A NEW PARTNER IN THE *DANSE MACABRE*: THE ROLE OF NITRIC OXIDE IN THE HYPERSENSITIVE RESPONSE

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Summary. Plant responses to abiotic and biotic stress are to a great extent coordinated by similar chemical signals. Thus, salicylic acid (SA) and reactive oxygen intermediates (ROIs) influence tolerance to heat and chilling as much as resistance to pathogens during the hypersensitive response (HR) form of cell death. We have shown that following elicitation the generation of SA and ROI is biphasic and each signal influences the production of the other in a positive feedback mechanism. When SA is applied alone this "potentiates" plant responses to be more effective following elicitation by either pathogenic challenge or abiotic stress. Nitric oxide (NO) has also been proposed to contribute towards plant defence. Using photoacoustic laser (PLA) detection NO generation was detected in tobacco following challenge with bacterial pathogens but was not produced in a biphasic manner. In contrast PLA measurements showed that C_2H_4 production during the HR was biphasic. This pattern of C_2H_4 production could be mimicked with the NO-donor sodium nitroprusside (SNP), acting part through the initiation of SA synthesis. A model is proposed where NO acts with SA to influence the biphasic production of cell death-eliciting signals including ROI and C_2H_4.

"So it is clear that in all these cases the thing does not move itself, but it contains within itself the source of motion" (Aristotle Physics 8.4; 255b 29-30).

Background

Resistance to pathogens in plants is often associated with the elicitation of a programmed cell death – the hypersensitive response (HR, Morel and Dangl, 1997). The initia-

tion of the HR is controlled by the recognition of a pathogen-encoded avirulence (*avr*) gene product by a plant derived resistance (*R*) gene protein (H. Kosack and Jones, 1997). Characterisation of bacterial pathogen recognition has suggested that the AVR protein is delivered into the plant cytoplasm via a pilus formed from proteins encoded by the *hrp*-operon (Alfano and Collmer, 1997). It is likely that other proteins, probably virulence determinants and not –recognised by the plant are also delivered via the HRP pilus (Fouts et al., 2002). AVR recognition rapidly initiates a calcium influx (Grant et al., 2000) and the generation of reactive oxygen intermediates (ROI) – the oxidative burst (Baker and Orlandi, 1995). The generation of ative oxygen species (AOS) in particular has been proposed to be intimately associated with the initiation of cell death (Levine et al., 1994) as altering the levels of superoxide anions and H_2O_2 affected the HR (Jabs et al., 1997, Chamnongpol et al., 1998).

Examination of the kinetics of the oxidative burst has suggested recognition events additional to the *avr*/ *R* gene interaction influence plant defence (Fig. 1). Typically, interactions leading to a HR exhibit a biphasic oxidative burst – with a transient rise at ~30 min following inoculation followed by a second rise which persists until cell death. As the first transient rise is also observed during interactions leading to
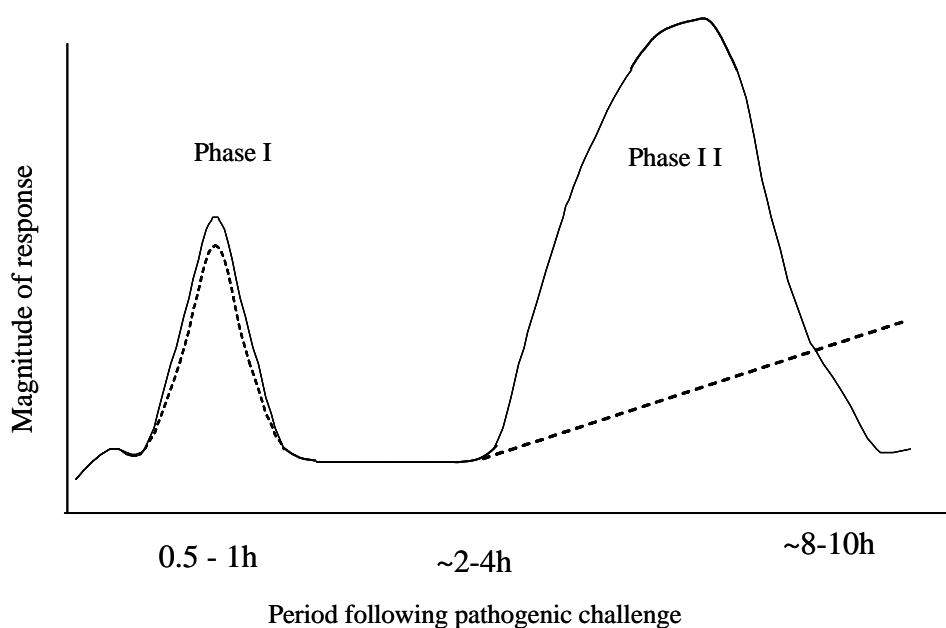

Fig. 1. The biphasic mode of plant responses to pathogenic challenge. Several plant responses to pathogens are biphasic exhibiting an initial transient response (phase I) that is exhibited by both hypersensitive response (HR, —) eliciting and disease forming (· – – ·) pathogens. Thus, elicitors other than the avirulence gene product have been proposed induced phase I. A second response (phase II) is primarily seen during the HR and was therefore supposed to be elicited following AVR product recognition. The phase II response persists until the initiation of the HR-associated tissue collapse (Shirasu et al., 1997; Draper 1997; Mur et al., 2000).

disease symptom development, it has been proposed that this is elicited by "non-specific" i.e. *avr*/*R* gene-independent interactions (Lamb and Dixon, 1997).

Potentiation, also known as priming (Conrath et al., 2002) or conditioning (Kauss et al., 1993) sets the cell so that responses to subsequent stimuli are augmented and are therefore more effective. The second stimuli could be pathogenic challenge, wounding (Mur et al., 1996; Shirasu et al., 1997) or indeed abiotic stress (Dat et al., 1998). Several other defensive features are potentiated including the oxidative burst and cell death, and expression from phenylalanine ammonia lyase (Katz et al., 1998), gluta-thione-*S*-transferase (Shirasu et al., 1997) and PR10-class pathogenesis-related protein (PR) genes (Mur et al., 1996). A potentiation mechanism has been proposed to mediate the biphasic oxidative burst with first transient rise (phase I) initiating SA synthesis to potentiate the second (phase II) rise in ROI, whilst this initiates substantial SA production (Draper, 1997; Shirasu et al., 1997). Significantly, the synthesis of SA was also found to be biphasic and correlated with oxidative burst (Draper, 1997). A focus of our groups work has been on the understanding the mechanisms underlying SA-mediated potentiation.

Salicylic acid sets the kinetics of the HR.

The roles of SA in plant responses to abiotic and biotic stress have been revealed by the isolation of mutants of *Arabidopsis* (reviewed by Dong, 2001) and the generation of *NahG* transgenic plants. *NahG* encodes salicylate hydroxylase [EC 1.14.13.1] an enzyme which catalyses the NADPH dependent decarboxylation and hydroxylation of SA (2-hydroxybenzoic acid) to form catechol (Fig. 2a). *NahG* forms part of the *nah* operon from *Pseudomonas putida* that metabolises the degradation of naphthalene to pyruvate via salicylate (Schell, 1985). Fusion of the *NahG* structural gene to the constitutive plant-active promoter CaMV35S, allowed the generation of transgenic tobacco (Gaffney et al., 1993; Bi et al., 1995) and *Arabidopsis* (Delaney et al., 1994) with reduced levels of SA. Challenging *NahG* transgenic lines with viral (Delaney et al., 1994; Mur et al., 1997), fungal (Lawton et al., 1995) and bacterial pathogens (Mur et al., 2000) demonstrated that HR mediated resistance was dependent on SA. Wild type tobacco exhibited a necrotic lesion (a HR) when inoculated with the avirulent bacterial strain *Pseudomonas syringae* pathovar (*P. s.* pv.) *phaseolicola* (Fig. 2b) but spreading necrosis and chlorosis (yellowing) when challenged with the disease forming species *P. s.* pv. *tabaci* (Fig. 2c). However inoculating 35S-*NahG* tobacco plants with *P. s.* pv. *phaseolicola* lead to the formation of lesions which resembled those elicited by *P. s.* pv. *tabaci* (compared Fig. 2c with Fig.2d) and was the most obvious indicator of a loss in HR-mediated resistance. NahG transgenics have also shown the importance of SA to senescence (Morris et al., 2000) tolerance to ozone (Rao et al., 1999) and heating (Larkindale et al., 2002).
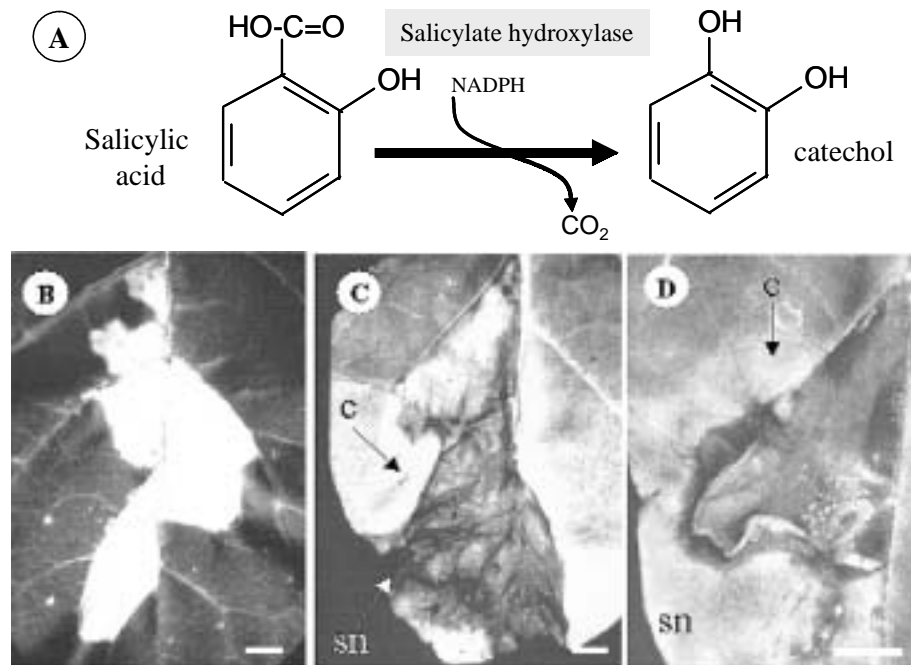
Fig. 2: The effects of salicylate hydroxylase expression on a bacterially elicited hypersensitive response in tobacco. **(A)** Salicylate hydroxylase [EC 1.14.13.1] catalyses the NADPH decarboxylation (yielding CO_2) and hydroxylation of 2-hydroxybenzoic (salicylic acid) to give catechol. Injection of wild type tobacco cv. Samsun NN leaves with **(B)** *Pseudomonas syringae* pathovar (*P. s.* pv.) *phaseolicola* gives rise by 7d to a defined non-spreading necrotic lesion but when challenging with **(C)** *P. s.* pv. *tabaci* to areas of spreading necrosis **(sn)** and chlorosis **(c)**. **(D)** Lesion phenotypes at 7d following injection of *P. s.* pv. *phaseolicola* into leaves of 35S-*NahG* cv. Samsun NN. Note regions of spreading necrosis **(sn)** and chlorosis **(c)**. Bars = 1 cm. Based on figures presented in Mur et al., 2000.

In order to elucidate the most crucial period in lesion development where SA was required for resistance, the *NahG* transgene was fused to promoters which were active at different stages in the elaboration of the HR. Using the viral pathogen, tobacco mosaic virus (TMV) it was discovered that SA accumulation during the oxidative burst was the most essential to plant resistance (Mur et al., 1997). Hence, it was hypothesised that a SA-potentiated oxidative burst could be a vital component in plant defence (Draper, 1997; Mur et al., 1997). This was further suggested by the observation that the kinetics of bacterially-elicited cell death and PR10 gene expression was delayed in 35S-*NahG* transgenic tobacco plants (Mur et al., 2000). The oxidative burst was measured in 35S-*NahG* transgenic tobacco plants during the HR by infiltration with cerium chloride, with the resulting production of electron dense cerium perhydroxides which were detected using electron microscopy (Bestwick et al., 1997, Fig. 2a). Comparison the density of cerium perhydroxide deposition in wild type and 35S-*NahG*

tobacco plants at different time points indicated that the oxidative burst was delayed but not abolished in the transgenic line (Mur et al., 2000, Fig. 2b).

Is nitric oxide part of the potentiation loop?

Although these data established that the importance of SA and ROI to resistance, reports of $^{\bullet}$NO production associated with the HR suggested that this could be an important additional component (Durner and Klessig, 1998). $^{\bullet}$NO has been extensively studied in mammalian systems where it acts in e.g. smooth muscle relaxation, the innate immune response and initiating cell death (reviewed by Wendehenne et al., 2001). $^{\bullet}$NO can interact with the superoxide anion (O_2^-) to form the potent oxidant peroxynitrite ($O_2^- + {^{\bullet}NO} \rightarrow ONOO^-$) and pernitrous acid (ONOOH) which can rapid decompose to form the hydroxyl and nitrogen dioxide radicals ($^{\bullet}$OH and NO_2). Both peroxynitrite and OH will initiate the formation of lipoxy radical and lipid hydroperoxides which could contribute to oxylipin signalling in plants. $^{\bullet}$NO can also affect signalling components by thiol group nitrosylation (SH $\rightarrow$ S-NO) or complex with haem centres within enzymic complexes, for instance guanyl cyclase, to alter protein activity (reviewed by Beck et al., 1999).

Measurements of $^{\bullet}$NO production in soybean single cell suspension cultures on inoculation with a bacterial pathogen suggested that generation was biphasic and broadly co-incident with the oxidative burst. Further, $^{\bullet}$NO donors were observed to initiate the synthesis of SA whilst the application of inhibitors of mammalian nitric oxide synthase (NOS) compromised HR-mediated resistance (Delledonne et al., 1998; Durner et al., 1998). Taking these data together suggested that $^{.}$NO could be an additional player in the SA/ROI potentiation loop.

However, the utility of inhibitors of mammalian NOS has been challenged given that no plant EST with significant homology to NOS has been isolated (Beligni and Lamattina, 2001). Additionally, many $^{\bullet}$NO assays utilized in plants are likely to yield equivocal data. $^{\bullet}$NO assays based on reduced haemoglobin (Delledonne et al., 1998) will also detect ROI (Dellebonne et al., 2001). $^{.}$ The $^{\bullet}$NO specific fluorescent dye, 4,5-diaminofluorescein-2-diacetate (DAF-2DA) will allow semi-quantitative measurements to be made using confocal scanning laser microscopy but this also limits the tissues to which it can be applied, for example cell cultures (Shkihama et al., 2002) or epidermal peels, (Foissner et al., 2000). Measurements may also be skewed due to differences in dye loading between tissue types and/or specific organelles (Foissner et al., 2000).

Greater accuracy in NO measurements must be based on direct assays of $^{\bullet}$NO emanation. Hence, we have employed a direct assay based on the photoacoustic laser detection (PLD) of $^{\bullet}$NO (Fig. 3a) Photoacoustic detection is based on measuring the resonance (sound) arising from absorption of flashes of specific wavelengths of laser
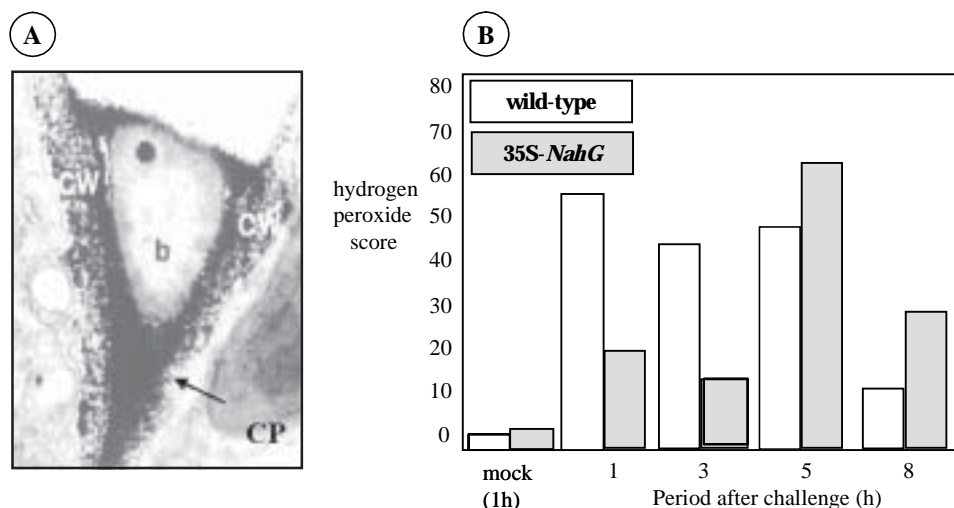
Fig. 3: The *Pseudomonas syringae* pv. *phaseolicola* –elicited oxidative burst in wild type and 35S-*NahG* transgenic tobacco cv. Samsun NN. **(A)** The addition of cerium chloride [$CeCl_3$] to sites of pathogen-challenge resulted in the deposition of cerium perhydroxide [$Ce(OH)_2OOH$ and $Ce(OH)_3$ OOH] at sites of H_2O_2 (Bestwick et al., 1997). The deposition of electron-dense cerium perhydroxide **(CP)** may be viewed using electron microscopy at sites of bacterial **(b)** interaction with the plant cell wall **(cw)**. **(B)** Cerium perhydroxide deposition scored at sites where *P. s.* pv. *phaseolicola* was present in intercellular spaces adjacent to cell wall interaction points (n=$\geq$22 for each time point) in wild type (white bars) and 35S-*NahG* cv Samsun NN (gray bars). A relative estimate of H_2O_2 accumulation at different time points was calculated by placing the observed response in a particular category, ("0" = no deposition; "1" = faint and patchy and "2" intense staining) based on which a "H_2O_2 score" was calculated as a percentage of sites in each category x category score/2. The maximum possible score was 100.x^2 contingency tests showed that the H_2O_2 scores at 1 and 3h following inoculation were significantly higher in wild type compared to 35S-*NahG* tobacco ($P < 0.001$). No significant effects were observed at other times ($P > 0.05$). Based on figures presented in Mur et al., 2000.

light by the gas under assay. The amplitude of the resonance is proportional to the concentration of the absorbing gas (te Lintel Hekkert et al., 1998, Fig. 3a). PLD has already been used to detect ethylene emanation from plant tissue using a CO_2 laser (De Vries et al., 1995) or acetaldehyde (Zuckermann et al., 1997) production using the CO laser. Our group is the first to detect ˙NO production from plant tissues by PLD,

˙NO production was detected using fully expanded five-week old tobacco leaves that had been injected with the HR-eliciting strain, *P. s.* pv. *phaseolicola,* the disease forming strain *P. s.* pv. *tabaci* and the *hrpL* mutant derivative of *P. s.* pv. *phaseolicola* that fails to deliver either avirulence or virulence determinants into the cell and was therefore used as a negative control (Fig. 3b). For each interaction, two rates of ˙NO generation were observed but this did not conform to the classical biphasic pattern (Fig. 1). Following inoculation there was an initial burst of ˙NO production that in each case apparently stabilised between 4-8h following inoculation. The magnitude
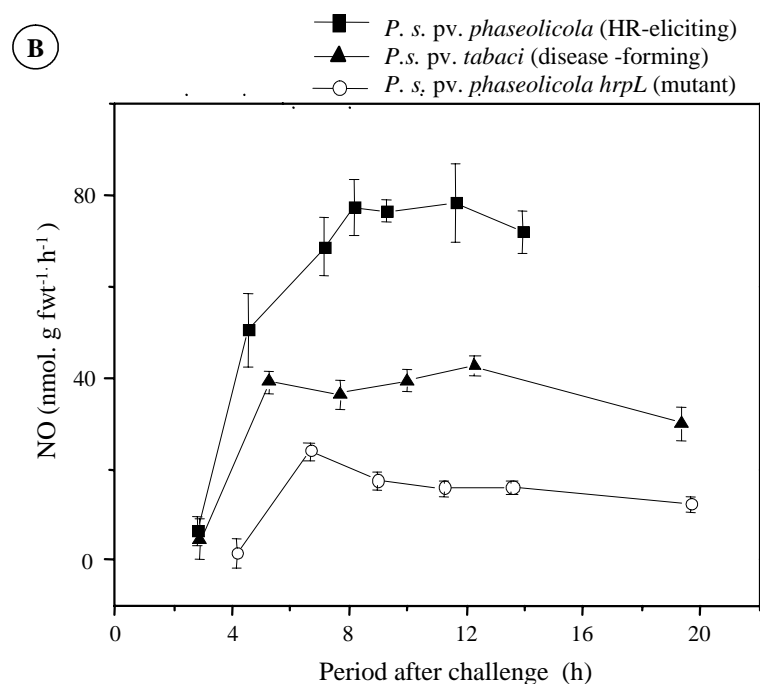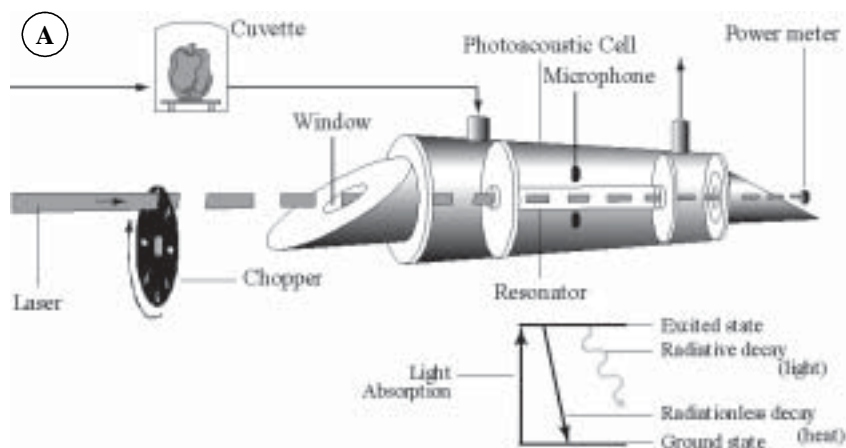
Fig. 4. Photoacoustic Laser detection of nitric oxide in pathogen-challenged tobacco. **(A)** The photoacoustic effect is based on the generation of acoustic waves as a consequence of light absorption. Absorption of an infrared photon excites a molecule into a higher ro-vibrational state. Collisions transfer the ro-vibrational energy to translational energy, i.e. heat. Modulation of the light intensity (turning the light on and off) causes the temperature of the sample to rise and fall periodically. Light modulation occurs by passing laser beam though a spinning disc within which windows have been cut – the chopper. The gas sample is passed from the biological sample held within a cuvette (here a pepper fruit) into the closed volume of the photoacoustic cell. Within the photoacoustic cell, the photon-induced temperature variation is accompanied by a pressure variation which creates a sound wave that can be detected

with a sensitive microphone. The pressure amplitude, and subsequently the microphone signal, is proportional to the number of absorbing molecules present in the gas (i.e. the trace gas concentration). Figure reproduced with permission from Harren (1998). Further details may be found at http://www-tracegasfac.sci.kun.nl/whatis.htm. **(B)** Estimation of nitric oxide emanation from tobacco cv Samsun NN leaves as measured using CO-laser based photoacoustic laser detection following injection with *Pseudomonas syringae* pathovar (*P. s.* pv.) *phaseolicola* (–■–), *P. s.* pv. *tabaci* (–▲–)and *P. s.* pv. *phaseolicola hrpL* (–□–) and retained on the plant for 2 h until the infiltrated liquid was observed to have dispersed. Results are given as mean NO production nmol.g fwt^{-1}.h^{-1} (n=3 leaves) ±SE.

of the initial burst of ˙NO and the final rate of production was greatest with the HR-eliciting strain but significant ˙NO was also generated by the disease-forming strain. These results were not due to the infiltration process since the control *hrpL* mutant induced relatively little ˙NO production.

The role of the detected ˙NO in each interaction was revealed by co-infiltration of the bacteria with the NOS inhibitor, N^G-methyl-L-arginine (L-NMMA). Using PLD this had previously been shown to suppress ˙NO production during the HR in tobacco plants. Co-application of L-NMMA with *P. s.* pv. *phaseolicola* increased bacterial populations within the lesions which was indicative of reduced resistance, though the overall phenotype was unaltered. However, with *P. s.* pv. *tabaci* inoculations, symptoms were more severe with L-NMMA treatment showing that ˙NO was contributing to plant defence mechanisms which suppress disease development. (data not shown, Mur et al., paper submitted).

Further work has showed that ˙NO production was unaltered in SA treated tobacco plant and in NahG transgenes (Mur et al., paper in prep). These data, together with the lack of biphasic generation pattern, suggest that NO is unlikely to integrated with the SA/ROI positive feedback mechanism

NO interacts with salicylic acid to potentiate ethylene signalling

Such interpretations notwithstanding ˙NO must influence the potentiation mechanism as it initiates SA synthesis (Durner et al., 1998). To examine the effects of ˙NO on the potentiation loop it was necessary to be able to measure accurately signals which are part of or are regulated by this mechanism. Serendipitously, using PLD it was discovered that the generation of C_2H_4 during the HR was biphasic and was suppressed in 35S-*NahG* transgenic tobacco plants. (Fig.5a). These data clearly indicated that SA potentiated C_2H_4 production and indeed, may be intimately associated in initiating H_2O_2 generation (De Jong et al., 2002). Further, injection with increasing concentrations of the˙ NO-donor sodium nitroprusside (SNP) resulted in proportionate increases in ethylene production (Fig. 5b). As SNP-induced ethylene production was suppressed in NahG tobacco and injection with SA alone did had no similar effect (data not shown) it seemed likely that SA acted with ˙NO to potentiate C_2H_4 production (Mur et al., paper in preparation). It remains to be established whether SA and NO act similarly to potentiate ROI generation.
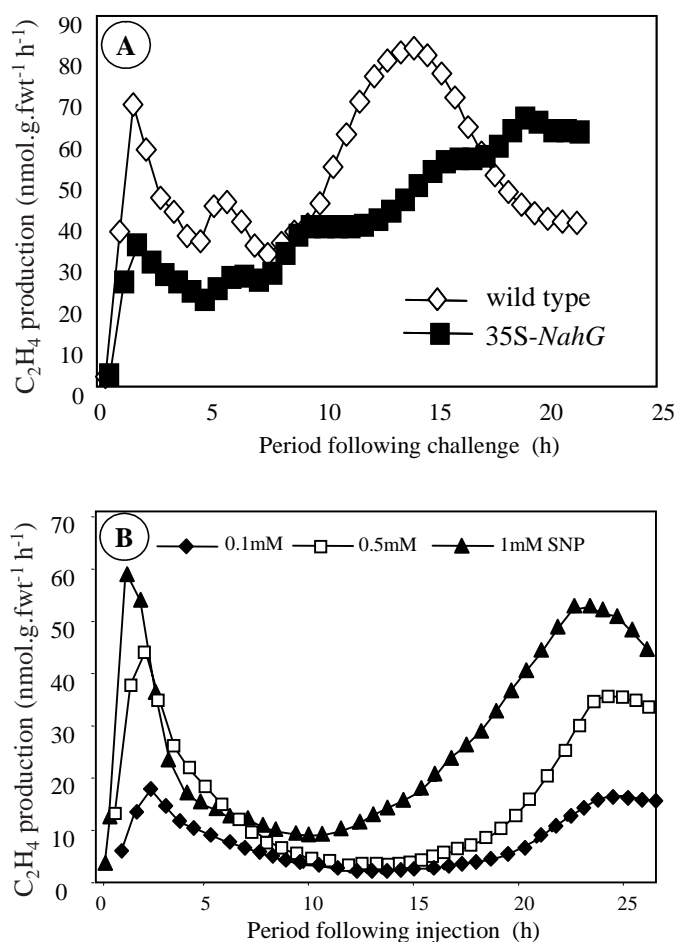
Fig. 5. Biphasic ethylene emanation following pathogenic challenge and injection with sodium nitroprusside. (**A**) C_2H_4 emanation following injection of wild type (◇) and 35S-*NahG* (■) DITTO tobacco cv. Samsun NN leaves with *Pseudomonas syringae* pathovars (*P. s.* pv.) *phaseolicola* as measured using CO_2-laser based photoacoustic laser detection (PLD). Results are given as mean C_2H_4 production nmol.g.fwt^{-1}.h^{-1} (n=3 leaves) ± se. (**B**) C_2H_4 production in tobacco leaves following injection with 0.1 (◆), 0.5 (□) and 1mM (▲)of the NO-donor sodium nitroprusside (SNP) measured using PLD. Results are given as mean C_2H_4 production nmol.g fwt^{-1}.h^{-1}. This experiment has been repeated yielding similar results.

Nitric oxide, the "unmoved mover"?

Perhaps the most surprising effect of the SNP injection is the generation of a biphasic pattern of ethylene production *without* the interaction of "non-specific" and *avr/R* – specific elicitors (see Fig. 1). This indicates that NO production a key feature in estab-

lishing the biphasic rise – most probably by acting in conjunction with SA. This hypothesis is developed in Figure 6. The proposed model highlights the importance of coupling HRP-independent and dependent elicitors to initiate both phase I and II. Where no HRP-delivered factors are recognised, only phase I is exhibited. However, AVR-recognition acts to increase the generation of $^{\bullet}$NO compared to the action of other HRP-delivered virulence factors (see Fig. 4a) and therefore increases the potency of phase II signals that include cell death inducers. Unlike ROI, SA and C_2H_4, $^{\bullet}$NO production is apparently unique in only being influenced by the eliciting interaction and not any downstream signals.

Clearly, much further work needs to establish how $^{\bullet}$NO and SA initiate biphasic rises in signal generation. In mammalian systems, biphasic signal generation has been
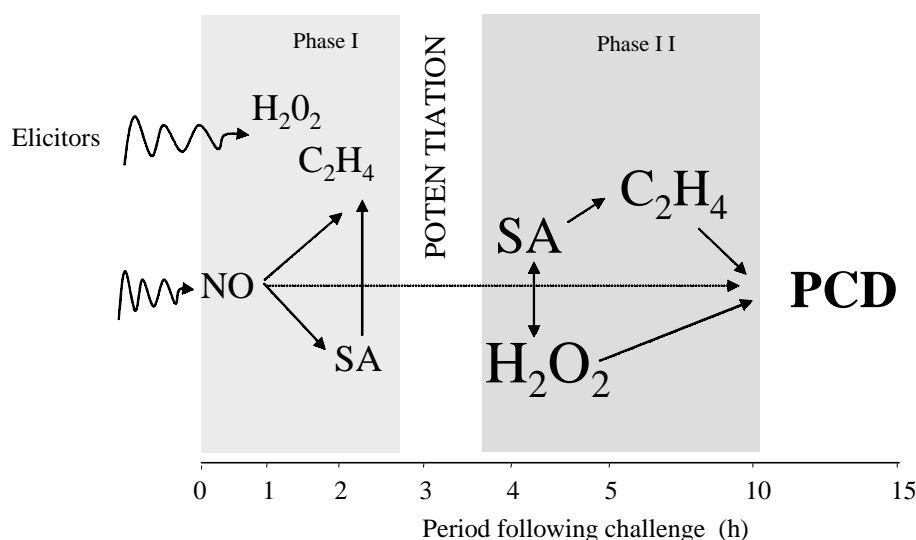

Fig. 6: Proposed model for biphasic events during the hypersensitive response. Based on data presented within this paper and elsewhere (Draper, 1997, Shirasu et al., 1997) the classical model for biphasic control of the hypersensitive response (see Fig. 1 and Lamb and Dixon, 1997) is reinterpreted. Two types of recognition of avirulence gene products (AVR) and of as yet uncharacterised non-AVR elicitors occurs within the first hour of pathogenic interaction. Non-AVR elicitation results in the transient (phase I) accumulation of H_2O_2, salicylic acid (SA) and C_2H_4. Hrp-dependent AVR-elicitation induces $^{\bullet}$NO production which contributes to the initiation of C_2H_4 and, with H_2O_2, (not shown) of SA. Rapid NO production initiates a potentiation mechanism whereby a second persistent phase is initiated. The potentiated phase II results in the augmented production of C_2H_4, H_2O_2 and other reactive oxygen intermediates for instance O_2^- which interact with $^{\bullet}$NO to produce the potent oxidant $ONOO^-$; all of which have been associated with the initiation of programmed cell death (PCD, Levine et al., 1994; Jabs et al., 1997, Beligni and Lamattina, 1999; de Jong et al., 2001). In the absence of AVR elicitation (as with disease forming pathogens) weaker $^{.}$NO production results in a reduced production of cell death initiating signals during a poor phase II. *Hrp* – mutants fail to elicit significant NO production and therefore do not exhibit a phase II response.

associated with the activation, de-activation and then the reactivation of the mono-meric GTP-binding protein (MGBP), p21^{ras}. In this system the biphasic pattern allows switching between MAPkinase (ERK) and phosphatidylinositol-3-kinase activity (Foschi et al., 1997). Intriguingly, biphasic rises in both MGBP and MAPkinase activities have been noted in pea epicotyls following treatment with ethylene (Moshkov et al., 2002a, b) and so a similar mechanism could also feature in plants. Ultimately, the influence of SA and ˙NO on such signalling components needs to be assessed, particularly how they could influence ROI generation by a NADPH oxidase complex (Mehdy, 1994) or ethylene production via ACC synthase (Bleecker and Kende, 2000).

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