

Flavo-haemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host

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Summary

Host cells respond to infection by generating nitric oxide (NO) as a cytotoxic weapon to facilitate killing of invading microbes. Bacterial flavohaemoglobins are well-known scavengers of NO and play a crucial role in protecting animal pathogens from nitrosative stress during infection. *Erwinia chrysanthemi*, which causes macerating diseases in a wide variety of plants, possesses a flavohaemoglobin (HmpX) whose function in plant pathogens has remained unclear. Here we show that HmpX consumes NO and prevents inhibition by NO of cell respiration, indicating a role in protection from nitrosative stress. Furthermore, infection of *Saintpaulia ionantha* plants with an HmpX-deficient mutant of *E. chrysanthemi* revealed that the lack of NO scavenging activity causes the accumulation of unusually high levels of NO in host tissue and triggers hypersensitive cell death. Introduction of the wild-type *hmpX* gene in an incompatible strain of *Pseudomonas syringae* had a dramatic effect on the hypersensitive cell death in soya bean cell suspensions, and markedly reduced the development of macroscopic symptoms in *Arabidopsis thaliana* plants. These observations indicate that HmpX not only protects against nitrosative stress but also attenuates host hypersensitive reaction during infection by intercepting NO produced by the plant for the execution of the hypersensitive cell death programme.

Keywords: flavohaemoglobin, HmpX, nitric oxide, hypersensitive cell death, *Erwinia chrysanthemi*.

Introduction

Flavo-haemoglobins have been isolated from phylogenetically distant micro-organisms and appear to be widely distributed among bacteria and fungi. The sequence of the *hmp* gene (Vasudevan *et al.*, 1991), encoding the prototype of this class of proteins, *Escherichia coli* Hmp, has revealed two major domains. The N-terminal domain has homology to haemoglobins while the C-terminal domain shows homology with ferredoxin-NADP⁺ oxidoreductase (Wu and Poole, 2003). Although globins may be broadly defined as haemoproteins that reversibly bind oxygen, it is now clear that flavohaemoglobins are involved in responding to nitric oxide (NO) and nitrosative stresses (Poole and Hughes, 2000) catalysing the production of nitrate from NO and O₂ in

the so-called NO denitrosylase reaction (Gardner *et al.*, 1998; Hausladen *et al.*, 2001).

Macrophages generate NO as a cytotoxic weapon to help destroy tumour cells or invading microbes (Stevanin *et al.*, 2002). Flavo-haemoglobins, such as those of bacterial and fungal human pathogens like *Salmonella enterica* and *Cryptococcus neoformans*, play crucial roles in the protection of these micro-organisms during infection (de Jesus-Berrios *et al.*, 2003). Reminiscent of the mammalian immune system in which reactive oxygen species (ROS) function together with NO in the macrophage killing of bacteria (Nathan, 1995), the plant hypersensitive disease resistance response (HR) is characterized by the rapid accumulation of

ROS and NO through the activation of enzyme systems similar to the neutrophil NADPH oxidase (Keller *et al.*, 1998) and NO synthase (NOS) (Guo *et al.*, 2003). This signal system triggers hypersensitive cell death, induces sets of defence genes and mediates a network involved in the establishment of systemic acquired resistance (Delledonne *et al.*, 1998; Durner *et al.*, 1998). Flavohaemoglobins-like proteins have been identified in numerous plant pathogens (Table 1), but whether or not their putative enzymatic activities correspond to a physiologically relevant process remains to be established.

Erwinia chrysanthemi causes macerating diseases in a wide variety of plants and plant organs (Barras *et al.*, 1994). However, *Saintpaulia ionantha* plants inoculated with *E. chrysanthemi* deficient in the flavohaemoglobin HmpX exhibit no symptoms or necrosis compared with maceration of the whole leaf and the spread of the disease on plants infected by the wild-type strain (Favey *et al.*, 1995). As mutation did not affect growth under either aerobic or micro-aerobic conditions, it was speculated that the loss of virulence is due to the increased sensitivity to ROS produced by the host (Favey *et al.*, 1995). In the present study, we demonstrate that HmpX exhibits a robust, oxygen-dependent NO consumption activity that provides a potential defence mechanism to *E. chrysanthemi* during infection and its proliferation within the host, when NO is generated for restricting survival of the pathogen. We also provide evidence indicating that the removal of NO by HmpX impairs the capacity of the plant to trigger a normal hypersensitive cell death of infected tissue. This latter function appears crucial for successful infection.

Results

E. chrysanthemi hmpX encodes a functional NO denitrosylase

The properties of *E. chrysanthemi* flavohaemoglobin HmpX were examined spectrophotometrically after heterologous expression in *E. coli* DH5 α carrying the plasmid pRK415-

Table 1 Occurrence of putative flavohaemoglobins in plant pathogens as determined by searching NCBI-annotated databases. Where possible, the accession number indicates the NCBI reference sequence (<http://www.ncbi.nlm.nih.gov/RefSeq/>)

Plant pathogen	Accession
<i>Botrytis cinerea</i>	CNS01CP4
<i>Cladosporium fulvum</i>	BE187718
<i>Erwinia carotovora</i>	YP_051340
<i>Erwinia chrysanthemi</i>	X75893
<i>Fusarium oxysporum</i>	AB016807
<i>Gibberella zeae</i>	XP_384634
<i>Pseudomonas aeruginosa</i>	F83311
<i>Xylella fastidiosa</i>	AE003859

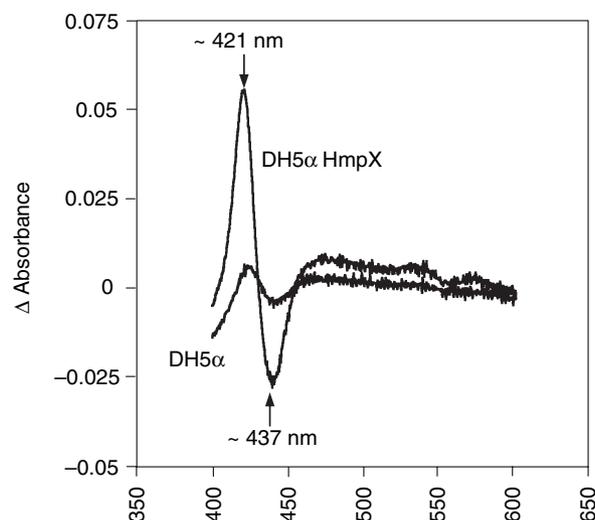


Figure 1. Detection of HmpX by visible absorbance spectroscopy. CO difference spectra were carried out on undiluted high-speed cell-free extracts of *Escherichia coli* strains DH5 α (DH5 α ; 5.9 mg protein ml⁻¹) and DH5 α -expressing HmpX (DH5 α HmpX; 7.2 mg protein ml⁻¹).

hmpX (Figure 1). As expected, the CO-difference spectra revealed features similar to those of purified *E. coli* Hmp and of an Hmp-overexpressing strain (Ioannidis *et al.*, 1992). The 421 nm peak is due to the CO adduct of ferrous HmpX, whereas the 437 nm trough arises from loss of the ferrous form on reaction with CO. Weaker α and β signals were evident at 530–580 nm. Cells expressing HmpX possessed a 10-fold higher concentration of flavohaemoglobins (0.05 nmol mg⁻¹) compared with the control strain constitutively expressing the endogenous Hmp.

In *E. coli* and *S. enterica*, expression of Hmp protects against NO inhibition of cell respiration (Crawford and Goldberg, 1998; Stevanin *et al.*, 2002). To test the ability of HmpX to consume NO and protect cell respiration, O₂ uptake rates were measured polarographically. At both high (approximately 65 μ M) and low (approximately 25 μ M) O₂ concentrations, the addition of NO (36 μ M) to glucose-stimulated respiring *E. coli* cells resulted in a rapid and immediate uptake of oxygen, which was followed by a period of inhibition of renewed respiration (Figure 2a, trace 1). Respiration in the *E. coli* strain expressing HmpX was inhibited only transiently (Figure 2b, trace 1) compared with control strains, as shown in Table 2. The prolonged period of respiratory inhibition correlated with an increased half-life of the added NO, as shown in trace 2 (Figure 2a,b) and Table 2. A comparison of the respiratory responses to NO added either at 65 or 25 μ M (Table 2) shows that the inhibition of respiration increased at lower oxygen tensions. This is consistent with previous results obtained for wild-type *E. coli* expressing its native Hmp and can be attributed to the competition by NO and oxygen for the active site(s) of the oxidases.

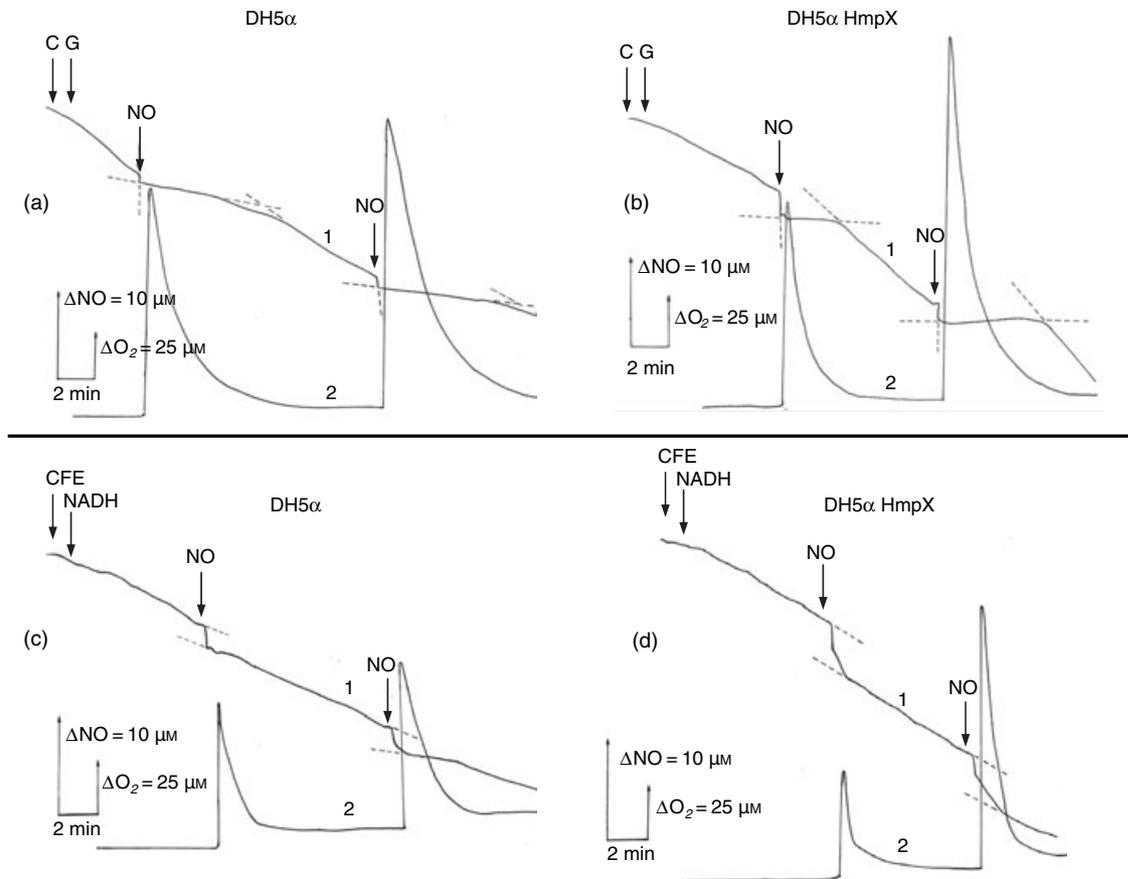


Figure 2. Effects of NO on oxygen uptake in *Escherichia coli*-expressing HmpX and evidence for NO-oxygenase activity.

(a and b) Measurements of respiration and NO concentrations in suspensions of washed whole cells were made using a closed chamber equipped with oxygen and NO electrodes. The chamber contained 2 ml 50 mM NaCl buffer. Cells (C) were added at the indicated points. Glucose (G; 5 mM final) was added to stimulate respiration; then 36 μM anoxic NO solution (40 μl) was added at the time indicated by the arrows on the oxygen tracings (1), both at high and low O_2 concentrations. In (a), 0.17 mg protein *E. coli* DH5 α cells (DH5 α) were used and in (b), 0.2 mg protein *E. coli* DH5 α -expressing HmpX (DH5 α HmpX) were used. Trace 1 shows O_2 concentration in the chamber and trace 2 shows NO concentration.

(c and d) Measurements of oxygen uptake and NO concentration of high-speed cell-free extracts (CFE) were made using a closed chamber equipped with oxygen and NO electrodes. The chamber contained 2 ml 50 mM NaCl buffer. NADH (500 μM) was added to stimulate oxygen uptake at the time indicated. Anoxic NO solution (36 μM , 40 μl) was added at the time indicated by the arrows on the oxygen trace (1) at both high and low oxygen concentrations. In (c) 0.52 mg *E. coli* DH5 α (DH5 α) protein extract were used and in (d), 0.53 mg of *E. coli* DH5 α -expressing HmpX (DH5 α HmpX) were used. Trace 1 shows O_2 concentration in the chamber and trace 2 shows NO concentration.

Table 2 Effects of NO on cells and cell-free extracts. O_2/NO ratios, periods of inhibition of O_2 uptake and half-times of NO decay were calculated after the addition of 36 μM NO to respiring whole cells or cell-free extracts of *Escherichia coli* strains DH5 α (DH5 α) and DH5 α -expressing HmpX (DH5 α HmpX). O_2 uptake was stimulated using glucose in intact cells or NADH in cell-free extracts

	High O_2 conc.			Low O_2 conc.		
	O_2/NO ratio	Period of inhib. (min) of O_2 uptake	Half-time for NO decay (min)	O_2/NO ratio	Period of inhib. (min) of O_2 uptake	Half-time for NO decay (min)
<i>E. coli</i> whole cells						
DH5 α	n/a	4.25 (2, 0.21)	1.04 (2, 0.2)	n/a	8.3 (2, 0.42)	1.59 (2, 0.17)
DH5 α HmpX	n/a	1.9 (2, 0.5)	0.53 (2, 0.29)	n/a	4.4 (2, 1.4)	0.21 (2, 0.08)
Cell-free extracts						
DH5 α	0.39 (2, 0.07)	0.95 ^a (2, 0.5)	0.85 (2, 0.07)	0.36 (2, 0.12)	4.4 ^a (2, 0.28)	1.5 (2, 0.42)
DH5 α HmpX	0.83 (5, 0.05)	0 ^a (3, 0)	0.3 (2, 0.14)	0.92 (4, 0.07)	0 ^a (3, 0)	0.6 (2, 0)

Values are mean (number of assays, standard deviation in brackets); inhib., period of inhibition of O_2 uptake; n/a, not appropriate.

^aInhibition of soluble oxidase activity.

In intact bacteria, the major contributor to O_2 uptake rates is respiration catalysed by membrane-bound oxidases. To study the consumption of O_2 and NO by the flavohaemoglobin, membrane-free extracts were used. O_2 uptake was stimulated by adding NADH and the effects of added NO on O_2 uptake were monitored (Figure 2c,d). Upon addition of NO, an immediate uptake of O_2 (trace 1) was accompanied by the decay of the NO signal (trace 2). The NO-stimulated O_2 uptake was quantified and used to determine the O_2 /NO ratio. *Escherichia coli* DH5 α -expressing HmpX exhibited greater 'NO-oxygenase' activity, as indicated by the higher O_2 /NO ratio and by the faster rate of NO decay compared with the control strain (Table 2).

In the absence of NO, the *E. coli* flavohaemoglobin Hmp generates O_2^- and H_2O_2 (Membrillo-Hernández *et al.*, 1996). Quenching of the fluorescent substrate scopoletin (Figure 3a) revealed that the cell-free extract of *E. coli* DH5 α -expressing HmpX generated H_2O_2 approximately five times faster than the control strain.

Although haemoglobins are soluble proteins with cytoplasmic functions, *Vitreoscilla* haemoglobin expressed in

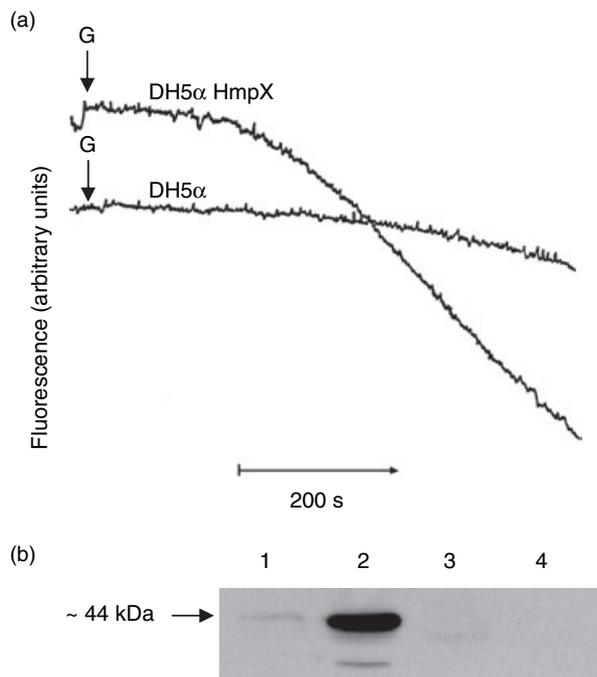


Figure 3. Evidence for H_2O_2 generation and HmpX localization in *Escherichia coli* cells expressing HmpX.

(a) Assay of H_2O_2 generation by quenching of scopoletin fluorescence was measured in cell-free extracts of *E. coli* DH5 α (DH5 α) and DH5 α -expressing HmpX (DH5 α HmpX). Additions of cell extract, horseradish peroxidase and scopoletin were made before the addition of glucose (G) as shown. Each assay contained about 1.4 mg protein.

(b) Western blot analysis of cytoplasmic (odd numbered lanes) and periplasmic fractions (even numbered lanes) from *E. coli* RKP4600-expressing HmpX (lanes 1 and 2) and RKP4600 (lanes 3 and 4) probed with antibody to Hmp. Approximately 25 μ g protein was used for each lane.

E. coli is localized, at least partly, in the periplasm (Khosla *et al.*, 1990). Moreover, the flavohaemoglobin Hmp is distributed between the periplasm and the cytoplasm in *E. coli* (Vasudevan *et al.*, 1995). Therefore, both the cytoplasmic and periplasmic fractions of *E. coli*-expressing HmpX were probed by immunoblotting. In order to remove interference by constitutively expressed Hmp, the plasmid pRK415-hmpX was used to transform the *E. coli* Hmp-deficient mutant strain RKP4600 (Mills *et al.*, 2001). The cytoplasmic and periplasmic fractions of *E. coli* RKP4600-expressing HmpX showed a weak band of approximately 44 kDa in the cytoplasm, but a much stronger band in the periplasm (Figure 3b). No bands were seen in the *E. coli* RKP4600 in either the cytoplasmic or periplasmic fractions. Catalase assays on all subcellular fractions showed that the total amount of catalase activity in the periplasmic fraction was <10% of that in the whole cell, indicating minimal contamination of periplasmic fractions.

As the recombinant HmpX primarily accumulated in the periplasm, we examined the periplasmic HmpX for evidence of NO consumption in the dual-electrode apparatus. NADH was added to stimulate the uptake of O_2 and a number of sequential additions of 36 μ M NO were made (traces not shown). The resulting rapid and immediate uptake of O_2 was measured to determine the O_2 /NO ratio. For *E. coli* DH5 α , this ratio was 0.48 (SD 0.076, eight assays), whilst for *E. coli* DH5 α expressing HmpX a ratio of 0.84 (SD 0.055, eight assays) was found. This near-stoichiometric consumption of O_2 and NO is consistent with the HmpX-catalysed NO denitrosylase activity in periplasmic fractions of *E. coli*.

E. chrysanthemi HmpX-deficient mutant induces an HR-like response in *S. ionantha*

The HmpX-deficient mutant of *E. chrysanthemi* strain 3937 was not pathogenic and produced no signs of maceration in *S. ionantha* plants as already reported (Favey *et al.*, 1995). Inoculation with 10^8 bacterial cells led to a 20-fold increase in colony-forming units 24 h after infiltrating the wild-type strain ($2 \times 10^9 \pm 0.12$ CFU) whereas only a twofold increase was observed after infiltrating the *E. chrysanthemi* HmpX-deficient mutant ($2 \times 10^8 \pm 0.21$ CFU). As HmpX possesses the characteristics of a NO denitrosylase, the sensitivity of the *E. chrysanthemi* HmpX-deficient mutant to nitrosative stress was tested by measuring bacterial growth in the presence of increasing concentrations of the nitrosating agent sodium nitroprusside (SNP) in the liquid medium. The HmpX-deficient mutant was more sensitive to SNP at any of the concentrations tested, growing between 50 and 75% to that of the wild-type strain (Figure 4).

As up to 50% of leaves inoculated with the HmpX-deficient mutant showed some necrotic spots 2–3 days after infection (Figure 5a), trypan blue was used to visualize dying cells during the early phase of infection. Despite the lack of

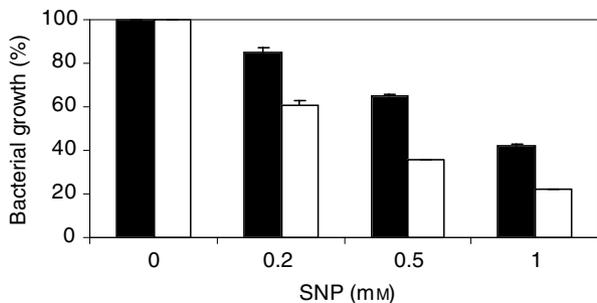


Figure 4. Bacterial growth of *Erwinia chrysanthemi* wild type (black bars) and *E. chrysanthemi* HmpX-deficient mutant (white bars) in liquid medium containing indicated amounts of the NO donor SNP.

macroscopic symptoms, leaves inoculated with the HmpX-deficient mutant presented numerous patches of blue cells as soon as 7 h after infiltration, which were not observed in leaves inoculated with the wild-type strain 3937 (data not shown). To test whether the HmpX-deficient mutant triggered cell death through activation of an HR-like response, ROS and NO production were examined. 3,3'-diaminobenzidine (DAB) polymerizes on contact with H₂O₂

in a peroxidase-dependent reaction, enabling the visualization of H₂O₂ *in situ* (Thordal-Christensen *et al.*, 1997). A strong, reddish-brown precipitate was observed in leaves infected with either the HmpX-deficient mutant or the wild-type strain (data not shown). NO accumulation was examined with the fluorophore 4,5-diaminofluorescein diacetate (DAF-2 DA), which is converted to the fluorescent triazole derivative DAF-2T in the presence of NO. By confocal laser scanning microscopy, green fluorescence was observed 1 h post-inoculation in *S. ionantha* leaves infected either with the wild-type strain or the HmpX-deficient mutant. DAF-2T fluorescence was maintained for several hours in leaves inoculated with the HmpX-deficient mutant, whereas in leaves inoculated with wild-type *E. chrysanthemi* fluorescence was no longer visible at 3 h after infiltration (Figure 5b). Quantification of DAF-2T fluorescence revealed that the accumulation of NO was significantly higher in leaves inoculated with the HmpX-deficient mutant compared with the wild-type strain already at 1 h (Figure 5c). Although this method does not allow a precise measurement of NO, the quantification of DAF-2T fluorescence emitted by leaves 1 h after infiltration with 2 mM SNP indicates that the amount of NO accumulated during infection with *E. chrysanthemi*

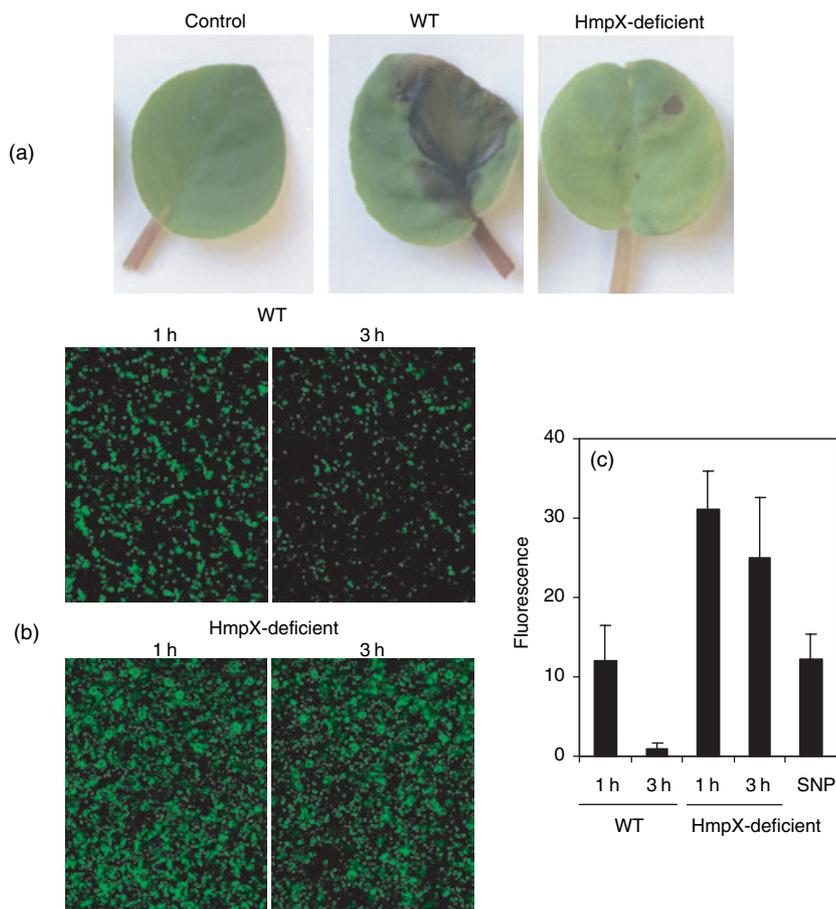


Figure 5. Analysis of *Erwinia chrysanthemi* pathogenicity in *Saintpaulia ionantha*.

(a) Macroscopic symptoms 3 days after inoculation with 10 mM MgCl₂ (control), 10⁸ CFU ml⁻¹ *E. chrysanthemi* wild type (WT) or 10⁸ CFU ml⁻¹ *E. chrysanthemi* HmpX-deficient mutant strain (Hmp-deficient).

(b) Confocal laser scanning microscopy of DAF-2 DA fluorescence 1 and 3 h after infection with 10⁸ CFU ml⁻¹ *E. chrysanthemi* WT or 10⁸ CFU ml⁻¹ *E. chrysanthemi* HmpX-deficient mutant strain (Hmp-deficient). Four independent samples were recorded for each condition, and representative leaf areas are shown.

(c) Quantification of DAF-2T fluorescence shown as mean green channel values of at least four leaves from different plants 1 and 3 h after infection with 10⁸ CFU ml⁻¹ *E. chrysanthemi* WT or 10⁸ CFU ml⁻¹ *E. chrysanthemi* HmpX-deficient mutant strain (Hmp-deficient) and 1 h after infiltration of 2 mM SNP.

unable to express HmpX is quite high (Figure 5c). The massive and sustained accumulation of NO together with the premature death of host cells observed during infection with the HmpX-deficient mutant suggests that HmpX scavenges NO, and that the detoxification of NO produced by the host is required for successful infection.

Expression of HmpX in the plant pathogen Pseudomonas syringae

For gain-of-function experiments, the plasmid pRK415-*hmpX* was mobilized into *P. syringae* pv. *glycinea* carrying the avirulence gene *avrA* (*Psg*) (Keen and Buzzell, 1991). Cells expressing HmpX exhibited a slower growth both on plates and in liquid medium compared with control cells. When grown in rich liquid media *Psg*-expressing HmpX exhibited a doubling time of roughly 2.4 h versus 2 h for wild-type *Psg*. As reported for other micro-organisms (Buisson and Labbe-Bois, 1998; Membrillo-Hernández *et al.*, 1996), overproducing flavohaemoglobin may be detrimental to cells, a finding consistent with plasmid loss experiments showing that in *Psg* the plasmid pRK415 is lost at very high frequency when it carries *hmpX* (data not shown). This loss is most likely a consequence of oxidative stress caused by generation of O_2^- in the absence of NO (Membrillo-Hernández *et al.*, 1996).

Expression of HmpX in avirulent P. syringae blocks hypersensitive cell death in soya bean cells

Psg carries the avirulence gene *avrA* and is recognized by soya bean cultivar Williams 82 that possesses the corresponding *Rpg2* resistance gene, leading to rapid induction of a hypersensitive response (Keen and Buzzell, 1991). Soya bean cell cultures were challenged with either *Psg* or the isogenic strain expressing HmpX. Whereas *Psg* induced a strong HR cell death at 24 h, *Psg*-expressing HmpX did not (Figure 6a). This lack of execution of the cell death programme was not determined by loss of pathogen recognition, as soya bean cells responded to challenge with *Psg*-expressing HmpX by activating a sustained oxidative burst, as demonstrated by a scopoletin assay of H_2O_2 accumulation (data not shown) and by massive accumulation of transcripts encoding cellular protectants such as glutathione S-transferase (GST; Figure 6b). However, careful analysis of H_2O_2 accumulation revealed that while *Psg* induced the typical avirulence gene-dependent oxidative burst characterized by a first transient burst of ROS accumulation followed by a second burst, sustained for 4–6 h, *Psg*-expressing HmpX induced a massive, single phase, oxidative burst that was maintained for at least 48 h (Figure 6c).

To determine whether the substantial accumulation of H_2O_2 in soya bean cell suspensions was caused by the direct enzymatic action of the flavohaemoglobin, we investigated

the contribution of *Psg*-expressing HmpX to the oxidative burst. The removal of soya bean cells during oxidative burst, either by centrifugation for 1 min at 800 *g* or by filtration, abolished the peroxidase-mediated scopoletin fluorescence quenching, indicating that H_2O_2 cannot be detected in the absence of host cells (Figure 6d). It should be noted, however, that this assay relies on the H_2O_2 -dependent degradation of scopoletin by extracellular peroxidases, which are continuously produced by soya bean cell suspensions. As HmpX generates O_2^- , the effect of exogenous superoxide dismutase (SOD) was tested. Whereas the addition of SOD to the filtered soya bean medium did not alter the levels of scopoletin fluorescence, the addition of H_2O_2 or xanthine/xanthine oxidase (generating O_2^-) to the medium caused the degradation of scopoletin (Figure 6d), indicating that soya bean-conditioned medium contains sufficient levels of both SOD and peroxidases for the detection of O_2^- potentially generated by HmpX. Therefore, the lack of ROS accumulation when soya bean cells were first removed from the liquid medium indicates that the massive oxidative burst detected was caused by the host cells and not by *Psg*-expressing HmpX. This data was further confirmed by examining the effect of Ca^{2+} blockers and protein kinase inhibitors on the oxidative burst. In soya bean cell suspensions challenged with *Psg*-expressing HmpX, either the Ca^{2+} channel blocker lanthanum (La^{3+}) or the protein kinase inhibitor K252-a abolished H_2O_2 production (data not shown).

The oxyhaemoglobin assay of NO accumulation revealed that soya bean suspension cells accumulate lower amounts of NO when challenged with *Psg*-expressing HmpX (Figure 6e). However, the amount of NO measured in the presence of higher concentrations of oxyhaemoglobin (10 μ M) was significantly higher (Figure 6e), suggesting that the amount of NO trapped is directly related to oxyhaemoglobin concentration. This phenomenon, markedly less pronounced when soya bean suspension cells were challenged with *Psg*, suggests that HmpX competes for NO with oxyhaemoglobin and therefore indicates that HmpX traps NO outside from host cells.

HmpX expression in avirulent P. syringae attenuates the HR in Arabidopsis thaliana

To test whether HmpX also affects hypersensitive cell death *in planta*, we examined the effects of its constitutive expression in a genetically well-defined host–pathogen interaction. Infiltration of leaves of *A. thaliana* ecotype Col-0, which contain the *RPM1* resistance gene, with *P. syringae* pv. *tomato* carrying the avirulence gene *avrB* (*Pst*) results in colourless, dry HR lesions at the infection site. The plasmid-enabled expression of HmpX in *Pst* markedly delayed the development of macroscopic symptoms and promoted the spreading chlorosis observed in the compatible interaction

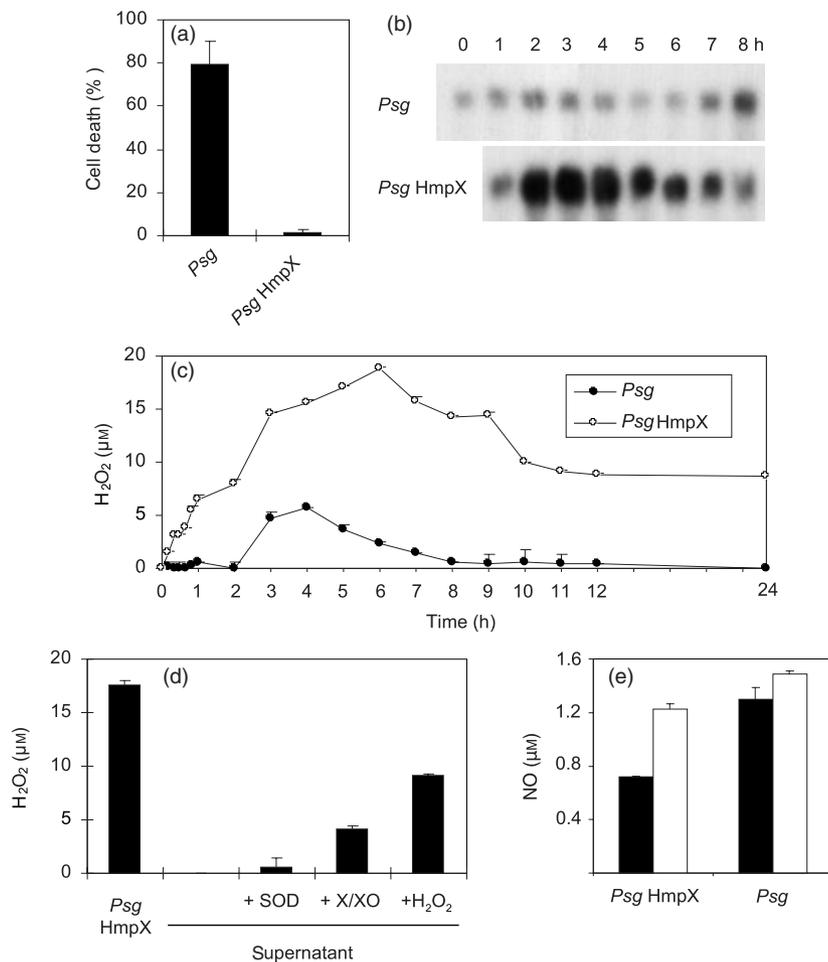


Figure 6. Analysis of the hypersensitive response in soya bean cell suspensions.

(a) Cell death was measured 24 h after challenge with *Pseudomonas syringae* pv. *glycinea* carrying the avirulence gene *avrA* (Psg) or with the isogenic strain expressing HmpX (Psg HmpX).

(b) Accumulation of transcripts encoding GST as monitored by Northern analysis of total cellular RNA isolated at indicated times after treatment of soya bean cells with *P. syringae* pv. *glycinea* carrying the avirulence gene *avrA* (Psg) or with the isogenic strain expressing HmpX (Psg HmpX). RNA loading was checked by gel staining with ethidium bromide (data not shown).

(c) Kinetics of ROS accumulation in response to *P. syringae* pv. *glycinea* carrying the avirulence gene *avrA* (●) or to the isogenic strain expressing HmpX (○).

(d) ROS accumulation 4 h after challenge with *P. syringae* pv. *glycinea* carrying the avirulence gene *avrA* and expressing HmpX. The contribution of HmpX to H₂O₂ accumulation was determined by removing soya bean cells and measuring ROS accumulation in the soya bean medium (supernatant). The assay was validated by adding to the supernatant 50 U of SOD (+SOD), 0.5 mM xanthine/0.5 U xanthine oxidase (+X/XO) or 10 µM H₂O₂ (+H₂O₂; see text for clarity).

(e) NO accumulation in response to *P. syringae* pv. *glycinea* carrying the avirulence gene *avrA* (Psg) or to the isogenic strain expressing HmpX (Psg HmpX) measured with 3 µM (black bars) or 10 µM (white bars) oxyhaemoglobin 4 h after bacterial infection.

with the isogenic virulent bacteria lacking *avrB* (Figure 7a). Trypan blue analysis of dead cells revealed that the number of HR patches decreased significantly when leaves were infected with bacteria expressing HmpX (Figure 7b,c). Moreover, as assessed by DAB staining, H₂O₂ accumulation during the oxidative burst was significantly lower when Col-0 plants were challenged with *Pst* (HmpX) compared with *Pst* (Figure 7d,e).

Discussion

Micro-organisms that evade host microbicidal activity are a prerequisite for invasive disease (Fields *et al.*, 1986). Inflammatory cells such as macrophages and neutrophils produce large amounts of ROS and NO (Beckman *et al.*, 1990). Therefore, during the infection process pathogens exhibit an increase in the expression of antioxidant enzymes such as SOD and catalase (De Groote *et al.*, 1997; Nunoshiba *et al.*, 1993) and increase their defences against nitrosative stress (Pathania *et al.*, 2002). The concerted mode of protection against reactive oxygen and nitrogen species produced by cells of the invaded host is also revealed in plant

pathogens by the essential role of both SOD and HmpX in *E. chrysanthemi* pathogenicity (Favey *et al.*, 1995; Santos *et al.*, 2001). HmpX is the first flavohaemoglobin from a plant pathogen shown to be an NO denitrosylase. CO difference spectra revealed that recombinant HmpX could be detected spectroscopically in a manner similar to *E. coli* flavohaemoglobin Hmp (Ioannidis *et al.*, 1992), and was detectable as a CO adduct closely resembling carbon monoxide-Hmp. HmpX was also found to consume NO and to prevent NO-mediated inhibition of cell respiration, thus demonstrating its function in protection from nitrosative stress. Although the possibility that HmpX is cytoplasmic unless over-expressed in a heterologous host cannot be ruled out, the fact that most HmpX was found in the periplasm suggests a role in the detoxification of NO accumulating extracellularly as part of the defence mechanism from the host plant. It is clear that NO *per se* (but not necessarily NO⁺ or NO⁻) can penetrate the outer and cytoplasmic membrane, as NO added externally is consumed (Stevanin *et al.*, 2002) and more tellingly, NO generated in the cytoplasm is readily detected in whole cell suspensions using an NO-selective electrode (Corker and Poole, 2003). It is there-

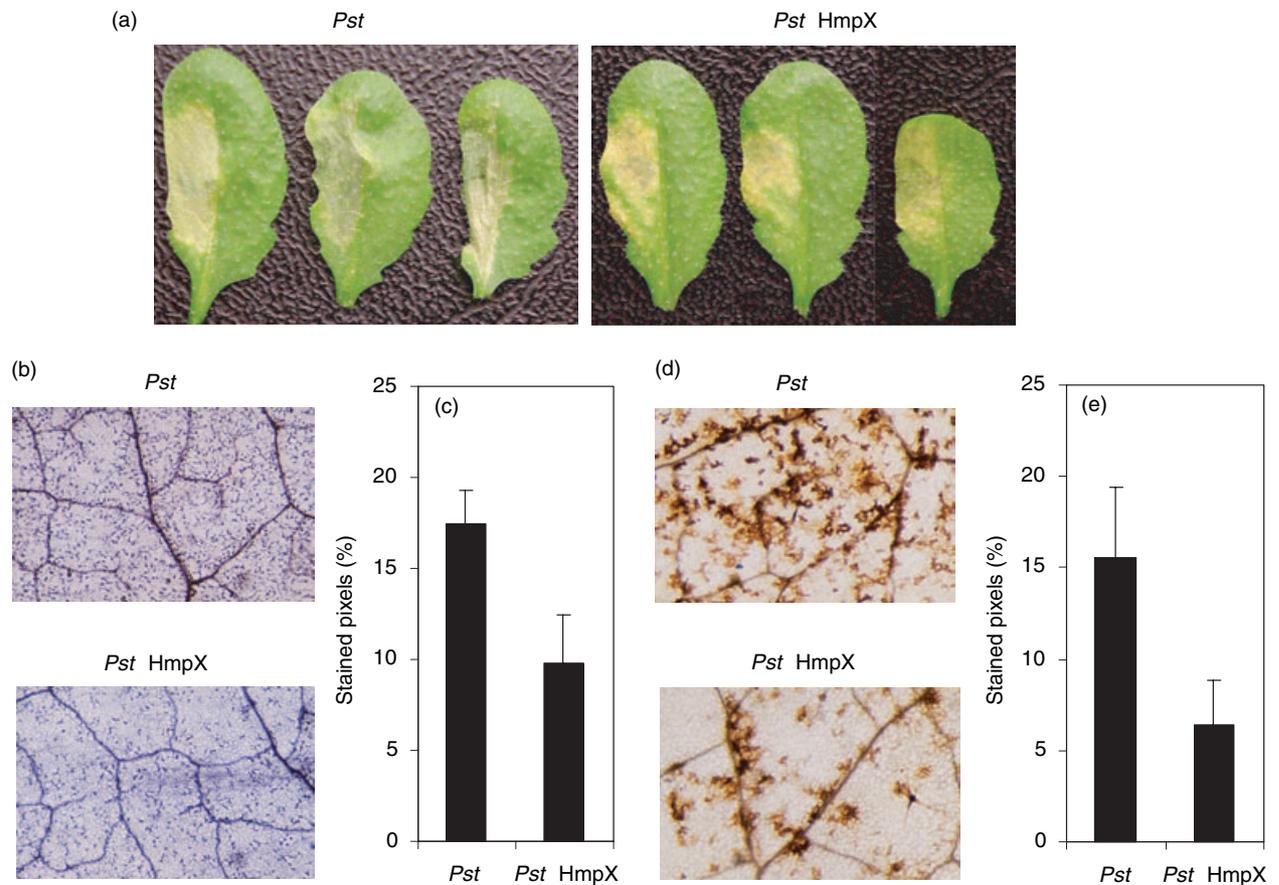


Figure 7. Analysis of the hypersensitive response in *Arabidopsis thaliana* leaves.

(a) Macroscopic HR symptoms 3 days after challenge with 5×10^6 CFU ml⁻¹ *Pseudomonas syringae* pv. *tomato* carrying the avirulence gene *avrB* (*Pst*) or with the isogenic strain expressing HmpX (*Pst HmpX*).

(b) Trypan blue staining (100-fold magnification) of representative *A. thaliana* leaves 24 h after challenge with 5×10^6 CFU ml⁻¹ *P. syringae* pv. *tomato* carrying the avirulence gene *avrB* (*Pst*) or with the isogenic strain expressing HmpX (*Pst HmpX*).

(c) Quantification of dead cells expressed as percentage of stained pixels inside the infiltration zone of at least five leaves from different plants.

(d) DAB staining (100-fold magnification) of representative *A. thaliana* leaves 4 h after challenge with 5×10^6 CFU ml⁻¹ *P. syringae* pv. *tomato* carrying the avirulence gene *avrB* (*Pst*) or with the isogenic strain expressing HmpX (*Pst HmpX*).

(e) Quantification of H₂O₂ accumulation expressed as percentage of stained pixels inside the infiltration zone. Bars represent mean ± SD of six leaves from different plants.

fore not surprising that loss of HmpX functionality causes loss of pathogenicity in *E. chrysanthemi* (Favey *et al.*, 1995) as revealed by the inability of the HmpX-deficient mutant to macerate *S. ionantha* leaves and by its very limited growth *in planta*, an effect commonly observed during incompatible interactions.

Infection by necrotrophic pathogens has been extensively shown to be associated with oxidative stress in the host to promote infection (Govrin and Levine, 2000). Recently, *Botrytis elliptica*, a necrotrophic pathogen with a very narrow host range, has been shown to kill lily cells through stimulation of their production of both ROS and NO, but only during compatible interactions. A partially purified *B. elliptica* culture filtrate was able to trigger ROS and NO accumulation as well as cell death, and to confer pathogenicity on lily to incompatible *Botrytis* species (Van Baarlen

et al., 2004). Similarly, *E. chrysanthemi* causes oxidative stress at an early stage of infection before the massive production of pectinolytic enzymes (Boccaro *et al.*, 1988; Santos *et al.*, 2001). However, *E. chrysanthemi* causes soft-rotting on a wide range of host plants due to the action of endopectate lyases that rapidly macerate host tissues, and the typical HR that causes tissue necrosis, phytoalexin accumulation and loss of virulence in non-host plants (Pouteau *et al.*, 1994; Toth *et al.*, 2003). Therefore, whereas necrotrophs typically induce plant cell death to facilitate colonization of the host tissue (Govrin and Levine, 2000), for *E. chrysanthemi* the HR correlates with the inability to establish successful infection (Shevchik *et al.*, 1998).

Saintpaulia ionantha leaves infected with *E. chrysanthemi* accumulate massive levels of H₂O₂ as detected by DAB staining. However, the same analysis carried out on plants

infected with the HmpX-deficient mutant produced very similar results, indicating that *S. ionantha* plants trigger the oxidative burst also in the absence of leaf maceration. The use of the NO-specific fluorescent dye DAF-2DA revealed that both the wild type and the HmpX-deficient mutant of *E. chrysanthemi* caused an early accumulation of NO. However, the burst of NO was transient when leaves were inoculated with the wild-type strain whereas it was sustained and massive when *S. ionantha* leaves were infected with the HmpX-deficient mutant, indicating that the lack of NO denitrosylase activity causes the maintenance of an unusually strong burst of NO in host tissue. Furthermore, the HmpX-deficient mutant strain showed a reduction in growth in liquid medium under nitrosative stress compared with the wild type that, alone, does not appear sufficient to cause the complete loss of virulence and dramatic reduction in bacterial growth when the mutant strain is inoculated to *S. ionantha* plants. These data do not contradict the widely accepted model of action of enzymatic defences against oxidative and nitrosative stress mounted by the host; they suggest that the loss of virulence of HmpX-deficient *E. chrysanthemi* involves increased sensitivity to nitrosative stress as well as host activation of an HR-like response. This hypothesis is supported by the sustained burst of NO and by the dramatic increase in the number of dying cells as early as 7 h after infection that accompanies the infection of *S. ionantha* plants with the HmpX-deficient mutant strain. To assess this function, HmpX was ectopically expressed in genetically well-defined host–pathogen interactions. Its constitutive expression in incompatible *P. syringae* dramatically suppressed hypersensitive cell death in soya bean cell suspensions and markedly delayed the development of macroscopic symptoms and promoted the spreading chlorosis observed in the compatible interaction when infiltrated in leaves of *A. thaliana*.

A remarkably similar defence behaviour was observed in *A. thaliana* expressing Hmp from *E. coli* (Zeier *et al.*, 2004). These transgenic plants had a diminished nitrate reductase-derived NO accumulation, and after challenge with avirulent *P. syringae* they showed an attenuated NO burst. Furthermore, the oxidative burst and induction of PAL transcripts were significantly attenuated, hypersensitive cell death was reduced, and the expression of PR-1 was delayed. Thus, flavohaemoglobin expression in transgenic plants had very similar effects on plant defences compared with its expression in the bacterial pathogen (Figure 7), and essentially confirmed previous pharmacological and genetic studies indicating a pivotal role of NO in the plant signalling network during pathogen infections (Romero-Puertas *et al.*, 2004; Zeidler *et al.*, 2004). However, whereas flavohaemoglobin expression in the host plant and *P. syringae* led to a decrease in H₂O₂ accumulation during the HR in the *A. thaliana*–*P. syringae* system, the dramatic enhancement of the oxidative burst and accumulation of GST transcripts when

soya bean cell suspensions were challenged with avirulent *P. syringae* pv. *glycinea*-expressing HmpX suggests that the molecular mechanisms underlying NO-mediated regulation of the oxidative burst might differ in the two systems.

NO signalling functions depend on many complex conditions affecting its reactivity, such as the rate of production and diffusion, with ROS being the key mediator in channeling NO into the cell death pathway (Murgia *et al.*, 2004) through a still unknown mechanism (Delledonne *et al.*, 2001). The evidence that flavohaemoglobin expression in transgenic plants has very similar effects on hypersensitive cell death compared with its expression in the bacterial pathogen suggests a crucial function of intracellular NO in the establishment of a proper defence reaction. This hypothesis is strengthened by our observation that, in soya bean suspension cells, Psg-expressing HmpX competes with exogenous oxyhaemoglobin for NO, indicating that HmpX intercepts NO in the extracellular space. Other recent reports support the view of NO as an intracellular signal that functions in cell-to-cell spread of the HR. In fact, *A. thaliana* plants challenged with avirulent *P. syringae* were found to initially accumulate NO exclusively in the extracellular space, after which NO was seen in the cytoplasm of nearby cells: many of these cells died soon after (Zhang *et al.*, 2003). Moreover, pharmacological studies indicated that NO does not elicit hypersensitive cell death but is required for the onset of apoptotic cell death in the adjacent cells during the defence response of oat to avirulent *Puccinia coronata* (Tada *et al.*, 2004).

In conclusion, NO and ROS play important antimicrobial roles in host defence, and pathogens counteract these molecules with specific detoxifying mechanisms. However, NO and ROS are also critical signalling molecules for the establishment of disease resistance mechanisms during HR, and elaborate enzymatic defences against oxidative and nitrosative stresses may scavenge the redox signals that host cells employ in disease resistance. It is therefore intriguing to hypothesize that the antioxidant apparatus of at least some plant pathogens may also function by suppressing the activation of defence reactions through interception of the crucial signalling molecules of the host.

Experimental procedures

Bacteria

Escherichia coli cultures were grown in Luria-Bertani medium (Miller, 1972) at 37°C unless specified. *Pseudomonas syringae* and *E. chrysanthemi* cultures were grown at 30°C in King's medium B (King *et al.*, 1954) and M9 synthetic medium (Miller, 1972) supplemented with 0.5% (w/v) glycerol. The HmpX-deficient mutant of *E. chrysanthemi* strain 3937 was described previously (Favey *et al.*, 1995). For plant infection, bacteria were pelleted, washed three times with 10 mM MgCl₂, resuspended, and diluted in 10 mM MgCl₂ to the indicated concentration.

Expression of HmpX in *E. coli* and *P. syringae*

The pRK415-*hmpX* vector plasmid contains a 1200-bp fragment that was amplified from the plasmid pB413 carrying the *hmpX* genomic region (Favey *et al.*, 1995). The gene was amplified using the PCR Supermix High Fidelity (Invitrogen, Carlsbad, CA, USA) with specific primers carrying restriction sites for directional cloning and proper reading frame (Forward: 5'-AAGCTTGGGATCCATGCTGGATCAACAAACCATCGC-3'; Reverse: 5'-GAGCTCCGGTTATATAACCTTGTCGGG-3'). The amplified fragment was cloned into pGEM-T vector (Promega, Madison, WI, USA), sequenced and then introduced into pRK415, a broad host range expression vector for Gram-negative bacteria (Keen *et al.*, 1988). The resulting plasmid (pRK415-*hmpX*) was transferred to *E. coli* strains DH5 α and RKP4600, the latter being a *hmp*-null mutant (Mills *et al.*, 2001) and then mobilized in *P. syringae* pv. *glycinea* race 4 carrying the *avrA* avirulence gene (Keen and Buzzell, 1991) and *P. syringae* pv. *tomato* DC3000 carrying the *avrB* avirulence gene (Innes *et al.*, 1993). Western blot analysis was performed using a polyclonal antibody to purified Hmp (Stevanin *et al.*, 2000) and the ECL chemiluminescent system (Amersham Biosciences, Little Chalfont, UK).

Periplasmic and cytoplasmic cell fractions

The method used for preparing periplasmic fractions was based on previous reports (French *et al.*, 1996; Poole *et al.*, 1994; Vasudevan *et al.*, 1995). Cells were grown to an OD₄₂₀ of approximately 0.4, divided into 200 ml aliquots and centrifuged for 10 min at 5000 *g* after the addition of 6 ml 1 M NaCl and 6 ml 1 M Tris-HCl, pH 7.3, to each aliquot. The pellets were resuspended together in 6 ml of their own supernatant and 6 ml buffer containing 40 % sucrose, 2 mM EDTA and 33 mM Tris-HCl, pH 7.3, was added. After 20 min at room temperature, the sample was centrifuged for 5 min at 10 000 *g*. The pellet was then resuspended in 6 ml ice-cold water after which 70 μ l 100 mM MgCl₂ was added and the sample was left on ice for 10 min. After centrifugation at 10 000 *g* for 5 min, the supernatant (periplasmic fraction) was concentrated in a 15 ml VivaSpin concentrator (VivaScience, Hannover, Germany) retaining proteins with molecular weight above 30 000 Da. The remaining pellet was sonicated and centrifuged for 1 h at 132 000 *g* to yield a supernatant (cytoplasmic fraction). Periplasmic fractions were also prepared by using lysozyme at a final concentration of 50 μ g ml⁻¹ as described (Vasquez-Laslop *et al.*, 2001).

Cell-free extracts

Cultures were grown until they reached the stationary phase, unless otherwise stated, and harvested by centrifugation for 10 min at 13 000 *g* and washing in PBS buffer. Cells were disrupted by sonication using an MSE/Sanyo Soniprep 150 sonicator (Sanyo Galenkamp Plc., Loughborough, UK) delivering four 20-sec periods at full power, with 1 min cooling intervals. The volume of cells used for sonication was typically 5 ml. The material was then centrifuged at 132 000 *g* for 1 h at 4°C. The amount of H₂O₂ produced by cell-free extracts was measured using the scopoletin assay as described (Søballe and Poole, 2000).

Polarography and spectroscopy

Polarographic studies were carried out as previously detailed (Mills *et al.*, 2001). The oxygen electrode was calibrated with air-saturated water and sodium dithionite crystals. The NO electrode was calibrated

with freshly prepared solutions of anoxic NO as described (Mills *et al.*, 2001). Experiments were carried out in 50 mM MOPS, 50 mM NaCl, pH 7.0. Electronic absorption spectra were recorded using a custom-built SDB4 dual-wavelength scanning spectrophotometer as described (Kalnenieks *et al.*, 1998).

Determination of bacterial growth under nitrosative stress

Freshly grown bacterial cultures were diluted to 10⁵ cells ml⁻¹ in 2.5 ml of appropriate medium, transferred to 10 ml tubes and incubated with vigorous shaking in the presence of different concentrations of the nitrosating agent SNP. After 16 or 24 h, bacterial growth was determined spectrometrically (OD₆₀₀) and expressed as a percentage of the OD reached by the same bacterial culture not exposed to SNP.

Soya bean cell suspensions

Experiments with *Glycine max* cv. Williams 82 cell suspensions were performed 3 days after subculture (Levine *et al.*, 1994). Cells were incubated in 12-well tissue culture plates (1 ml/well), agitated at 60 rpm and challenged with 10⁸ bacteria. The accumulation of H₂O₂ was assayed by measuring quenching of scopoletin fluorescence (Levine *et al.*, 1994). Cell death was assayed 24 h after the indicated treatments by spectrophotometric measurement of the uptake of Evan's blue stain (Levine *et al.*, 1994). NO accumulation was assayed by monitoring the conversion of oxyhaemoglobin to methaemoglobin as previously described (Delledonne *et al.*, 1998). Oxyhaemoglobin was added to the indicated final concentrations. After 2 min, changes in absorbance of the medium at 421 and 401 nm were measured and NO levels were calculated using an extinction coefficient of 77 mm⁻¹ cm⁻¹ [A₄₀₁ (methHb)–A₄₂₁ (HbO₂)].

Plant inoculations

Growth of *S. ionantha* cv. Blue Rhapsody, infiltration of leaves with *E. chrysanthemi* (100 μ l of a bacterial suspension containing 10⁸ CFU ml⁻¹) and measurement of bacterial growth *in planta* were as described (Favey *et al.*, 1995). Growth of *A. thaliana* ecotype Col-0, infiltration of leaves with *P. syringae* pv. *tomato* DC3000 (5–10 μ l of a bacterial suspension containing 5 \times 10⁶ CFU ml⁻¹) and measurement of bacterial growth *in planta* were as described (Cameron *et al.*, 1994). Dead cells were visualized by trypan blue staining as described (Koch and Slusarenko, 1990). The production of H₂O₂ was visualized *in situ* with DAB staining as described (Thordal-Christensen *et al.*, 1997). The number of stained pixels inside the infected leaf area was quantified by reduction of microscopic photographs to greyscale mode followed by quantification of pixels with a grey tone value <125 as described (Zeier *et al.*, 2004). The production of NO was visualized using dDAF-2 DA as described (Foissner *et al.*, 2000). Fluorescence for DAF-2DA-infiltrated leaves was quantified by determining the mean green channel values for captured images with the histogram function of Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA). To account for background fluorescence, the corresponding mean value for non-infected leaves infiltrated with the fluorescent dye (8.2 \pm 1.4) was subtracted.

RNA blot hybridization

Total RNA was isolated using Trizol reagent (Invitrogen). RNA blot hybridization was performed with the Gmhs26 *gst* cDNA probe (Levine *et al.*, 1994). Equal loading was verified by gel staining with

ethidium bromide and by hybridization with an rRNA gene sequence (Pepper *et al.*, 1994).

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