Effects of urate, a natural inhibitor of peroxynitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*

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Summary

Urate, a natural peroxynitrite scavenger, has been used to investigate the possible role of peroxynitrite during plant-pathogen interactions. Urate greatly reduced lesion formation in *Arabidopsis* leaves treated with an abiotic peroxynitrite-generating system or with a peroxynitrite solution, indicating that it can act as an effective scavenger in planta. In the interaction with the avirulent *Pseudomonas syringae* pv. *phaseolicola* (*avr*RPM1$^+$), cell death in the inoculated area was strongly reduced by urate, without compromising disease resistance. In contrast, urate promoted discrete cell death in response to an isogenic *Pseudomonas syringae* (*avr*RPM1$^-$), which did not trigger an HR when inoculated alone, and it induced resistance and arrest of pathogen growth. Scavenging of peroxynitrite did not modify the response of *Arabidopsis* to an avirulent strain of *Xanthomonas campestris* pv *campestris*, that showed a high resistance to NO and peroxynitrite. Our data indicate that peroxynitrite plays a significant role in the responses of plants to *Pseudomonas syringae*.

Keywords: *Arabidopsis*, disease-resistance, *Pseudomonas*, peroxynitrite, scavenger, urate.

Introduction

Plants defend themselves from invading pathogens by activating a number of defence responses, which include reinforcement of the cell wall, synthesis of phytoalexins and induction of various defence-related genes (Hammond-Kosack and Jones, 1996). Among the most rapid responses triggered upon recognition of an avirulent pathogen is the production of reactive oxygen intermediates (ROIs), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Baker and Orlandi, 1995; Lamb and Dixon, 1997; Levine *et al.*, 1994). ROIs are believed to perform multiple roles during plant defence, acting as signalling molecules that trigger host cell death in the hypersensitive response (HR) (Dangl *et al.*, 1996; Jabs *et al.*, 1996; Levine *et al.*, 1994) or as possible direct inhibitors of pathogen growth (Lamb and Dixon, 1997).

In animals, ROIs cooperate with nitric oxide (NO) in some pathological conditions, such as inflammation, acute phase responses and programmed cell death (Stamler, 1994). NO may lead to the generation of peroxynitrite ($ONOO^-$), a potent oxidant that is formed by the combination of NO with O_2^-. Peroxynitrite formed during the inflammatory response causes a variety of toxic effects, including lipid peroxidation and cell death (Hooper *et al.*, 1998; Radi *et al.*, 1991; Stamler *et al.*, 1992; Stamler, 1994).

Recently, it has been shown that in plant cells NO acts synergistically with ROIs in the promotion of cell death and in the activation of defence responses (Delledonne *et al.*, 1998; Durner *et al.*, 1998), and elicitor treatment induces ROIs and NO production with almost identical time-courses (Allan and Fluhr, 1997; Foissner *et al.*, 2000). The simultaneous synthesis of both NO and ROIs may generate levels of peroxynitrite that could contribute to host cell death and to pathogen inhibition (Bolwell, 1999; Durner and Klessig, 1999), as in the mammalian inflammatory response.

Urate, a naturally occurring product of purine metabolism, is a strong inhibitor of peroxynitrite effects (Balavoine and Geletii, 1999; Hooper *et al.*, 1998; Regoli and Winston, 1999; Whiteman and Halliwell, 1996). Urate inhibits the effects of peroxynitrite, but not NO, in lipopolysaccharide-stimulated mammalian cells, acting as a strong inhibitor of cell death (Hooper *et al.*, 1998). In humans, urate is the last

product of purine degradation and, when accumulated above normal levels, becomes a clinical problem (hyperuricemia and gout). In plants, purines are oxidized to NH_4^+ and CO_2, and urate concentration is usually maintained at low levels, except in some leguminous plants, called 'ureide-plants', that accumulate higher levels of purines (Schubert, 1986).

We have exploited the peroxynitrite-scavenging ability of urate to investigate the possible role of peroxynitrite in the response of plants to bacterial pathogens. We show that whereas urate significantly attenuates cell death during the HR of *Arabidopsis* plants in response to an avirulent *Pseudomonas syringae* strain (*avr*RPM1$^+$), without compromising resistance, it promotes discrete cell death and pathogen growth inhibition when infiltrated together with an isogenic bacterial strain that lacks the *avr* gene. These results unveil a role of peroxynitrite in these plant–pathogen interactions.

Results

Urate protects against the phytotoxic effects of peroxynitrite

To validate the use of urate as a tool to investigate the role of peroxynitrite in the death of plant cells, an experiment with an exogenously added peroxynitrite-generating system was carried out. In this experiment, the NO donor sodium nitroprusside (SNP) was used alone or in combination with the superoxide-generating system hypoxanthine/xanthine oxidase (HX/XO). In presence of the enzyme, these reagents generate, in an abiotic manner, a sustained synthesis of peroxynitrite, a product that should be removed and its effects attenuated if urate is also added. The NO donor was used at a concentration (0.5 mM) that has been demonstrated to induce plant cell death, provided that oxidative stress is also occurring (Delledone *et al.*, 1998). The concentrations of HX and XO used (1 mM and 0.1 U ml^{-1}, respectively) were within the range that has been shown to produce cell death in lesion-mimic mutants (Jabs *et al.*, 1996). Urate was used at an equimolar concentration with respect to HX. To discriminate among the effects due to peroxynitrite or to the synergims of SNP plus the H_2O_2 that could be originated by dismutation of superoxide, catalase (50 U ml^{-1}) was added to the peroxynitrite generating system. Leaves were infiltrated with 1 mM H_2O_2, as a control for the possible peroxide damage in absence of NO.

Figure 1 shows that when *Arabidopsis* leaves were infiltrated with SNP plus HX/XO, most of the treated leaves showed severe lesions at 24 h post infiltration. This effect was essentially prevented when urate was also present, as only a much lower number of leaves showed lesions, and most of these were only incipient chlorotic ones, instead of necrotic (Figure 1). In contrast to the protective urate effects, catalase did not reduce either the number, or the severity of the lesions in the treated leaves. Glucose (G; 0.5 mM) plus glucose oxidase (GO; 0.5 U ml^{-1}), an *in vitro* system to generate H_2O_2, was also used, both alone and in combination with SNP. The effects of urate in the damage originated by these substances was investigated. The number of leaves treated with G/GO plus SNP that presented severe lesions was not reduced when urate was added to the mixture, further demonstrating that urate does not exert any protective effects against damage originated from H_2O_2 plus SNP (lesions appeared in 17 out of 36 leaves treated with G/GO plus SNP, and in 19 out of 36 leaves when urate was included in the treatment). Complete absence of lesions were observed not only in control *Arabidopsis* leaves (mock), but also in tissue infiltrated with urate, or with H_2O_2, and only incipient lesions were present in a small number of leaves when using either SNP, HX/XO (Figure 1a,b), or G/GO (not shown).

To demonstrate that the effects of urate were specific, a commercial solution of peroxynitrite was directly infiltrated into *Arabidopsis* leaves. Peroxynitrite used at 1 mM produced necrotic lesions in all the treated leaves, and lesions were still observed after treatment with 0.1 mM peroxynitrite. Urate, applied together with the peroxynitrite solution did reduce both the severity and the number of leaves that showed visible lesions (Figure 2a,b). Thus, urate specifically protects against peroxynitrite damage.

In contrast with the results obtained with commercial peroxynitrite, treatment of *Arabidopsis* leaves with the peroxynitrite donor, 3-morpholinosydnonimine (SIN-1) had a toxic effect only when used at concentrations above 2 mM (not shown). As a control, NaOH 1 mM used to prepare the diluted peroxynitrite solutions was used without any visible damage in the treated leaves (Figure 2).

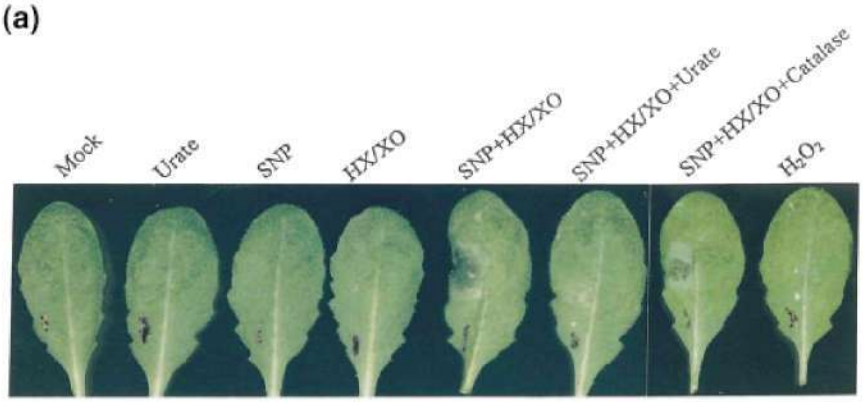
Opposite effects of urate on cell death caused by P. syringae *with and without the avrRPM1 gene*

Typical HR responses were observed after local inoculation of *Arabidopsis* plants ecotype Col-0 with suspensions of the incompatible *Ps. syringae* pv *phaseolicola* strain 411 (avrRPM1$^+$), with clear necrosis affecting the infiltrated area at 24 h post infiltration, as shown in Figure 3. In contrast, in leaves infiltrated with the same bacterium plus 1 mg ml^{-1} urate, small necrotic foci were intercalated with healthy and with chlorotic tissue within the infiltrated area, greatly reducing the severity of the macroscopic lesion obtained when this pathogen was inoculated alone (Figure 3).

To further assess the effect of urate on cell death caused by the avirulent *Pseudomonas* strain, fluorescence microscopy was used. Plant cells that undergo HR display a

Figure 1. Urate protection against damage produced by a peroxynitrite-generating system.
Arabidopsis leaves were hand infiltrated with: urate 1 mM; sodium nitroprusside (SNP) 0.5 mM; hypoxanthine/xanthine oxidase (HX/XO) 1 mM HX plus 0.1 U ml^{-1} XO; catalase 50 U ml^{-1}; hydrogen peroxide (H_2O_2) 1 mM. When several products were used simultaneously, they were mixed at the adequate concentration before infiltration of 15–20 µl into each leaf. (a)Visible symptoms on representative leaves, photographed at 24 h after infiltration. (b) Visible symptoms scored at 24 h. Black spots on the base of the leaves are ink marks.

(a)

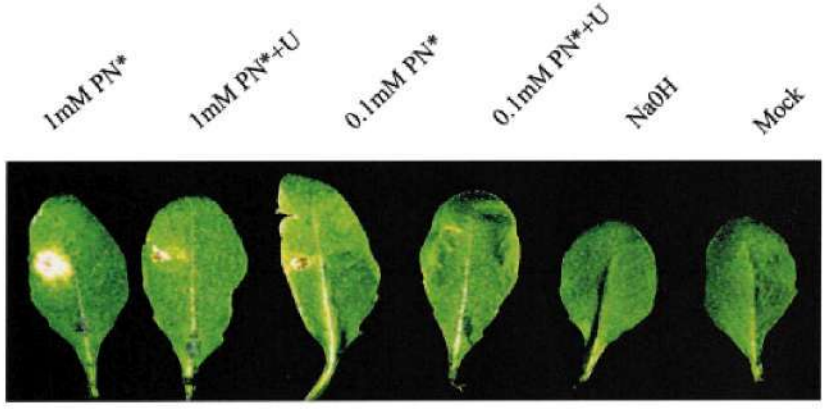


(b)

TREATMENT	N° Leaves tested	LESION		
		absent	trace	severe
Mock	25	25	-	-
Urate	25	25	-	-
SNP	38	33	5	-
HX/XO	36	33	3	-
SNP+HX/XO	56	2	12	42
SNP+HX/XO+Urate	56	16	24	16
SNP+HX/XO+Catalase	48	-	6	42
H_2O_2	18	18	-	-

Figure 2. Urate protection against peroxynitrite damage.
Arabidopsis leaves were hand infiltrated with peroxynitrite (PN*) at: 1 mM or 0.1 mM, with or without the addition of 1 mM urate. As controls, NaOH 1 mM and mock solutions were used. (a) Visible symptoms on representative leaves, photographed at 48 h after infiltration. (b) Visible symptoms scored at 24 h.

(a)



(b)

TREATMENT	N° Leaves tested	LESION		
		absent	trace	severe
Peroxynitrite 1mM	24	-	-	24
Peroxynitrite 1mM+Urate	25	2	12	12
Peroxynitrite 0.1mM	24	3	15	6
Peroxynitrite 0.1mM+Urate	25	18	4	3
NaOH 1mM	14	12	2	-
Mock	14	14	-	-

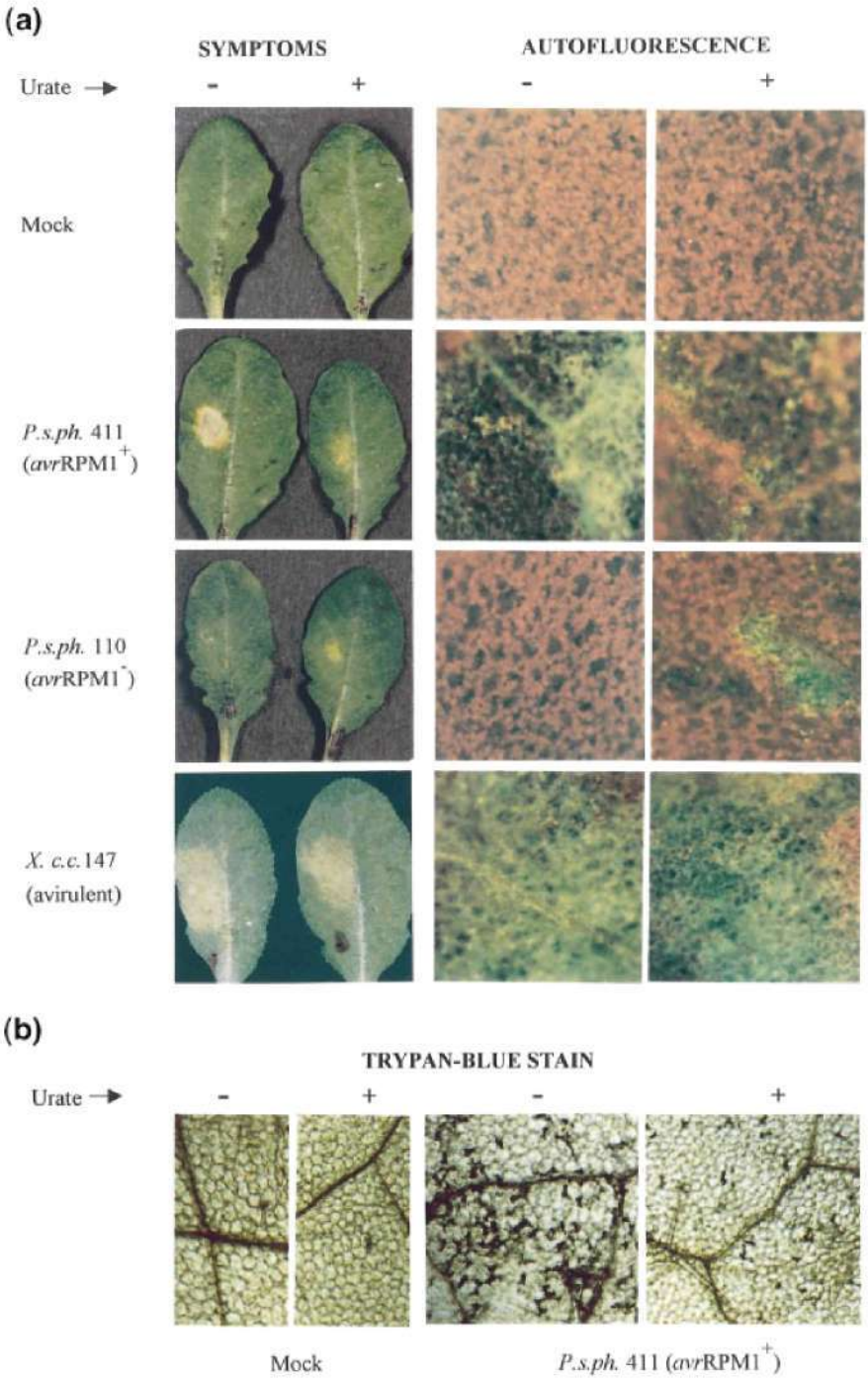


Figure 3. Effects of urate on the response to bacterial pathogens.
(a) Col-0 *Arabidopsis* plants were inoculated with 10^6 cfu/ml of: *P.s.ph.* 411, *P.s.phaseolicola* 411 (*avr*RPM1$^+$); *P.s.ph.* 110, *P. s. phaseolicola* 110 (*avr*RPM1$^-$), with or without 1 mg ml^{-1} uric acid. After 24 h, leaves were harvested and examined for visible symptoms (left) or ultraviolet-stimulated autofluorescence (right). For avirulent *X.c.campestris* 147 strain (X.c.c. 147), 5×10^6 cfu ml^{-1} were used as inoculum, and the leaves were examined at 48 hp.i. As controls, leaves were infiltrated with mock or mock plus 1 mg ml^{-1} uric acid (upper panels). Black spots on the base of the leaves correspond to ink marks. (b) Microscope photographs of control and *P.s.ph.* 411 inoculated leaves, with and without urate, at 24 hp.i., stained with the Trypan-blue dye (Keogh *et al.*, 1980).

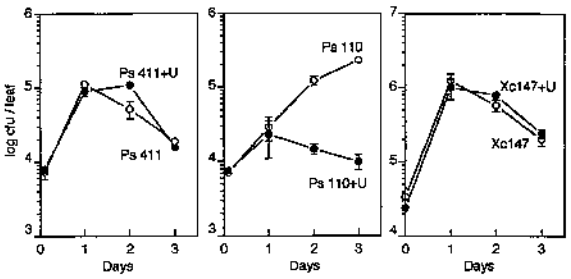


Figure 4. Urate effects on pathogen growth *in planta*.
Mature *Arabidopsis* leaves (4–6-weeks-old) were inoculated with bacteria as described in Experimental procedures. Inocula of 0.5×10^6 cfu ml^{-1} were used for the *P.s.phaseolicola* strains: Ps 411, *P.s.phaseolicola* 411 (*avr*RPM1$^+$); Ps 110, *P.s.phaseolicola* 110 (*avr*RPM1$^-$). *X.c.campestris* strain 147 (Xc 147) was inoculated at 5×10^5 cfu ml^{-1}. For each data point, 4–6 replicates containing 3 infected leaves were used. Colonies were counted after incubation at 28°C for 2 days. The results are the average from 3 to 4 independent experiments. Open symbols: bacteria alone; filled symbols: bacteria plus 1 mg ml^{-1} urate.

marked increase in fluorescence, primarily due to the production and release of phenolic compounds upon cell death (Bennett *et al.*, 1996; O'Neil and Mansfield, 1982). In our experiments, *P.s. phaseolicola* 411 (avrRPM1$^+$) was inoculated at a relatively low dose (1×10^6 cf.u./ml). As expected, at 24 h post inoculation, most of the cells within the infiltrated area showed a marked autofluorescence with confluence of cell death in the whole area (Figure 3a). In contrast, when the same strain was inoculated with urate, the number of autofluorescent cells observed within the infiltrated area at 24 h post infiltration was highly reduced (Figure 3a). To confirm this result, the inoculated leaves were stained with trypan-blue and observed microscopically. The results shown in Figure 3(b) further support the protective effect of urate against the HR-mediated cell death. Staining of individual dead cells was found in the leaves inoculated with the avirulent pathogen plus urate, whereas groups of dead cells were observed in absence of urate (Figure 3b). However, confluent autofluorescence of the inoculated area was observed both with or without urate by increasing the bacterial titre two- to fourfold (data not shown), suggesting that urate was not effective in preventing the death of cells in direct contact with the bacteria.

Since the peroxynitrite formed in the interaction with a pathogen could have direct antimicrobial activity, we investigated whether scavenging of this toxic compound could stimulate bacterial growth *in planta*. The *P.s.phaseolicola* strain 110, which is isogenic to the *P.s.phaseolicola* 411 strain, but lacks the *avr*RPM1 gene, was appropiate to this end because, under favourable conditions, it is able to grow slowly on *Arabidopsis* Col-0. More vigorous growth was to be expected if the peroxynitrite putatively formed during the interaction was limiting growth and its effects were then inhibited by the urate added. As expected, infiltration with strain *P.s.phaseolicola* 110 did not produce any visible symptoms at 24 h post inoculation, but, surprisingly, small necrotic lesions were readily observed at this time when leaves were coinoculated with urate. In agreement with the macroscopic observations, leaves infiltrated with the bacterium alone did not show any fluorescent foci, whereas single autofluorescent cells or groups of cells where found within the whole inoculated area when urate was present (Figure 3a). At 6–8 days post inoculation, leaves inoculated with *P.s.phaseolicola*110 (*avr*RPM1$^-$) alone started to become chlorotic (data not shown), in agreement with the low virulence of this pathogen in *Arabidopsis*, while no symptoms, other than the small necrotic patches, were observed in the leaves coinoculated with urate (data not shown)

In contrast to the above observations, urate had no effect on the incompatible interaction with *Xanthomonas campestris* pv. *campestris* strain 147, that is known to induce HR in *Arabidopsis* Col-0 (Lummerzhein *et al.*, 1993). Macroscopic HR symptoms produced by this strain were first visible at about 24 h post inoculation, and the lesions were clearly necrotic and highly autofluorescent by 48 h (Figure 3a). Co-inoculation of urate with this bacterium did not reduce the development of macroscopic lesions, nor did it affect fluorescence within the inoculated area at any of the tested inoculation levels (5×10^5 cf.u./ml to 5×10^6 cf.u./ml) (Figure 3a).

The upper panel in Figure 3(a) shows that control leaves infiltrated with mock solution or with 1 mg ml^{-1} urate did not show any macroscopic or microscopic symptom.

Restriction of pathogen growth in planta

To determine whether the reduction by urate of the cell death caused by the *P.s.phaseolicola* 411 (*avr*RPM1$^+$) strain in *Arabidopsis* plants was associated with compromised disease resistance, bacterial growth was monitored. Figure 4 shows that the bacterial population of this strain *in planta* decayed after initial active growth, and this growth restriction was not significantly affected by urate.

Under our experimental conditions, strain *P.s.phasolicola* 110 (*avr*RPM1$^-$) was able to grow in *Arabidopsis* Col-0 leaves, although at a lower rate than the initial growth rate of the *P.s.phaseolicola* 411 strain (Figure 4). Co-inoculation of the *avr*RPM1$^-$ strain with urate led to a severe restriction of bacterial growth, which occurred at a lower population level than in the case of the *avr*RPM1$^+$ strain (Figure 4). This result was also obtained when a higher bacterial titre was used for inoculation (not shown). Bacterial growth *in planta* of the avirulent *X.cacampestris* 147 strain was unaffected by the presence of urate (Figure 4).

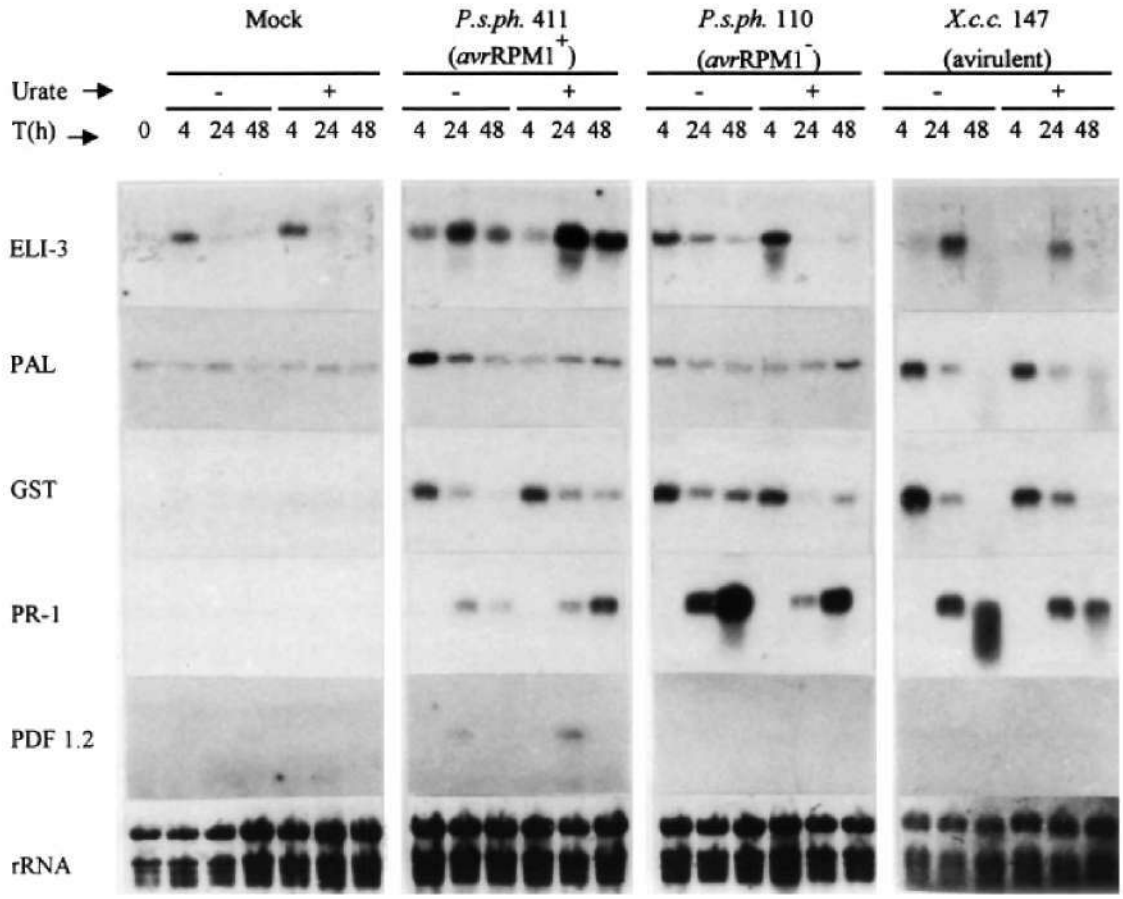


Figure 5. Urate effects on the expression of defence-related genes in response of bacterial pathogens.
Bacterial pathogens were infiltrated in *Arabidopsis* leaves with or without 1 mg ml^{-1} urate. Total RNA (9 μg) from mock or pathogen-inoculated *Arabidopsis* leaves was separated by electrophoresis, blotted and hybridized with the ELI-3, PAL, GST, PR-1, PDF 1.2 *Arabidopsis* cDNA probes, as described in Experimental procedures. RNA loading was monitored by EtBr-staining of rRNA.

To exclude a direct effect of urate on bacterial growth, sensitivity to this reagent was tested both in liquid media and in solid media (data not shown). Urate concentrations up to 2 mg ml^{-1} (above saturation), had no effect on the bacteria tested.

Effects of urate on the expression of defence-related genes

We have used specific probes for key marker genes to investigate whether the observed effects of urate are mediated by the activation of one or more of the known defense pathways in plants. Mannitol dehydrogenase (ELI-3) and phenylalanine-amonnia lyase (PAL) were monitored because of their known early induction in the HR; glutathione S-transferase (GST), as an oxidative-stress marker; pathogenesis-related protein PR-1, as a marker for the salycilate-mediated pathway; and defensin (PDF1.2), as a gene that is activated by ethylene and jasmonate.

The most relevant conclusion from the observations summarized in Figure 5 is that none of these marker genes was induced by urate in the absence of the pathogen. Therefore, the effects of urate could not be explained by its ability to activate any of the defense pathways represented by the markers directly. However, defence genes whose expression was induced upon inoculation with pathogens were modulated by urate in several ways. A transient peak of PAL mRNA observed upon challenge with the *avr*RPM1$^+$ strain at 4 h post infection did not occur in the presence of urate (Figure 5), suggesting that peroxynitrite is required for the early induction of PAL. Increases in the levels of PAL mRNA were found at 48 hp.i. in both the *avr*RPM1$^+$ and the *avr*RPM1$^-$ *Pseudomonas* strains when urate was coinoculated with these bacteria (Figure 5). The induction of PR-1 mRNA expression in response to the *avr*RPM1$^+$ *Pseudomonas* strain was also slightly increased by urate at 48 hp.i., whereas PR-1 levels were clearly reduced in the case of coinoculation of urate with the *avr*RPM1$^-$ strain

(Figure 5). In contrast, the ELI-3 mRNA, that peaked in response to the *avr*RPM1$^+$ at 24 hp.i., showed a marked increase when the pathogen was coinoculated with urate (Figure 5). Besides the ELI-3, PDF1.2 mRNA showed a slight increase in its response to the *avr*RPM1$^+$ strain plus urate. This gene was not induced by either the *avr*RPM1$^-$ or by the incompatible *X.c.campestris* 147 strains. Urate also slightly increased the expression of the oxidative-stress-related GST gene in response to the *avr*RPM1$^+$ strain, but reduced the expresion of this gene in response to the *avr*RPM1$^-$ *Pseudomonas* strain, as compared to the inoculation without urate (Figure 5). In general, the coinoculation of urate with the *avr*RPM1$^+$ strain resulted in an slight increase of defense gene expression at late time points, whereas in response to the *avr*RPM1$^-$ *Pseudomonas* caused a decrease of their expression levels.

Interestingly, the induction of defence genes upon infection with the incompatible *X.c.campestris* 147 was not modified by urate treatment, which further indicated that urate did not have any effect in the incompatible interaction between this *Xanthomonas* strain and *Arabidopsis*.

Peroxidase activity in response to P.s.phaseolicola *and urate*

Peroxidases (POX) have been involved in the crosslinking of phenolic compounds onto cell walls and increases in POX activity have been demonstrated as one of the early responses of plants to avirulent pathogens (McLusky *et al.*, 1999). As urate had a strong effect on HR necrosis, we decided to analyse its effects on POX activity induction caused by the virulent and the avirulent *Pseudomonas* strains. Figure 6 shows that a marked activity increase observed at 24 hp.i. in response to the avirulent strain *P.s.phaseolicola* 411 (*avr*RPM1$^+$) was attenuated by urate. In contrast, urate did not affect POX activity upon infiltration with the *P.s.phaseolicola* 110 (*avr*RPM1$^-$) strain. Infiltration with urate alone did not affect POX activity (Figure 6). These results were essentially reproduced when the main protein band that stained for POX activity (at pH > 8.5) in IEF gels was quantified by densitometry (data not shown).

Differential pathogen sensitivity to NO

The sensitivity of the strains used in this study to NO was investigated by bacterial counting after incubation with increasing concentrations of the NO-releasing compound sodium nitroprusside (SNP). Figure 7(a) shows that this compound, which quantitatively releases NO, was moderately toxic to both *Pseudomonas* strains, independently of the presence or absence of the *avr*RPM1 gene, whereas it

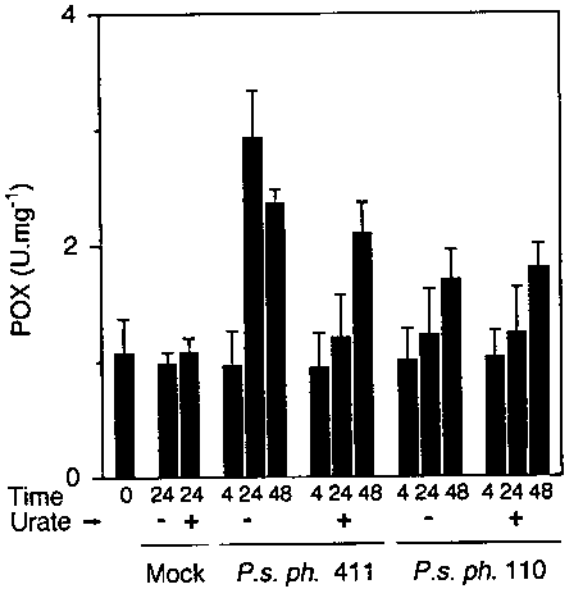


Figure 6. Urate effects on peroxidase activity in response to bacterial pathogens.
Arabidopsis leaves infiltrated with a mock solution or with a suspension of the bacteria: *P.s.ph.* 411, *P.s.phaseolicola* 411 (*avr*RPM1$^+$); *P.s.ph.* 110, *P.s.phaseolicola*) 110 (*avr*RPM1$^-$), with or without 1 mg ml^{-1} urate, were collected at the times indicated. Protein extracts and determination of peroxidase activity of the samples was done according to Polle *et al.* (1994). The results are means of three independent experiments and activity measurements were always done in duplicate. Bars represent the standard deviations.

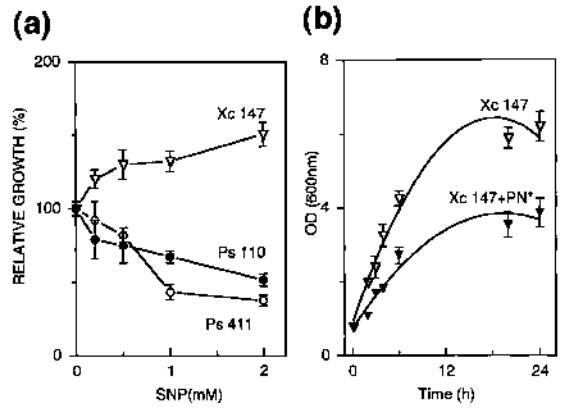


Figure 7. Bacterial sensitivity to NO and peroxynitrite *in vitro*.
(a) The bacteria used in this study were incubated for 24 h in a solution containing several concentrations of the NO donor sodium nitroprusside (SNP). Serial dilutions were plated on adequate media, and colonies counted at 48 h. Bacterial strains were as in Figure 3; (b) Growth of *X.c.campestris* strain 147 with the peroxynitrite (PN*) generating system SNP + HX/XO. The concentration of the chemicals was as in Figure 1.

slightly stimulated growth of the avirulent *Xanthomonas* strain. Besides resistance to NO, the ability of this strain to grow in the presence of the peroxynitrite generating system SNP + HX/XO was investigated. As shown in Figure 7(b), growth of this bacteria was only inhibited by

about 40%, whereas growth of the two *Pseudomonas* strains used in this study was totally prevented by these compounds (data not shown). This suggested that the *X.c.campestris* 147 strain has mechanisms to detoxify the NO and, probably, to avoid peroxynitrite production, leading to a lack of urate effect *in planta*.

Discussion

We have used urate as a pharmacological tool to analyse the effects of peroxynitrite in plants. Urate significantly reduces lesion formation caused by an exogenous peroxynitrite-generating system and by direct application of peroxynitrite in *Arabidopsis* leaves (Figures 1 and 2). Peroxynitrite is generated from the reaction of nitric oxide (NO) and ROIs, and these species are known to cooperate in cell death both in animals (Stamler, 1994) and in plants (Delledonne *et al.*, 1998; Durner *et al.*, 1998). To produce peroxynitrite, we used the NO donor SNP and the superoxide generating system HX/XO. Lesion formation in Arabidopsis leaves by the combination of these substances was effectively prevented by urate (Figure 1). In this experimental system, we can not exclude that the protective effects of urate may be due to the scavenging of phytotoxic substances other than peroxynitrite (i.e. ROIs). Several treatments were tested to further assess the specificity of these effects. In these experiments urate was totally unable to protect against the toxic effects of hydrogen peroxide plus NO (Figure 1 and effects of SNP + G/GO, not shown), whereas it clearly protected against the high toxicity of direct application of pure peroxynitrite (Figure 2).

Triggering of lesion formation by treatment with the peroxynitre generating compound SIN-1 was not observed, although it is known to generate equimolar amounts of superoxide and NO, but it is possible that a slow release of these compounds might facilitate detoxification and prevent their induction of cell death. It should be kept in mind that, as discussed above, hydrogen peroxide plus an NO-generating system do produce lesions that are not prevented by urate. Other speculative explanations of this negative observation are possible. However, we believe that the results obtained by the direct application of peroxynitrite should convincingly show that peroxynitrite does produce necrotic lesions in plants, and that urate is able to attenuate or to protect against the phytotoxic effect of peroxynitrite (Figure 2).

Based on this evidence, as well as precedents from studies in animal systems, we have used urate as a tool to assess the possible role of peroxynitrite in the plant defense response to pathogens. Synergistic effects of exogenous NO and ROIs in triggering the death of plant cells open the possibility that peroxynitrite toxicity may be responsible, at least in part, for cell death in plant–pathogen interactions (Bolwell, 1999). This has been already demonstrated in mammalian cells in which inhibition of peroxynitrite actions by urate had a clear effect in preventing their death under pathological conditions, such as those produced in LPS-stimulated cells (Hooper *et al.*, 1998). Urate has also been shown to be a strong protector against peroxynitrite-dependent tyrosine nitration of proteins (Regoli and Winston, 1999; Whiteman and Halliwell, 1996).

The effects of urate on the interaction of *Pseudomonas syringae* with *Arabidopsis* shown in Figure 3 suggest that this compound is able to interfere, at least in part, with the cell death triggered during the defense response. Our results suggest that, during the incompatible interaction, urate is not able to inhibit the fast death of those cells in direct contact with the pathogen (hereafter, primary cell death). In contrast, urate seemed to prevent the death of cells within the infiltrated area that were not in contact with the pathogen (hereafter, secondary death), suggesting a possible role for peroxynitrite in the death of these cells. This interpretation is in line with previous reports in which it was shown, by using appropriate inoculum sizes, that only those plant cells in direct contact with the pathogen undergo fast cell death. According to these reports, two types of death may coexist in the lesion formed in an incompatible interaction, the typical HR death and a senescence-like death (Pontier *et al.*, 1999; Turner and Novacky, 1974).

Cell death is a central feature in gene-for-gene disease resistance. However, there are some reports in which a significant reduction in cell death does not imply a lower level of resistance. The *dnd1* (defense-no death) mutant of *Arabidopsis* shows a fully active defense against avirulent pathogens in absence of HR-mediated cell death (Yu *et al.*, 1998). And, in *Arabidopsis* leaves in which SAR has been induced by an incompatible *Pseudomonas* strain, a 90% reduction of necrosis upon a new challenge with the same avirulent pathogen is observed, without any effect in the bacterial titter (Summermatter *et al.*, 1995). Similarly to these precedents, urate-mediated reduction of cell death in response to *P.s.phaseolicola* 411 (*avr*RPM1$^+$) did not lead to a significant reduction in the resistance.

Urate also has a significant effect on the interaction with the *P.s.phaseolicola* 110 (avrRPM1$^-$) strain. In this particular interaction urate promoted primary cell death in a discrete number of cells within the inoculated area, and led to arrest of pathogen growth and resistance (Figures 3 and 4). This result further indicated that primary cell death was crucial for plant defense, and suggested that the number of target cells that need to go through the cell death program to induce resistance may be relatively low. Our observations were consistent with the function of micro-HR in leaves undergoing a SAR response, in which death of isolated cells mediates the amplification of the defense

response (Alvarez *et al.*, 1998). According to our results, we suggest that resistance would essentially depend on primary cell death and peroxynitrite could be responsible for most of the secondary death. Peroxynitrite is a highly toxic molecule that is able to travel short distances reaching subcellular compartments and neighbouring cells (Marla *et al.*, 1997), therefore it may have a significant role in the amplification of the signal during the incompatible interaction. Nevertheless, due to the limitations of pharmacological studies, direct or indirect estimations of the peroxynitrite formed during the plant defense responses will be needed before a conclusive role could be assigned to this toxic compound.

Despite the apparent opposition in the effects of urate on the *avr*RPM$^+$ and the *avr*RPM$^-$ *Pseudomonas* strains, our results are in line with several reports that argue about the protective and toxic functions of NO, peroxynitrite and other NO-related species (reviewed by Stamler, 1994). In systems in which the toxicity is mainly dependent on ROIs damage, NO might act, at least up to a certain concentration, as a ROIs-scavenging compound and thus it will limit damage (Wink *et al.*, 1993). In contrast, high NO concentrations are generally toxic. Under these conditions, the formation of peroxynitrite might have a deleterious effect when combined with even low amounts of the superoxide anion (O_2^-) (Beligni and Lamattina, 1999; Lipton *et al.*, 1993). Enhanced NOS activities and subsequent elevated NO levels have been observed when resistant, but not susceptible, tobacco was infected with tobacco mosaic virus (Durner *et al.*, 1998), or when *Arabidopsis* leaves or soybean cultured cells were infected with avirulent pathogens but not with virulent ones (Delledone *et al.*, 1998). The effects of urate on the interaction with the *avr*$^-$ strain suggest that possibly peroxynitrite, and therefore NO, were also produced in the compatible interactions, although at levels that have not been detected by the methods used.

Although peroxynitrite has been suggested as responsible for direct pathogen killing (Durner and Klessig, 1999), the use of urate did not lead to a higher growth of strain *P.s.phaseolicola* 110 (*avr*RPM1$^-$) (Figure 4), suggesting that peroxynitrite toxicity was not limiting bacterial growth *in planta*. On the contrary, addition of urate resulted in a severe growth restriction. As urate was not directly toxic to the bacterial pathogens and, by itself, did not induce any of the monitored defense pathways (Figure 5), the observed growth inhibition should have been mediated by some mechanisms associated with the observed primary cell death. Nitrosation of urate has been reported (Skinner *et al.*, 1998; Vasquez-Vivar *et al.*, 1996) and a urate-derived radical has been demonstrated to actively release NO in animals (Skinner *et al.*, 1998). If the same radical is formed during plant–pathogen interactions in the presence of urate, the NO released from the modified urate may affect the signalling responses, and could explain the results obtained with the *avr*RPM1$^-$ strain.

When applied together with the bacteria, urate did modulate the induction of the defense-related genes, albeit in a minor, apparently indirect way. Although we can not exclude the activation of defense pathways in a discrete number of cells, which would have escaped our observation, it is unlikely that urate might mediate the onset of resistance through the induction of SAR, wounding or antioxidant responses (Figure 5). In this context, the only gene whose behaviour may merit further discussion was ELI-3. This gene was induced early in the HR response (Kiedrowski *et al.*, 1992) and encodes a mannitol dehydrogenase activity (Williamson *et al.*, 1995). Under adequate conditions, this activity produces NADPH, which could be used by the burst NADPH oxidase. According to this precedent, the activation of the ELI-3 by urate could affect the signalling pathways in which the NADPH oxidase is implicated (Blumwald *et al.*, 1998; Keller *et al.*, 1998; Torres *et al.*, 1998).

In line with the inhibition of necrosis, the increase of peroxidase (POX) activity in response to the incompatible *P. syringae* was also reduced in the presence of urate (Figure 6). Increases in POX activity are involved in the cross-linking of the cell wall during incompatible interactions, and may, in some interactions, be a direct generator of ROIs (Allan and Fluhr, 1997; Bestwick *et al.*, 1997; Bolwell *et al.*, 1995; Brown *et al.*, 1998). Despite the actual role of POX in these interactions, the low increases of POX activity in the presence of urate would suggest the participation of peroxynitrite in HR-mediated alterations of the cell wall.

Preliminary results indicated that the observed effect of urate on the *P. syringae* interactions with *Arabidopsis* could be reproduced with other *Pseudomonas* strains in a different host. When leaves of *Nicotiana tabacum* cv Xanthi were inoculated with the incompatible strain *P. syringae* pv. *syringae* or with the compatible *P. syringae* pv. *tabaci* in the presence of urate, the effects observed clearly resembled those shown in the interaction between *Arabidopsis* and the *P. s. phaseolicola* strains (J.M. Alamillo and F. García-Olmedo, unpublished results). However, the lack of effect of urate on the infection by *X.c.campestris* and the resistance of this strain to NO, which is probably metabolized or detoxified by this bacterium (Figure 7), suggests that NO might not be relevant in some plant–pathogen interactions in which it could be readily eliminated. Mechanisms to avoid the bactericidal activity of NO have been reported in *Echerichia coli* and *Salmonella typhimurium* and a flavohaemoglobin protein has been identified as responsible for most of the adaptative defense against NO toxicity (Crawford and Goldberg, 1998; Gardner *et al.*, 1998). A similar system seems to work in the plant pathogen

Erwina chrysanthemi, in which mutants of a gene encoding a flavohaemoglobin protein have reduced virulence (Favey *et al.*, 1995), further supporting a role for NO in plant defense.

In summary, we show that urate can be a useful tool to investigate peroxynitrite effects in plants, in a similar way as has been shown in animal systems. According to the effects of urate on the interaction of *P. syringae* with *Arabidopsis* we suggest that peroxynitrite may play a significant role during the plant defense responses.

Experimental procedures

Growth of A. thaliana *and bacterial inoculation*

Arabidopsis thaliana L. (Columbia-0 ecotype) seeds were sown on a mixture of vegetal substrate and vermiculite (3 : 1), vernalized for 3 days at 4°C and grown at 24°C and 65% relative humidity (RH), with 8 h light at 120 μE m^2 s^{-1}, and 16 h dark. At least 24 h before inoculation with the bacterial pathogen, plants of 4–6 weeks were placed at 90% RH, and these conditions were maintained until the end of each experiment. Bacterial suspensions (15–20 μl) with appropriate inocula were infiltrated into leaf mesophyll cells of intact plants using a hypodermic syringe without a needle.

Chemical treatments

Chemicals were hand-infiltrated into *Arabidosis* leaves and the effects scored 24 h after treatment. When using several products, they were mixed at the adecuate concentration prior to infiltration. The chemicals used were: urate 1 mM; sodium nitroprusside (SNP) 0.5 mM; hypoxanthine/xanthine oxidase (HX/XO) 1 mM HX plus 0.1 U ml^{-1} XO; catalase 50 U ml^{-1}; glucose/glucose oxidase (G/GO) 0.5 mM glucose plus 0.5 U ml^{-1} glucose oxidase; hydrogen peroxide (H_2O_2) 1 mM. Commercial peroxynitrite obtained from CALBIOCHEM^R (San Diego, CA, USA) as aqueous solution in 4.7% NaOH at 170–200 mM, was diluted to 1 mM or 0.1 mM in precooled and degased 1 mM NaOH solution. Diluted samples were prepared just before use and were protected from light, heat and air during this short time. When urate was added, peroxynitrite and NaOH were prepared at twice their concentrations and mixed 1 : 1 with urate. 3-Morpholinosydnonimine (SIN-1) was also purchased from CALBIOCHEM^R, and used as aqueous solution at 1, 2 and 5 mM. NaOH 1 mM, used to prepare the diluted peroxynitrite solutions was also infiltrated, as control.

Bacterial cultures

Pseudomonas syringae pv. *phaseolicola* race 6, strain 110, and the isogenic strain 411 carrying a plasmid with the *avr*RPM1 avirulence gene, that interacts with the *Arabidopsis* Col-0 resistance gene RPM1, were a gift from J.L. Dangl (University of North Carolina, NC, USA). These bacteria were grown in King's B medium overnight at 28°C, with the appropriate antibiotics (King *et al.*, 1954). Avirulent *Xanthomonas campestris* pv. *campestris*, strain 147 was from the laboratory of M. Van Montagu (Gent University, Gent, Belgium). This strain was grown overnight using Kado's medium (Kado and Heskett, 1970) with the adequate antibiotic, as previously described (Lummerzhein *et al.*, 1993). Overnight cultures were centrifuged and resuspended in 10 mM $MgSO_4$ at the appropriate dilution before infiltration into *Arabidopsis* leaf tissues.

Determination of bacterial growth in plants

Plants were infected with the bacteria suspended in 10 mM Mg SO_4. Inocula of 0.5–1 × 10^6 cfu/ml were used for the *Pseudomonas* strains, whereas *Xanthomonas* was inoculated at 5 × 10^6 cfu/ml. For each data point, 3 infected leaves were excised and ground with a plastic pestle in a microcentrifuge tube containing 1 ml of 10 mM Mg SO_4. This material was then diluted and aliquots were spread on plates. Plates were incubated at 28°C for 2 days and colonies counted. Each data point was obtained as means of 4–6 replicates and the average results from 3 to 4 independent experiments were represented.

Microscopy

To monitor cell death, bacteria were infiltrated in 25–30% of the mesophyll leaf space of individual leaves, at the indicated bacterial dose. Co-inoculation with urate was done by the addition of 1 mg ml^{-1} urate to the bacterial suspension just before inoculation. Leaves were examined for HR-associated autofluorescence by using a Zeiss (Oberkochen, Germany) microscope with a fluorescein filter set (Ex. 460 nm; Em. 478 nm). Lactophenol trypan blue staining of inoculated leaves was used to detect necrotic lesions at 24 hp.i., as previously described by Keogh *et al.* (1980). Leaf areas damaged by physical handling were not considered for the evaluation of the results.

Gene expression studies

Arabidopsis leaves infiltrated with bacterial suspensions, urate or mock solutions were collected at the indicated time. Total RNA was obtained from frozen leaf material by phenol-chloroform extraction followed by lithium chloride precipitation (Lagrimini *et al.*, 1987). Total RNA (10 μg) was separated by electrophoresis through formaldehyde-agarose gels, blotted onto nylon membranes (Hybond N; Amersham, UK), and hybridized with ^{32}P-labelled DNA probes, essentially as described by Ausubel *et al.* (1994). *Arabidopsis* cDNA probes for GST, and PAL were obtained by PCR using specific primers. ELI-3 probe was from J.L. Dangl. *Arabidopsis* PR-1 cDNA was from Uknes *et al.* (1992). PDF 1.2 was from the ESTs database at the ABRC (Ohio State University, OH, USA).

Protein extracts and peroxidase activity

Preparation of protein extracts and POX activity measurement of treated and control *Arabidopsis* leaves were done according to Polle *et al.* (1994). POX activity was measured by the increase in absorbance at 470 nm, using guaiacol as substrate, or by staining of bands separated on IEF gels (Liu *et al.*, 1990).

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