoton emission in *ndr1* leaves challenged with DC3000(*avrRpm1*), but consistent with the above observations, BG was delayed by

>1 h relative to Col-0 plants undergoing an RPM1-induced HR (data not shown).

BG is abolished by inhibitors of nitric oxide synthase.

Recently NO has been demonstrated to play a key role in gene-for-gene resistance, although the contribution appears to depend upon the particular pathogen. The inducible NO inhibitors, P-BITU (*S,S*-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea) and the arginine analogue N^6 -nitro-L-arginine (L-NNA), prevent RPM1-specified HR (Delledonne et al. 1998). Figure 6B shows BG can be inhibited in a dose-dependent manner following coinoculation of DC3000(*avrRpm1*) (0.5×10^7 CFU per ml) with L-NNA. P-BITU also attenuated biophoton emission but required coinfiltration at a much higher concentration (3 mM). Interestingly, neither coinfiltration with NO scavengers cPITO (carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide) nor urate (Alamillo and Garcia-Olmedo 2001; data not shown) was able to

Fig. 5. Biophoton generation (BG) requires increased cytosolic calcium but not increased reactive oxygen species. **A** and **B**, Coinfiltration of LaCl₃ (1.5 mM) but not diphenyl iodonium (9 μ M) inhibits BG following DC3000(*avrRpm1*) challenge (photons integrated from 2 to 3.5 h post-inoculation [hpi]). **C** and **D**, Coinfiltration of catalase (25 μ g/ml) fails to abolish BG (integration from 2.5 to 3.5 hpi). **E** and **F** In planta generation of superoxide or hydrogen peroxide by xanthine/xanthine oxidase (X/XO; 0.5 mM/0.05 unit ml⁻¹) or glucose/glucose oxidase (G/GO; 2.5 mM/25 units ml⁻¹) did not generate detectable photon emission at any stage over a 24-h period, while control plants with or without the NADPH oxidase inhibitor diphenyl iodonium responded within 2 hpi. **G** Plants carrying a transposon insertion in one of the *Arabidopsis* NADPH oxidase respiratory burst subunits, *AtrbohD* (Torres et al. 2002), fail to accumulate H₂O₂ following RPM1 elicitation but still generate biophotons (integration time 2.5 to 3.5 hpi).

Fig. 6. Biophoton emission is attenuated by the inducible nitric oxide synthase inhibitor L-NNA. **A** L-NNA was coinfiltrated with DC3000(*avrRpm1*) at 0, 0.5, 1, and 3 mM. Biophoton generation (BG) is abolished in a dose-dependent manner (photon integration over 2.5 to 3.25 h postinoculation [hpi]). Another nitric oxide synthase inhibitor, P-BITU (*S,S*-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea), coinfiltrated at 1, 2, and 3 mM was less effective at blocking photon emission, with attenuation of BG not occurring until coinfiltrated at 3 mM.

quench BG, suggesting the NO generated may rapidly interact with other cellular components. L-NNA also compromises RPS2- and RPS4-mediated biophoton emission and subsequent HR (data not shown). In summary, our data suggest both cytosolic Ca^{2+} influx and NO generation but not ROS are necessary for bioluminescence generation.

DISCUSSION

To understand *R*-gene signaling and the subtle effects of genetic modifiers or pharmacological reagents, it is important to be able to measure timing of key biochemical events in a non-destructive manner. We have previously reported the use of the calcium-sensitive bioluminescence probe, aequorin to report changes in intracellular calcium early in the RPM1 elicitation process. However, this assay required a specific transgenic line and was not able to report other gene-for-gene combinations tested (Grant et al. 2000). Here, we describe a robust, facile, and noninvasive method to measure a key feature of an incompatible host-pathogen response, the sustained generation of biophotons. BG appears intimately associated with hypersensitive cell death orchestrated through a typical gene-for-gene interaction. All functional gene-for-gene combinations examined, irrespective of the class of R protein (TIR or CC-NBS-LRR) or plant species, generated a temporally distinct biophoton signature, unique for the specific *avrR* recognition. BG was genetically dependent upon a functional *R*-gene signaling pathway. In the *avrRpm1/RPM1* interaction, BG was independent of SA levels and occurred subsequent to increases in intracellular calcium concentrations and approximately coincident to increases in ROS, as determined by electron microscopy (Grant et al. 2000). However, photon emissions were independent of ROS, as determined by pharmacological and genetic approaches.

Biophoton imaging provides a nondestructive assay that will be of general utility in genetic and pharmacological approaches to the study of *R*-gene-dependent resistance responses.

Biophoton emission may arise from lipid peroxidation, a major consequence of oxidative stress (Havaux 2003). In plants, the most likely location for the majority of lipid peroxidation events would be the highly unsaturated biomembranes of chloroplasts and other plastid bodies. If lipid peroxides caused BG, scavengers of lipid peroxides would be expected to abolish bioluminescence. We tested this possibility by coinfiltration of leaves with avirulent bacteria and the lipid scavenger butylated hydroxytoluene (BHT). BHT concentrations over the range of 0.2 to 2 mM failed to prevent BG (data not shown). Additionally, we also measured the levels of lipid peroxides before, during, and after BG by measuring malondialdehyde (MDA) levels. MDA is a secondary end product of the oxidation of polyunsaturated fatty acids and is widely considered an indicator of general lipid peroxidation. Only small, statistically insignificant increases in MDA levels between DC3000(*avrRpm1*) and DC3000 were recorded at 2.5 and 3.5 hpi. It is unlikely these absolute differences themselves are sufficient to account for the dramatic levels of bioluminescence detected during the incompatible interaction, although we cannot rule out the possibility a specific class of hydroperoxide is involved in BG.

We cannot rule out the possibility biophotons themselves constitute an endogenous signaling mechanism specifying initiation of the HR. Spatial patterns of biophoton emission evident in Figure 2 suggest the wave of bioluminescence through the iterative generation and emission of photons could act as local signaling network in leaves undergoing the HR. Analysis of available microarray data for defense responses suggest a strong correlation between transcriptional activation and BG

(W. Truman *unpublished data*). Moreover, transgenic plants that exhibit enhanced resistance in the absence of an RPM1-specified HR do not show biophoton emission (A. Al-Daoud and M. Grant *unpublished data*).

Reactive nitrogen species have recently been demonstrated to play a role in orchestrating the HR (Delledonne et al. 1998; Durner et al. 1998). We demonstrated L-NNA, an inhibitor of mammalian inducible nitric oxide synthase (NOS), was capable of quenching BG (and the HR) in a dose-dependent manner but was unable to prevent BG (or the HR) using the NO scavenger cPTIO, even at concentrations in excess (3 mM) of those previously shown to inhibit NO generation. Similarly, urate, which has recently been shown to interfere with the *RPM1*-mediated cell death but not resistance following delivery of *avrRpm1* by *P. syringae* pv. *phaseolicola* 411 (Alamillo and Garcia-Olmedo 2001) was ineffective at abolishing BG. These data suggest that a small amount of NO may be sufficient for generating the biophoton signal.

While aware that the NOS inhibitors and NO scavengers do not identify the specificity of the nitrosative stress, our data allow speculation that NO radicals ($\text{NO}^{\bullet}$), nitrosonium cations (NO^+), or nitroxyl radicals (NO^-) may contribute to the BG. The reaction of NO and reactive oxygen intermediates can generate ONOO^- , a potent oxidative and nitrating agent, even in the presence of low amounts of the superoxide anion (O_2^-) (Beligni and Lamattina 1999; Lipton 1999). Free radicals formed during the peroxynitrate reaction can react with various organic compounds in particular membrane lipids. In addition to lipid peroxides, protein radicals can abstract an H^+ from tryptophan residues (Halliwell et al. 1999) to propagate a long-lived radical pathway capable of generating the high and prolonged levels of photon emissions seen in this study (Fig. 2). Moreover, the ability of ONOO^- to move short distances between cells (Marla et al. 1997) would allow signal amplification and enhanced BG. Although ONOO^- can be generated through the NO donor, sodium nitroprusside (SNP) and the superoxide generating system xanthine/xanthine oxidase (X/XO), NO generation by SNP is light-dependent and therefore not a suitable pharmacological agent for this study.

Although we have yet to define the mechanisms underlying for BG, this provides a robust and readily accessible technique to compare timing of defense responses across different R-protein functions. We are currently investigating using this technique to analyze incompatible plant-fungal interactions at the single cell level. The demonstration that conditional induction of *avrRpt2* and *avrRpm1* faithfully replicated the R-protein-specific biophoton signature generated through delivery and intracellular processing of the cognate Avr protein will facilitate forward genetic screens for identifying modified responses in defense signaling pathways.

MATERIALS AND METHODS

Chemicals.

All chemicals were from Sigma (Gillingham, Dorset, U.K.), unless specified.

Plant growth.

Arabidopsis thaliana seed was vernalized for 2 days at 4°C, prior to sowing in Levingtons F2 compost. Plants were grown under short-day conditions in a controlled environment chamber (12 h of light, 100 to 150 microeinsteins at 22°C days, 20°C nights) for five to six weeks to prior to imaging.

Stable DEX-inducible lines were generated by cloning the open reading frame of *avrRpm1* or *avrRpt2* into the steroid-inducible vector pTA7002 (Aoyama and Chua 1997) modified to contain BASTA (resistance to the herbicide phosphinothricin)

selection. Constructs were sequence-verified, were moved into *Agrobacterium tumefaciens* GV3101, and were used to transform *Arabidopsis* Col-5 by floral dip. Homozygous T2 lines were selected by segregation for BASTA and were characterized for HR and transcript induction following DEX application (brushing leaves with a 15- μ M DEX solution in 0.005% silwet L-77 [OSi Specialties Europe, Antwerp, Belgium]) prior to their use in imaging experiments.

***Pseudomonas* strain maintenance and pathogen challenge.**

Pseudomonas strains, with or without the appropriate avirulence genes cloned into the broad host range vector pVSP61 (Innes et al. 1993), were maintained under selection on King's B agar and were cultured as described (Grant et al. 2000). Inoculum were prepared from appropriate *Pseudomonas* overnight cultures, were washed once in 10 mM MgCl₂, and were resuspended in 10 mM MgCl₂ to the required density (0.5×10^7 CFU/ml, unless otherwise stated). Leaves of 4- to 5-week-old plants were pressure-infiltrated on the abaxial surface, using a needleless 1-ml syringe.

Pharmacological challenges. BHT, P-BITU, urate, L-NNA, DPI, or cPITO were coinfiltrated with DC3000(*avrRpm1*) at the concentrations indicated.

For reactive oxygen generation, leaves were syringe-infiltrated with either the X/XO (0.5 mM/0.05 unit ml⁻¹) or glucose/glucose oxidase (G/GO; 2.5 mM/25 units ml⁻¹ [Calbiochem, Merck Biosciences Ltd., Nottingham, U.K.]) in 10 mM sodium phosphate buffer, pH 7.0, to generate superoxide or H₂O₂ respectively (Jabs et al. 1996; Overmyer et al. 2000).

Plant imaging.

Up to four pathogen-challenged plants were placed inside a dark box, and digital monochrome images were captured from a Hamamatsu ORCAII ER CCD camera with a 35-mm f2.8 Nikkor lens. Photons were counted for 15 min at 2x2 binning mode, and images were acquired over the appropriate time period using Wasabi imaging software (Hamamatsu Photonics, Welwyn Garden City, U.K.).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Nottingham Arabidopsis Stock Centre (NASC) Affymetrix Experiment:
Impact of Type III effectors on plant defense responses webpage:
affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=59