loss of fluorescence for a given bouton was calculated as the difference between the average of 10 data points of baseline before a stimulus and the average of the last 10 data points of the time-lapse sequence.

Modelling. Exocytosis of releasable vesicles under a sustained stimulus was lumped into a single rate α (see Fig. 4a). In the fused state, dye could either departition with rate δ or be taken up again by means of fast endocytosis with rate β. Freshly endocytosed vesicles were recycled back to the readily releasable pool either with a single rate γ (Fig. 4b) or through a series of n consecutive steps, each having a rate constant γ/n (Fig. 4c). The variables S_x represent the relative amounts of dye incorporated into vesicle membrane pools, with subscripts 0, 1 and 2 denoting readily releasable, fused and retrieved vesicles. The state vector $\mathbf{S} = (S_0 S_1 S_2)^T$ represents the filling of the three states, and its evolution in time is described by the matrix equation

$$\frac{d\mathbf{S}}{dt} = \begin{bmatrix} -\alpha & 0 & \gamma \\ \alpha & -(\beta+\delta) & 0 \\ 0 & \beta & -\gamma \end{bmatrix} \mathbf{S}$$

with the sum of the states S_0, S_1 and S_2 corresponding to the observable fluorescence. The amount of retrieved dye can be obtained by integrating the flux through the transition β (Fig. 4a, bottom).

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Nitric oxide functions as a signal in plant disease resistance

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Recognition of an avirulent pathogen triggers the rapid production of the reactive oxygen intermediates superoxide (O_2^-) and hydrogen peroxide (H_2O_2)[1]. This oxidative burst drives cross-linking of the cell wall[2], induces several plant genes involved in cellular protection and defence[3,4], and is necessary for the initiation of host cell death in the hypersensitive disease-resistance response[1,3]. However, this burst is not enough to support a strong disease-resistance response[4,5]. Here we show that nitric oxide, which acts as a signal in the immune, nervous and vascular systems[6], potentiates the induction of hypersensitive cell death in soybean cells by reactive oxygen intermediates and functions independently of such intermediates to induce genes for the synthesis of protective natural products. Moreover, inhibitors of nitric oxide synthesis compromise the hypersensitive disease-resistance response of *Arabidopsis* leaves to *Pseudomonas syringae*, promoting disease and bacterial growth. We conclude that nitric oxide plays a key role in disease resistance in plants.

The oxidative burst required for hypersensitive cell death in soybean cell suspensions inoculated with avirulent *P. syringae* pv. *glycinea* gives a sustained accumulation of 6–10 μM H_2O_2 (ref. 7). Addition of the H_2O_2-generating system glucose (0.5 mM)/glucose oxidase (0.5 U ml^{-1}) to these cell suspensions resulted in a steady-state accumulation of ~6 μM H_2O_2 for >3 h, and hence closely mimics the oxidative burst induced by the avirulent pathogen. However, *in situ* generation of H_2O_2 in this fashion, or equivalent generation of O_2^- by addition of xanthine/xanthine oxidase, induced only a relatively weak cell-death response (Fig. 1a).

In the immune system, reactive oxygen intermediates (ROI) often function together with nitric oxide (NO), for example in macrophage killing of bacteria and tumour cells[6,8]. We therefore tested whether NO might be the second factor required for a strong hypersensitive disease-resistance response (HR). Treatment of cell suspensions with 0.5 mM sodium nitroprusside generated a steady-state NO concentration of ~2 μM (see below), which in the absence of ROI did not induce cell death. However, this NO donor markedly potentiated the induction of cell death by exogenous H_2O_2 or O_2^- (Fig. 1a). The interaction was synergistic, with NO promoting a 5–10-fold increase in ROI-induced cell death. Potassium ferrocyanide, an analogue of sodium nitroprusside that does not release NO, had no effect on ROI-induced cell death.

NO also potentiated cell death induced by endogenous ROI. Activation of the oxidative burst following pathogen recognition involves a protein kinase cascade[3,9]. Cantharidin, an inhibitor of

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type-2a protein phosphatases, partially activates this signalling pathway in the absence of pathogen avirulence factors[3]. Salicylic acid, which functions in signal amplification[7], enhances this effect, resulting in the accumulation of >30 μM H_2O_2 (Fig. 2i; ref. 7). This massive oxidative burst caused only a weak induction of cell death, but simultaneous addition of nitroprusside strongly potentiated the response (Fig. 1a). Likewise, a yeast cell-wall elicitor, which rapidly stimulates a strong oxidative burst[10] (Fig. 2j), did not induce cell death without exogenous NO (data not shown). Rapid agitation of plant cells also causes ROI production[11] and, under these conditions, which gave a steady-state H_2O_2 concentration of ~1 μM in the soybean cultures, nitroprusside induced cell death without added ROI (Fig. 1b). This response was blocked not only by the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), but also by diphenylene iodonium, an inhibitor of the neutrophil NADPH oxidase that inhibits the plant oxidative burst[3], and by catalase, which destroys H_2O_2, thereby confirming the involvement of endogenous ROI together with the added NO (Fig. 1b). Two other NO donors, *S*-nitroso-*N*-acetylpenicillamine and diethylamino NO, gave a similar response (data not shown).

The synergistic interaction between exogenous NO and ROI suggested that NO might have a physiological function in the HR. Endogenous NO was assayed by the conversion of haemoglobin to methaemoglobin[12]. ROI can interfere with this assay, and although haemoglobin conversion was observed with cells treated with yeast elicitor, pretreatment of the assay sample with superoxide dismutase plus catalase abolished this spurious signal (Fig. 2a). Neither yeast

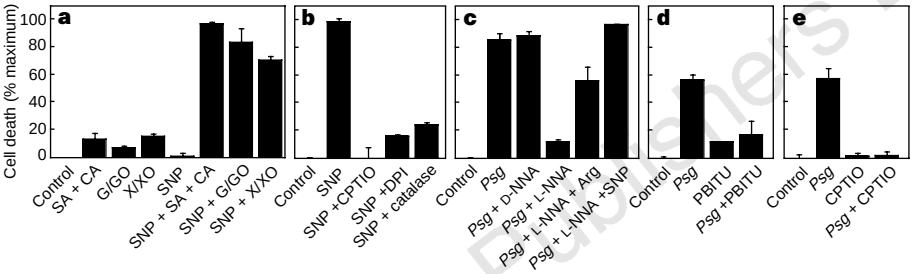

Figure 1 Function of NO in the induction of hypersensitive cell death in soybean cell suspension cultures. **a**, **b**, NO potentiation of ROI-induced cell death. **c**, **d**, NO function in *P. syringae* pv. *glycinea* (*Psg*)-induced hypersensitive cell death. In **b**, cell suspensions were rapidly agitated (100 r.p.m.) to stimulate ROI generation sufficient to maintain a steady-state H_2O_2 concentration of ~1 μM. In **a**, **c**–**e**, cell suspensions were shaken slowly (60 r.p.m.) and the basal level of H_2O_2 was <0.05 μM. Reagents were added as indicated at the following final concentrations: 50 μM salicylic acid (SA), 4 μM cantharidin (CA), 0.5 mM glucose + 0.5 U ml^{-1} glucose oxidase (G/GO), 0.5 mM xanthine + 0.5 U ml^{-1} xanthine oxidase (X/XO), 0.5 mM nitroprusside (SNP), 5 mM (**b**) and 50 μM (**d**) CPTIO, 10 μM diphenylene iodonium (DPI), 300 U ml^{-1} catalase, 200 μM L-NNA or D-NNA, 1 mM PBITU, 2 mM arginine. Cell death was assayed after 24 h by spectrophotometric determination of Evans blue uptake relative to that in equivalent cell suspensions killed by ethanol.

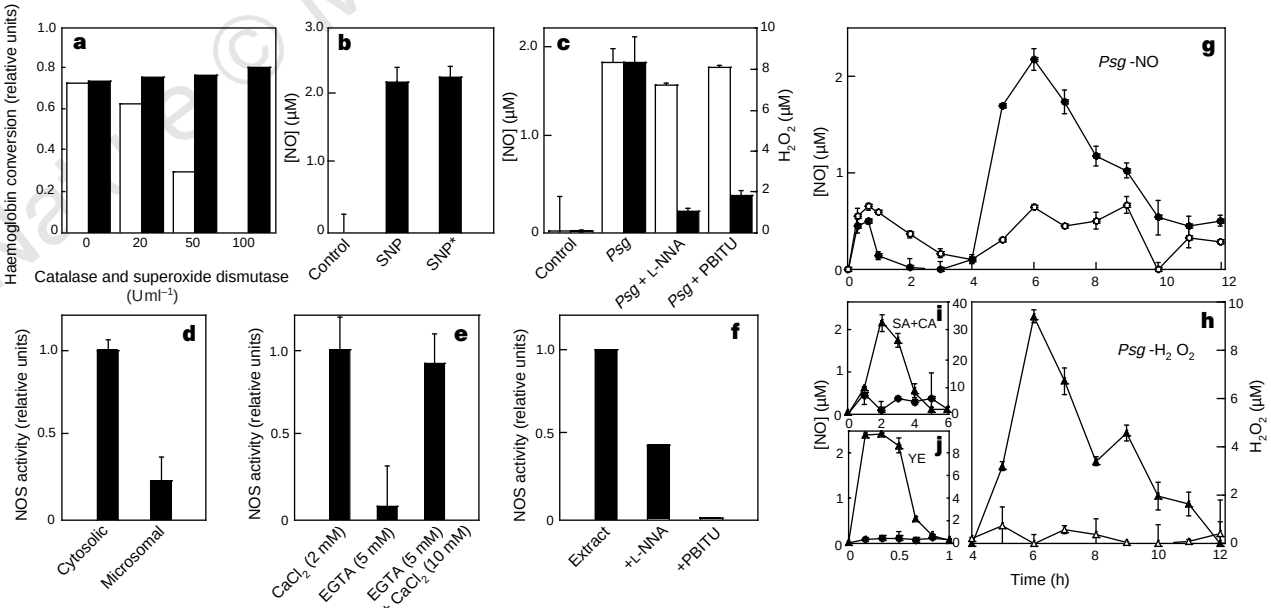

Figure 2 NO production in soybean cell suspensions. **a**, Validation of NO assay. The effects of pretreatment of assay samples with the indicated activities of superoxide dismutase and catalase on haemoglobin conversion were measured in samples from cells induced with yeast elicitor for 20 min (white bars) or *P. syringae* pv. *glycinea* (*Psg*) for 6 h (black bars). **b**, NO accumulation in cells 2 h after treatment with 0.5 mM nitroprusside (SNP). Asterisk denotes cells that were rapidly shaken to give mechanically induced accumulation of ROI. **c**, Effect of 0.2 mM L-NNA or 1 mM PBITU on *Psg* induction of H_2O_2 (white bars) and NO (black bars) accumulation after 6 h. **d**, Distribution of NOS activity between cytosolic and microsomal fractions of soybean whole-cell extracts. Relative activities are presented on a cell-equivalent basis. **e**, Calcium dependence of cytosolic NOS activity. **f**, Effect of 0.2 mM L-NNA or 1 mM PBITU on NOS activity in whole-cell extracts **g**–**j**, NO (circles) and H_2O_2 (triangles) accumulation in soybean cell suspensions treated with: **g**, **h**, *Psg* (filled symbols: avirulent strain; open symbols: isogenic virulent strain); **i**, cantharidin (CA, 1 μM) + salicylic acid (SA, 50 μM); **j**, yeast elicitor (YE, 100 μg glucose equivalents ml^{-1}).

elicitor nor salicylic acid plus cantharidin stimulated NO accumulation, as measured by haemoglobin assay after superoxide dismutase and catalase pretreatment (Fig. 2i, j), consistent with the inability of these stimuli to trigger cell death despite activation of a strong oxidative burst. In contrast, inoculation of soybean cell suspensions with *P. syringae* pv. *glycinea* stimulated NO production, as measured by assay of samples depleted for ROI by pretreatment with superoxide dismutase plus catalase (Fig. 2a, g). A rapid, relatively weak stimulation by both avirulent and virulent strains was followed by several-fold greater NO production specifically in cells inoculated with the avirulent strain, concomitant with the avirulence gene-dependent oxidative burst (Fig. 2g, h). The maximal accumulation of 2 μM NO and of 10 μM H_2O_2 closely matched their respective levels in the experiments with nitroprusside and glucose/glucose oxidase (Fig. 2b), and hence would be sufficient to account for the induction of hypersensitive cell death by avirulent *P. syringae*.

In animals, NO is generated by the reaction arginine → citrulline + NO, which is catalysed by NO synthase (NOS)[6]. Plants also have NOS activity[13,14], and in our soybean cell extracts NOS activity was calcium-dependent and found primarily in the cytosolic fraction (Fig. 2d, e). The NOS inhibitors N^{ω}-nitro-L-arginine (L-NNA) and *S*,*S*′-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea (PBITU)[15,16], which inhibits this reaction in soybean cell extracts (Fig. 2f), markedly inhibited (at concentrations comparable to those used with animal cells) the accumulation of endogenous NO in bacterially induced cells, but had no effect on the accumulation of H_2O_2 (Fig. 2c). Both PBITU and L-NNA, but not the inactive enantiomer D-NNA, blocked bacterial induction of hypersensitive cell death (Fig. 1c, d). L-NNA inhibition could be partially competed with exogenous arginine or completely bypassed with nitroprusside (Fig. 1c). Moreover, the induction of hypersensitive cell death by avirulent *P. syringae* was also inhibited by the NO scavenger CPTIO (Fig. 1e), which blocks NO accumulation by a different mechanism.

H_2O_2 drives oxidative crosslinking of tyrosine-rich cell-wall structural proteins[2] and the oxidative burst also induces protective cellular genes encoding glutathione *S*-transferase (*gst*) and glutathione peroxidase[3]. In soybean cells, however, ROI are not the primary signals for rapid induction of defence genes encoding enzymes of phenylpropanoid biosynthesis involved in the synthesis of antibiotics, lignin and salicylic acid[15]. Whereas L-NNA had little effect on the initial induction of *gst* by avirulent *P. syringae*, the accumulation of transcripts encoding phenylalanine ammonia-lyase (*pal*), the first enzyme of the phenylpropanoid pathway[17], was markedly reduced (Fig. 3). Bacterial induction of transcripts encoding chalcone synthase (*chs*), the first enzyme of the branch specific for flavonoids and isoflavonoid-derived antibiotics[17], showed a similar dependence on endogenous NO (data not shown). Moreover, sodium nitroprusside caused a rapid induction of *pal* and *chs* transcripts that was blocked by CPTIO but not by catalase. In contrast, nitroprusside caused only a slow accumulation of *gst* transcripts and this was sensitive to catalase as well as to CPTIO (Fig. 3).

To test whether NO functions *in planta*, we examined the effects of inhibitors of NO accumulation in a genetically well defined host–pathogen interaction. Infiltration of leaves of *Arabidopsis* ecotype Col-0, which contains the *RPM1* resistance gene, with *P. syringae* pv. *maculicola* carrying the corresponding *avrRpm1* avirulence gene, results in a localized HR lesion which is first apparent ~1 d after inoculation[18]. Co-infiltration of the NOS inhibitors L-NNA or PBITU blocked this early HR and promoted the spreading chlorosis observed in the compatible interaction with the isogenic virulent bacteria lacking *avrRpm1* (Fig. 4a, b). L-NNA and PBITU alone did not cause disease symptoms (Fig. 4a) and had no effect on axenic bacterial growth (data not shown). Development of disease symptoms in leaves blocked for NO accumulation was accompanied by enhanced bacterial growth, although not to the extent seen with the isogenic virulent strain (Fig. 4c).

In the vertebrate native immune system, NO potentiates the antimicrobial and cytotoxic activities of ROI[6,8]. We have shown by several independent approaches that NO also functions in the plant HR and appears to be critical for preventing pathogen spread from the inoculation site. Moreover, our results reveal a striking complementarity between NO and ROI signal functions, involving not only synergistic induction of hypersensitive cell death, but also independent activation of complementary gene sets. This arrangement provides for flexible deployment of specific defences and binary control of progression to hypersensitive cell death.

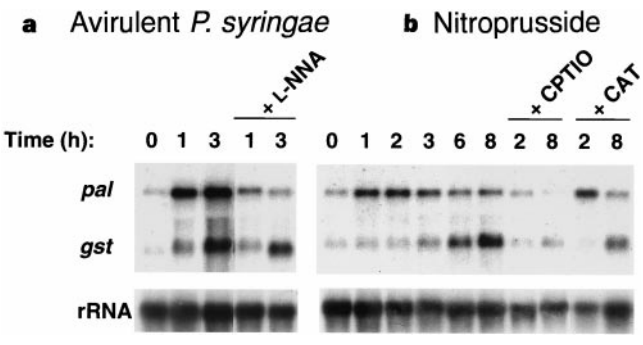

Figure 3 NO induction of *pal*. Northern blot analysis of **a**, the effects of the NO synthase inhibitor L-NNA on *pal* and *gst* induction by avirulent *P. syringae* pv. *glycinea*, and **b**, NO induction of *pal* and *gst*. RNA loading was checked by hybridization of rRNA. Reagents were added as indicated at the following final concentrations: 0.5 mM nitroprusside, 200 μM L-NNA, 50 μM CPTIO, 300 U ml^{-1} catalase (CAT).

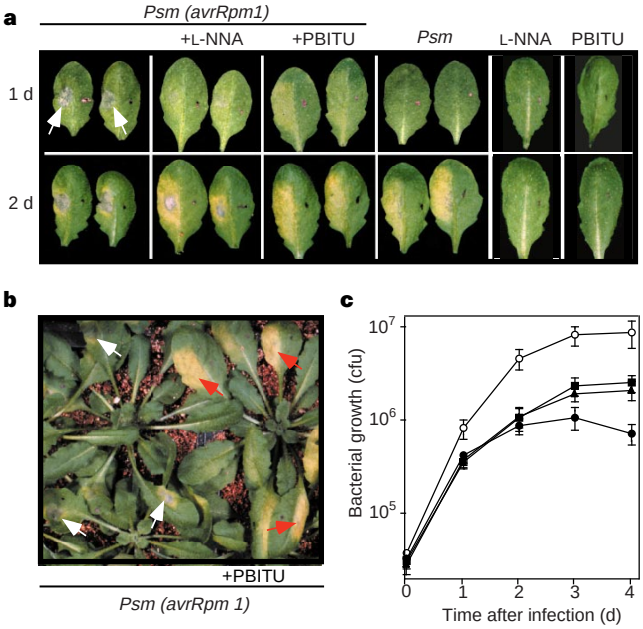

Figure 4 Function of NO in the hypersensitive disease-resistance response: effects of L-NNA and PBITU on: **a**, **b**, visible symptoms, and **c**, bacterial growth. *Psm* represents virulent *P. syringae* pv. *maculicola* (open circles); *Psm* (*avrRpm1*), the isogenic avirulent strain of *P. syringae* pv. *maculicola* inoculated alone (filled circles), with L-NNA (triangles) or with PBITU (squares). PBITU was 1 mM in all experiments, L-NNA was 0.1 mM in **a**, 0.2 mM in **c**. Red arrows mark disease; white arrows, HR. In **c**, data points are the mean and standard deviation of three replicates, each from three leaf discs. These experiments were repeated three times with similar results.

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In animals, many physiological effects of NO are mediated by cyclic GMP, following direct activation of guanylyl cyclase[6,8]. In plants, cGMP functions in the phytochrome-mediated light induction of *chs* associated with the formation of phenylpropanoid-derived pigments[19]. Recent results indicate that cGMP also functions in the induction of *pal* by exogenous NO (ref. 29), associated with the synthesis of phenylpropanoid products with defence functions[20]. Moreover, ROI induce hypersensitive cell death by raising cytosolic calcium[21] and NO can reduce the threshold for calcium signalling by modulation of cGMP-gated ion channels[22]. Hence, NO may activate cGMP pathways for both defence gene induction and potentiation of ROI-induced cell death.

An enzyme system similar to the neutrophil NADPH oxidase apparently contributes to the oxidative burst in plants[23,24]. Although NOS homologues have not yet been reported in plants, a plant complementary DNA sequence with pronounced similarity to PIN, a protein inhibitor of NOS, has been identified[25], suggesting that functional parallels between the native immune system and the plant HR may be maintained by conserved molecular mechanisms. □

Methods

Soybean (*Glycine max* cv. Williams 82) cell cultures, virulent *P. syringae* pv. *glycinea* race 4 and the isogenic strain carrying the *avrA* avirulence gene, which interacts with the soybean resistance gene *Rgm2* (ref. 26) were grown as described[3,7]. Three days after subculture, aliquots of the soybean cell suspension were transferred to 12-well tissue-culture plates (1 ml per well) agitated at 60 or 100 r.p.m. Bacteria (10^8 colony forming units (CFU) per ml) and reagents at the indicated concentrations were added simultaneously as appropriate. The growth of *A. thaliana* ecotype Col-0, infiltration of leaves with virulent *P. syringae* pv. *maculicola* or the isogenic avirulent strain carrying *avrRpm1* (5–10 μl of a bacterial suspension containing 5×10^6 CFU ml^{-1}), together with PBITU or L-NNA as indicated, and measurement of bacterial growth *in planta* have all been described[27].

Cell death was assayed by spectrophotometric measurement of the uptake of Evans blue stain[7], and H_2O_2 accumulation by fluoresence determination of scopoletin destruction[3]. For NO assay, soybean cell suspensions were incubated with 100 U catalase and 100 U superoxide dismutase for 5 min to remove ROI before addition of oxyhaemoglobin (5 μM final). After 2 min incubation, NO was quantified by spectrophotometric measurement of the conversion of oxyhaemoglobin to methaemoglobin[12]. Each cell death, H_2O_2 and NO datum point is the mean and standard deviation of three to five replicates. NOS activity was assayed by the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline[28] in whole-cell extracts, and microsomal and cytosolic fractions[24]. Northern blot hybridization was done as described[3]. Cantharidin, diphenylene iodonium, glucose oxidase and PBITU were from Calbiochem; CPTIO, diethylamino NO, *S*-nitroso-*N*-acetylpenicillamine were from Molecular Probes; all other reagents and enzymes were from Sigma.

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Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus

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Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus 8, is invariably present in Kaposi's sarcoma lesions[1,2]. KSHV contains several viral oncogenes and serological evidence suggests that KSHV infection is necessary for the development of Kaposi's sarcoma, but cellular transformation by this virus has not so far been demonstrated. KSHV is found in the microvascular endothelial cells in Kaposi's sarcoma lesions and in the spindle 'tumour' cells[3,4], which are also thought to be of endothelial origin. Here we investigate the biological consequences of infecting human primary endothelial cells with purified KSHV particles. We find that infection causes long-term proliferation and survival of these cells, which are associated with the acquisition of telomerase activity and anchorage-independent growth. KSHV was present in only a subset of cells, and paracrine mechanisms were found to be responsible for the survival of uninfected cells. Their survival may have been mediated by upregulation of a receptor for vascular endothelial growth factor. Our results indicate that transformation of endothelial cells by KSHV, as