

Nitric Oxide Interacts with Salicylate to Regulate Biphasic Ethylene Production during the Hypersensitive Response^{1[W]}

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C₂H₄ is associated with plant defense, but its role during the hypersensitive response (HR) remains largely uncharacterized. C₂H₄ production in tobacco (*Nicotiana tabacum*) following inoculation with HR-eliciting *Pseudomonas syringae* pathovars measured by laser photoacoustic detection was biphasic. A first transient rise (C₂H₄-I) occurred 1 to 4 h following inoculation with HR-eliciting, disease-forming, and nonpathogenic strains and also with flagellin (flg22). A second (avirulence-dependent) rise, at approximately 6 h (C₂H₄-II), was only seen with HR-eliciting strains. Tobacco leaves treated with the C₂H₄ biosynthesis inhibitor, aminoethoxyvinylglycine, suggested that C₂H₄ influenced the kinetics of a HR. Challenging salicylate hydroxylase-expressing tobacco lines and tissues exhibiting systemic acquired resistance suggested that C₂H₄ production was influenced by salicylic acid (SA). Disrupted expression of a C₂H₄ biosynthesis gene in salicylate hydroxylase tobacco plants implicated transcriptional control as a mechanism through which SA regulates C₂H₄ production. Treating leaves to increase oxidative stress or injecting with SA initiated monophasic C₂H₄ generation, but the nitric oxide (NO) donor sodium nitroprusside initiated biphasic rises. To test whether NO influenced biphasic C₂H₄ production during the HR, the NO synthase inhibitor N^G-nitro-L-arginine methyl ester was coinoculated with the avirulent strain of *P. syringae* pv *phaseolicola* into tobacco leaves. The first transient C₂H₄ rise appeared to be unaffected by N^G-nitro-L-arginine methyl ester, but the second rise was reduced. These data suggest that NO and SA are required to generate the biphasic pattern of C₂H₄ production during the HR and may influence the kinetics of HR formation.

Resistance to pathogens is often associated with localized cell death, the hypersensitive response (HR). The HR is initiated following host recognition of the pathogen-encoded avirulence (*avr*) gene product by a plant resistance (*R*) gene (Martin et al., 2003). In bacterial pathogens, AVR proteins are *R* gene product recognized members of a population of virulence effectors that are delivered into the plant via a hrp-pilus, a type III secretion system (TTSS). However, in the absence of an interacting *R* gene, this AVR protein will act as a virulence effector (Alfano and Collmer, 2004). Other non-AVR elicitors act on basal resistance mechanisms to influence plant defense, which can be suppressed by TTSS-delivered effectors to establish disease (Kim et al., 2005). Some non-TTSS-delivered

elicitors appear analogous to pathogen-associated molecular patterns (PAMPs; Parker, 2003; also as microbial-associated molecular patterns; Ausubel, 2005) and include flagellin (flg22; Zipfel et al., 2004) and lipopolysaccharide (Zeidler et al., 2004).

The HR is initiated and regulated by calcium (Grant et al., 2000) and reactive oxygen species (ROS)—mainly the superoxide anion and H₂O₂ (Lamb and Dixon, 1997) and nitric oxide (NO; Delledonne et al., 1998). NO has been shown to activate proteases that appear to contribute to a HR-type cell death (Clarke et al., 2000; Belenghi et al., 2003), most likely by interacting with ROS-associated signals (Delledonne et al., 2001) as well as inducing defense gene expression (Grun et al., 2006).

The HR is influenced by the interaction of PAMP and AVR elicitors. This has been classically described within the context of the biphasic generation of H₂O₂ during the pathogen-elicited oxidative burst (Lamb and Dixon, 1997). Here, there is an initial transient rise in H₂O₂ (H₂O₂-I), induced by non-AVR elicitors, followed by a more persistent AVR-dependent rise in H₂O₂ (H₂O₂-II) some hours later. The kinetics and amplitude of H₂O₂-II influences the rate of HR cell death and, hence, the effectiveness of the associated defenses (Shirasu et al., 1997; Mur et al., 2000). Similar elicitation events and biphasic kinetics have also been noted for pathogen-elicited calcium fluxes (Grant

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et al., 2000). The synthesis of salicylic acid (SA) may be initiated by oxidative stress (Chamngpol et al., 1998) and it may be that H₂O₂-I-initiated SA synthesis augments H₂O₂-II.

Ethylene has many roles in plant physiology and its biosynthesis and associated signaling have been extensively characterized (Bleecker and Kende, 2000). The biosynthetic pathway involves the conversion of *S*-adenosylmethione to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and then to ethylene by ACC oxidase. Ethylene production in response to pathogens has been stated as being rapid (<4 h; Ecker and Davis, 1987), but only in treatments with isolated defense elicitors (Tong et al., 1986; Bailey et al., 1991; Kenyon and Turner, 1992). In response to pathogens, increased ethylene production was not observed until at least 24 h following challenge (Lund et al., 1998; Penninckx et al., 1998; Chen et al., 2003; O'Donnell et al., 2003).

Ethylene has diverse roles in plant defense, mostly associated with resistance to pathogens that adopt a necrotrophic lifestyle (Thomma et al., 1999; Norman-Setterblad et al., 2000; Berrocal-Lobo et al., 2002). Ethylene-mediated resistance can be exhibited through the induction of antimicrobial pathogenesis-related protein genes (van Loon et al., 2004), Hyp-rich protein genes (Ecker and Davis, 1987), and genes encoding key enzymes in the phenylpropanoid pathway leading to the production of antimicrobial phytoalexin compounds (Dixon et al., 2002). Ethylene has been shown to initiate cell death through the initiation of ROS generation and proteolytic enzyme activation (de Jong et al., 2002); however, analysis of a HR forming in the ethylene insensitive *ein2* mutant in *Arabidopsis* (*Arabidopsis thaliana*) has suggested that ethylene plays, at best, a minor role in this type of cell death (Bent et al., 1992; Ciardi et al., 2000). Conversely, with many biotrophic (or partly biotrophic) pathogens, ethylene is a virulence factor. Host ethylene production is required for the full virulence of *P. syringae* pv (*P. s.* pv) *glycinea*, *P. s.* pv *tomato*, *Xanthomonas campestris* pv *vesicatoria*, *Verticillium dahlia*, and *Cucumber mosaic virus* on their hosts (Lund et al., 1998; van Loon et al., 2006). Some strains of *P. syringae* and *X. campestris* pathovars derive their own ethylene to serve a virulence function. Within these pathogens, ethylene is not synthesized from ACC, but from 2-oxoglutarate, by an ethylene-forming enzyme (EFE; Weingart and Volksch, 1997). The roles of ethylene in plant defense are therefore context specific, reflecting differing types of pathogen challenge and differing interactions with other defense signals.

We here extend our previous investigations on signal production during the nonhost HR elicited by *P. s.* pv *phaseolicola* (*PspH*) in tobacco (*Nicotiana tabacum*; Kenton et al., 1999; Mur et al., 2000, 2005a, 2005b) to describe the kinetics of ethylene production during interaction with various *P. syringae* pathovars, using laser photoacoustic detection (LPAD). These revealed biphasic patterns of ethylene production reflecting

elicitation by PAMP and AVR elicitors in a manner reminiscent of biphasic H₂O₂ and calcium production (Lamb and Dixon, 1997; Grant et al., 2000). Further characterization suggested that the biphasic pattern of ethylene production could arise through the interaction of NO with SA and possibly H₂O₂.

RESULTS

Biphasic Ethylene Production in Tobacco in Response to Avirulent Bacterial Pathogens Contributes to the HR

Publicly held transcriptomic data suggest that genes encoding ethylene biosynthetic enzymes were up-regulated in *Arabidopsis* following challenge with avirulent bacteria (Supplemental Fig. S1). To investigate this further, we sought to exploit LPAD to measure online ethylene production in a tobacco-based pathosystem as the larger tobacco leaves offer a readily inoculable target tissue.

Examining ethylene production in tobacco leaves following inoculation with *PspH* strain 1448A revealed a biphasic pattern of ethylene production (Fig. 1A; Supplemental Table S1). The first rise in ethylene production (designated C₂H₄-I) appeared to be peak at about 2 h before declining. The second increase in ethylene production (designated C₂H₄-II) occurred 6 to 8 h after inoculation (hai) and persisted until at least 14 hai. *P. s.* pv *tabaci* (*Pt*) causes wild-fire disease symptoms in tobacco cv Samsun NN and elicited only a single peak in ethylene production, which corresponded closely in amplitude and timing to the C₂H₄-I seen when inoculating with *PspH*. A similar, single peak was measured when inoculating with a *hrpL* mutant (*HrpL* regulates expression of many genes involved in the *Hrp*/TTSS protein secretion machinery; Fouts et al., 2002) of *PspH*, which fails to elicit a HR. C₂H₄ production following inoculations with the *flg22*, which would be present in the *PspH hrpL* strain, was examined (Fig. 1B). Inoculating with various concentrations of *flg22* initiated monophasic C₂H₄ production, which corresponded in timing to C₂H₄-I seen with the HR (Fig. 1A).

To investigate whether C₂H₄-II was specific to the HR, a *Pt* derivative into which the avirulence gene *avrRpm1* had been introduced was generated. Several features of inoculation with *Pt avrRpm1* suggested a HR was being elicited. Lesions formed when inoculating with *Pt avrRpm1* lacked the chlorotic wild-fire symptoms seen with *Pt* (Fig. 2A) and in planta bacterial growth was only observed with populations of *Pt*, but not *Pst avrRpm1* (Fig. 2B). Inoculation of tobacco with *Pt avrRpm1* led to a major second period of production (Fig. 2C), suggesting that C₂H₄-II correlated with recognition of the *avr* gene that did not differ significantly ($P = 0.272$) from that elicited by *PspH* (Supplemental Table S1).

Given the apparent AVR dependence of C₂H₄-II, a possible contribution of C₂H₄ to the HR was investigated. HR tobacco leaves were infiltrated with *PspH*

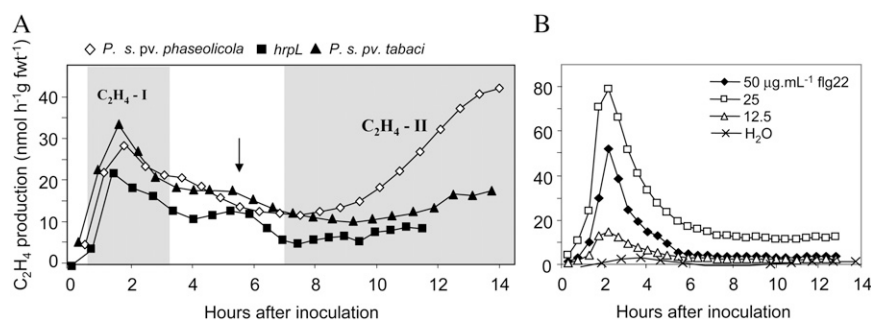


Figure 1. Ethylene production in tobacco following challenge with *P. syringae* pathovars and flagellin. A, C_2H_4 production in tobacco leaves infiltrated with strains of *P. syringae* pathovars as detected using LPAD. The intercellular spaces in tobacco leaves were infiltrated with bacterial suspensions (2×10^6 colony-forming units mL^{-1}) with the avirulent strain *Psp*, (\diamond), the nonpathogenic and non-HR-eliciting derivative *Psp hrpL* (\blacksquare), and the virulent strain *Pt* (\blacktriangle). The two rises associated with challenges with avirulent bacteria are indicated by shading and annotation as C_2H_4 -I and C_2H_4 -II. An increase in ethylene production, which may be elicited by the wounding associated with the injection procedure, is arrowed. B, C_2H_4 production elicited following infiltration with 50 (\blacklozenge), 25 (\square), and 12.5 (\triangle) $\mu g mL^{-1}$ *Psp*, flg22 with water (\times) representing the negative control.

with ACC or an ACS inhibitor, aminoethoxyvinylglycine (AVG). At 6 hai, AVG significantly ($P < 0.01$) decreased, whereas ACC increased ($P < 0.05$), electrolyte leakage elicited by *Psp* (Fig. 2D).

Because the inoculation procedure involved piercing with the syringe needle, the pattern of wound-associated ethylene was determined. Leaves were wounded by piercing with a wire brush and ethylene production increased after approximately 3 h and peaked at approximately 5 h, declining thereafter (Fig. 2E). This may correspond to a subsidiary peak in ethylene production seen at approximately 6 h following inoculation with *Pt avrRpm1* (Fig. 2C, arrow).

Kinetics of Ethylene Production Is Influenced by SA

The biphasic pattern of the oxidative burst is influenced by SA (Shirasu et al., 1997; Mur et al., 2000); hence, the effects of SA on ethylene production were assessed. Different concentrations of SA were injected into tobacco leaves and stimulated a rapid rise in ethylene production, which declined after approximately 2 h (Fig. 3A). To examine the effect of SA on ethylene production within a HR, *Psp* was inoculated into leaves of cauliflower mosaic virus 35S salicylate hydroxylase (SH) transgenic tobacco plants, which degrade SA to catechol. Ethylene production was perturbed in SH tobacco leaves with C_2H_4 -I being reduced and C_2H_4 -II somewhat delayed and reduced in amplitude compared to wild-type plants (Fig. 3B).

SA could influence ethylene production, at least in part, by altering ACS transcription. Hence, ACS expression following *Psp* challenge in wild-type and SH tobacco was investigated by northern blotting. An Arabidopsis gene probe for ASC6 was used because this exhibits the highest homology to the stress-responsive *NtACS2* gene (Lei et al., 2000). *Psp* induced ACS expression 6 hai, apparently peaking at approximately 12 h before reducing by 24 hai (Fig. 3C). In SH

tobacco, the baseline expression of ACS appeared to be greatly reduced and only weak up-regulation was detected at 9 and 12 hai, although expression was much higher at 24 hai.

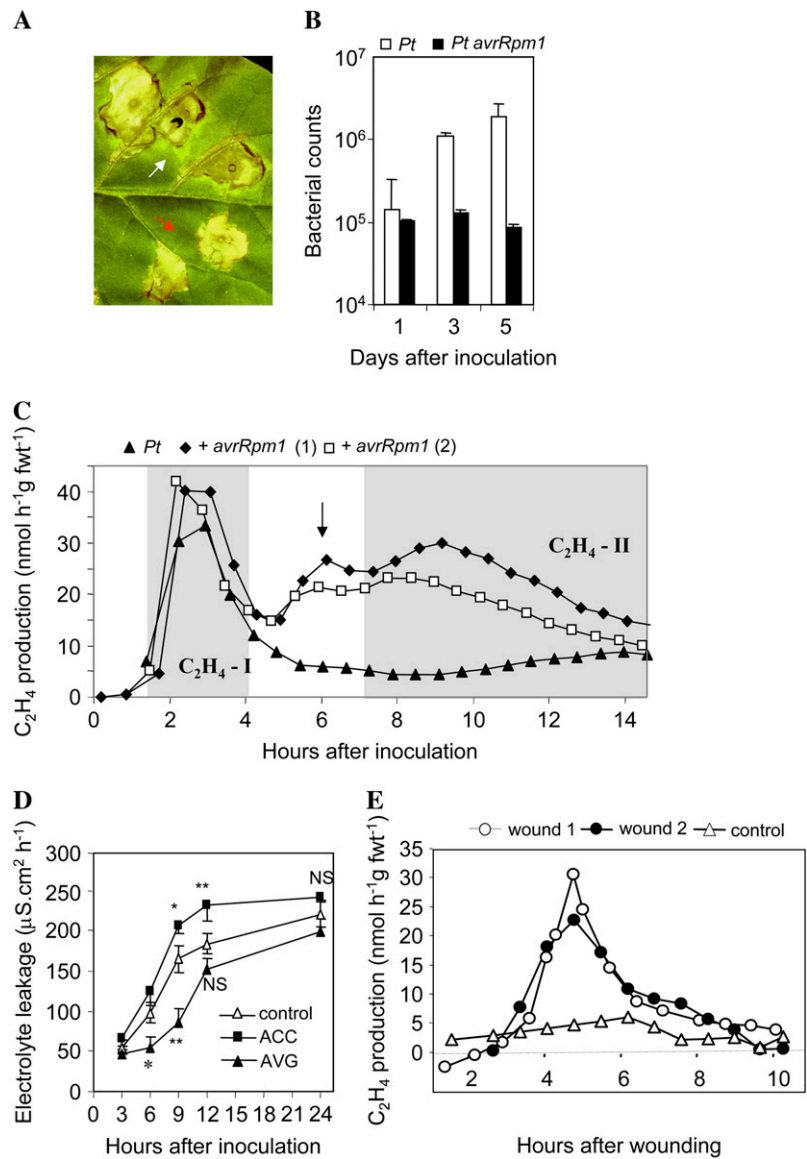
Some workers (e.g. Heck et al., 2003) have cautioned against relying solely on NahG transgenic lines to indicate SA effects. Therefore, ethylene production was examined in leaves exhibiting systemic acquired resistance (SAR), a SA-mediated phenomenon, following inoculation with *Psp*. The lower-most leaves of tobacco were inoculated with *Psp* or mock inoculated with water and then maintained under controlled environmental conditions for 5 d after inoculation (dai). *Psp*-inoculated tobacco exhibits SAR after 3 dai (Mur et al., 1996); hence, at 5 dai, single upper leaves of SAR-exhibiting tobacco plants and water-inoculated controls were injected with *Psp*. In *Psp* inoculation of SAR-exhibiting tissue, C_2H_4 -I was significantly ($P < 0.001$) augmented compared to *Psp* inoculation of control plants. C_2H_4 -II in SAR tissue was not significantly ($P = 0.09$) different from controls (Fig. 3D; Supplemental Table S2).

SA can act by influencing the generation of ROS to which C_2H_4 could act to augment (de Jong et al., 2002) or increase production in response to oxidative stress (Supplemental Fig. S1), likely in a positive feedback loop. Injections of either 1 mM methyl viologen or Glc:Glc oxidase (G:GO) elevated ethylene production (Supplemental Fig. S2). Hence, the observed patterns of ethylene production are likely to be linked to the well-established H_2O_2 -SA interaction during defense (Lamb and Dixon, 1997).

NO Cooperates with SA to Initiate C_2H_4 -II Ethylene Production during a *Psp*-Elicited HR in Tobacco

Along with SA and the oxidative burst, the generation of NO is also a feature of the HR. To investigate the effects of NO on ethylene production, various

Figure 2. The contribution of ethylene to the *Psp*-elicited HR in tobacco. A, Lesion phenotypes at 7 d following inoculation with *Pt* (white arrow; note, chlorotic halo) and a derived *Pt avrRpm1* transconjugant (red arrow; note lack of chlorotic halo). B, Bacterial populations of *Pt* (□) and *Pt avrRpm1* (■) within 1-cm-diameter cores within infiltrated leaf areas of tobacco. Data are given as mean colony-forming units ($n = 5 \pm \text{SE}$). The black arrow indicates a subsidiary increase in ethylene production that may be due to wounding. See main text for details. C, Ethylene production in tobacco leaves infiltrated with *Pt* (▲) and *Pt avrRpm1*, for which the results of two replicates (+ *avrRpm1*, 1 [◆] and + *avrRpm1*, 2 [□]) are presented. The two rises associated with challenges with avirulent bacteria are indicated by shading and annotation as C₂H₄-I and C₂H₄-II. D, Electrolyte leakage from explants of tobacco leaves inoculated with either only the avirulent strain *Psp* (△) or with either 0.1 mM ACC (■) or 0.1 mM AVG (▲). E, Ethylene production from tobacco leaves that had been multiply wounded by being struck with a wire brush. Data from two wounded leaves are given (wound 1, ○; wound 2, ●) and from a detached leaf that had not been wounded with the wire brush (△).



concentrations of the NO⁺ donor, sodium nitroprusside (SNP), were inoculated into tobacco leaves (Fig. 4A). Notably, injections of SNP, especially 1 and 0.5 mM SNP, induced a biphasic ethylene generation pattern. Injecting NO-exhausted solutions of SNP did not initiate ethylene production (data not shown). SNP was observed to up-regulate ACS expression, suggesting that, at least in part, increased ethylene production reflected up-regulation of biosynthetic genes (Fig. 4B). NO initiates SA synthesis (Durner et al., 1998) and SNP increased levels of SA accumulation at 24 h (data not shown). When injecting SNP into SH tobacco, C₂H₄-I was unaffected, but C₂H₄-II was clearly perturbed compared to SNP injected into wild-type tobacco controls (Fig. 4C). Given that NO generation from SNP will be unaffected by the SH transgene, we hypothesized that, during the HR, NO could influence the biphasic pattern of ethylene production through a SA-

independent mechanism during C₂H₄-I and an SA-dependent mechanism during C₂H₄-II.

NO Production during the *Psp*-Elicited HR in Tobacco Influences C₂H₄-II

The effects of NO generation during the *Psp*-elicited HR on ethylene production were tested using the mammalian NO synthase (NOS) inhibitor N^G-nitro-L-Arg methyl ester (L-NAME). This has proven to effectively suppress *Psp*-elicited NO generation, whereas the stereoisomer D-NAME had no detectable effect (Mur et al., 2005b). Addition of 1 mM L/D-NAME had no effect on the growth of *Psp* nutrient broth cultures and therefore was judged not to directly affect the bacteria (data not shown). When L-NAME was co-infiltrated with *Psp*, C₂H₄-I was not affected; however, C₂H₄-II was greatly suppressed compared to

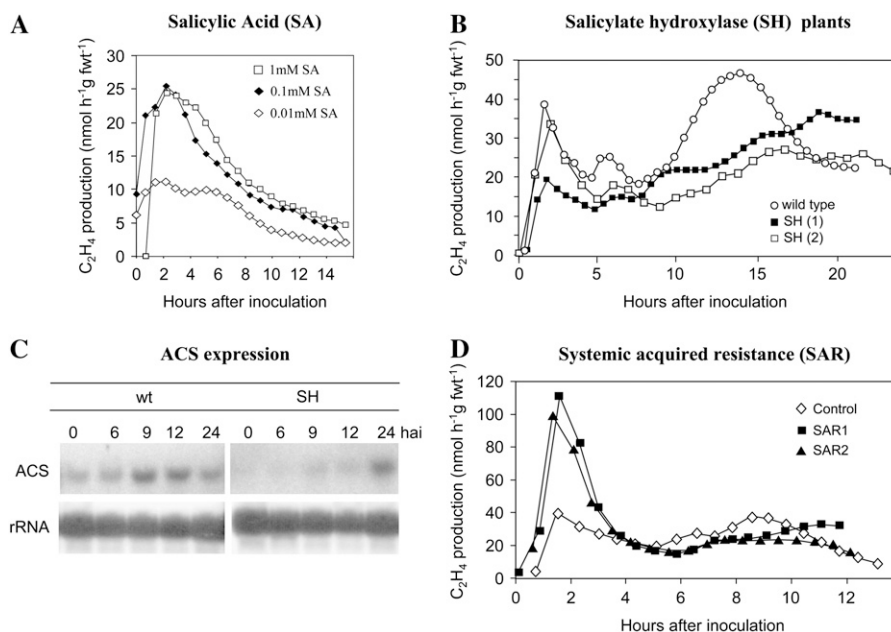


Figure 3. The influence of SA on *PspH*-elicited ethylene production and ACS expression in tobacco. A, Ethylene production in leaves of wild-type cv Samsun NN tobacco leaves injected with 0.01 (\diamond), 0.1 (\blacklozenge), and 1 (\square) mM SA. B, Ethylene produced from wild type (\circ) and two replicates of SH (35S-SH, 1 [\blacksquare]; 35S-SH, 2 [\square]) expressing tobacco plants challenged with *PspH* (C). Expression of ACS in tobacco at different hai of wild type and SH tobacco with *PspH* as detected using northern hybridization. A gene probe for the Arabidopsis ACS6 (At4g11280) was used as this exhibited high homology to stress-activated tobacco *NtACS2*. D, The lower-most leaves of tobacco plants were inoculated with *PspH* at several points over the leaf to form a patchwork of HR lesions. In control plants, the lower leaves were inoculated with water. After 7 d, the upper leaves of all plants were inoculated with *PspH* and ethylene production was measured. Results are given from two leaves from plants which had been first inoculated with *PspH* (SAR1, \blacksquare ; SAR2, \blacktriangle) and leaf from a plant which had been first injected with water (control, \diamond). Some of the data presented in Figure 3A have been published in conference proceedings (Mur et al., 2003).

D-NAME-treated controls (Fig. 5A). To investigate whether only early NO generation was important for C_2H_4 -II control, either L-NAME or D-NAME was injected into *PspH*-challenged tobacco leaves after C_2H_4 -I (Fig. 5B) or just before the first signs of increased ethylene production linked to C_2H_4 -II (Fig. 5C). When injecting with L-NAME at either time point, *PspH*-elicited C_2H_4 -II was suppressed, indicating that contemporaneous NO generation was required for biphasic ethylene production. However, C_2H_4 -II was more effectively suppressed when L-NAME was coinjected with *PspH* (Fig. 5A) or just after C_2H_4 -I (Fig. 5B). Indeed, application of L-NAME appeared to only delay the C_2H_4 -II rise (Fig. 5C). These series of experiments were repeated with the NO scavenger, CPTIO, which yielded similar data except that the extent of suppression was not as great as seen with L-NAME, most likely due to the photolability of CPTIO (Supplemental Table S2). Analysis of the L-NAME-perturbed patterns of ethylene production suggested that this reduced the effects of AVR recognition (Supplemental Fig. S3).

DISCUSSION

The roles of ethylene in the HR remain somewhat obscure. Different groups have noted normal HR

formation in Arabidopsis (Bent et al., 1992) and tomato (*Solanum lycopersicum*; Hoffman et al., 1999; Ciardi et al., 2000) lines where ethylene signaling was perturbed. However, HR-elicited ethylene production has also been frequently noted (Knoester et al., 1995; Lasserre et al., 1997) and tobacco mosaic virus-elicited HR lesion formation was delayed in ethylene-insensitive tobacco plants (Knoester et al., 2001). We noted that the kinetics of *PspH*-elicited HR, as revealed by electrolyte leakage, could be modulated by either adding the C_2H_4 precursor ACC or the ethylene biosynthesis inhibitor AVG (Fig. 2D).

To substantiate its link with *PspH*-elicited HR in tobacco, we determined C_2H_4 production following whole-leaf inoculation with bacterial suspensions. LPAD is a particularly appropriate method to do this because it allows online in planta measurements. LPAD indicated that C_2H_4 production from tobacco leaves challenged with avirulent *PspH* bacteria conformed to two main phases. This pattern was reminiscent of the biphasic oxidative burst and appeared to reflect similar elicitory steps (Lamb and Dixon, 1997). The first rise (C_2H_4 -I) was common to leaves inoculated with avirulent (*PspH*, *Pt avrRpm1*), virulent (*Pt*), and *hrp*-compromised strains (Figs. 1A and 2C), implying a non-AVR elicitation event. Indeed, the PAMP-flg22 was an effective initiator of ethylene production,

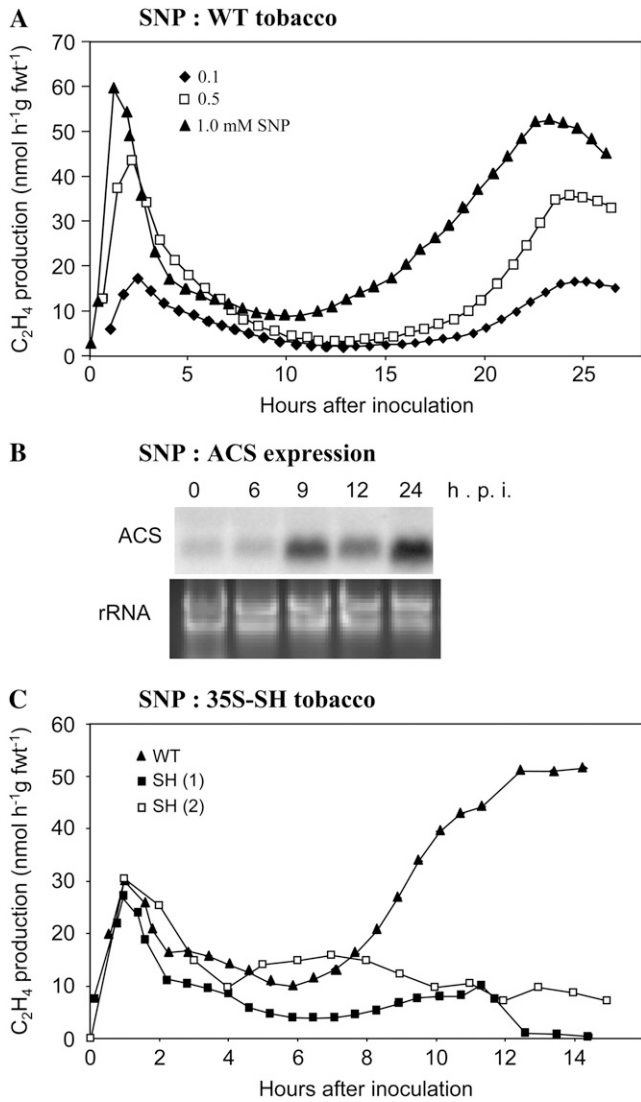


Figure 4. SNP-elicited ethylene production in tobacco leaves. A, Ethylene production was measured using LPAD from tobacco leaves following injection with 0.1 (◆); 0.5 (□), and 1 (▲) mM SNP. Inset, *NtACS* expression in tobacco following the application of SNP. B, Expression of ACS in tobacco at different hours postinjection of wild-type tobacco with 1 mM SNP. A gene probe for the Arabidopsis ACS6 (At4g11280) was used because this exhibited high homology to stress-activated tobacco *NtACS2*. C, Ethylene generation in wild-type Samsun (▲) and in two replicate leaves (SH, 1 [■] and SH, 2 [□]) at various hours after 1 mM SNP. Some of the data presented in Figure 5A have been published in conference proceedings (Mur et al., 2003).

which was similar in its kinetics to C_2H_4 -I (Figs. 1A and 2C; Supplemental Fig. S4). Another prediction of the biphasic model is that the second rise is AVR dependent. Data to support this were obtained from the C_2H_4 -II observed with a *Pt avrRpm1* transconjugant strain, but was not detected with the parental *Pt* strain (Fig. 2C).

It is possible that the bacteria contributed to the observed ethylene production. In *P. syringae* patho-

vars, ethylene can be produced by EFE acting on 2-oxoglutarate (Fukuda et al., 1993). With certain *P. s. pv glycinea* interactions with soybean (*Glycine max*), the majority of ethylene is derived from the pathogen and acts as a virulence factor, contributing to symptom development (Weingart et al., 2001). However, mutation of the *Psph efe* gene did not affect virulence and, more pertinently, screens of bean-adapted *Psph* strains failed to find evidence of ethylene production (Weingart and Volksch, 1997). Further, there is no *efe*-annotated open reading frame in the *Psph* 1448A genome sequence (<http://pseudomonas-syringae.org>); hence, it is probable that the contribution of *Psph* to the

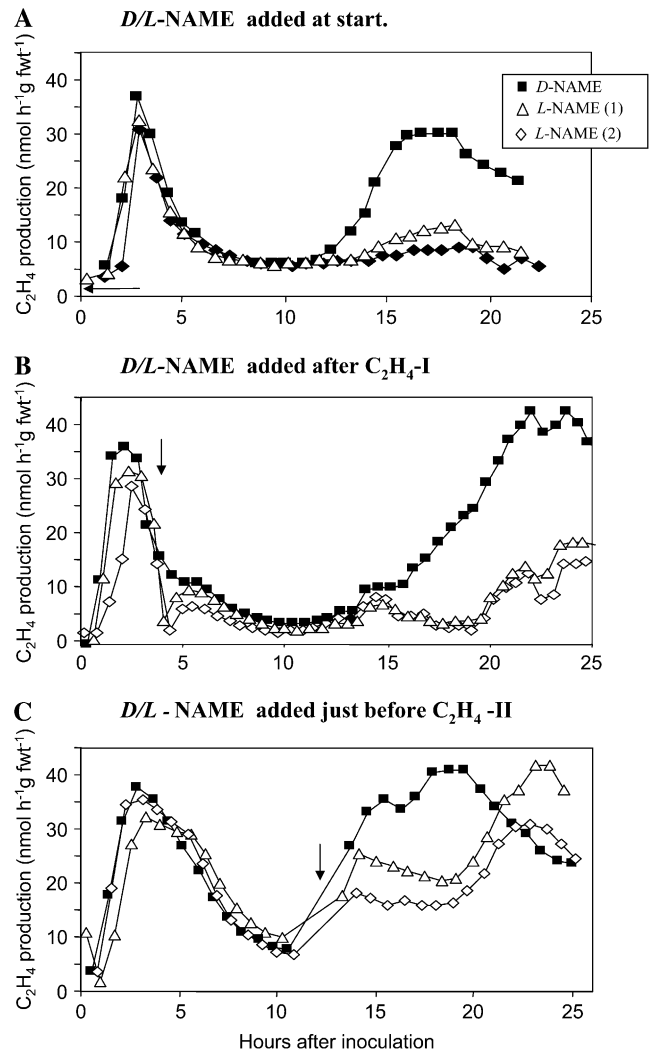


Figure 5. The effects of *D/L*-NAME on ethylene production elicited in tobacco following challenge with *Psph*. The leaves of wild-type tobacco leaves were inoculated with the avirulent *Psph* strain and the ethylene production measured by LPAD. The effects of adding 1 mM *D*-NAME (■) or in two replicates 1 mM *L*-NAME, 1 [△]; *L*-NAME, 2 [◇]) at first injection with *Psph* (A), just after the first transient peak in ethylene generation (C_2H_4 -I; B), or at the first sign of the second rise in production (C_2H_4 -II; C). The times of injection with either *L*- or *D*-NAME are indicated with arrows.

observed ethylene production was likely to be negligible. The paucity of *Pt* genomic sequence data means that ethylene production by this species cannot be ruled out, although Weingart and Volksch (1997) found no evidence of in vitro ethylene production in the four strains of *Pt* that they screened.

A feature of the biphasic oxidative burst is its modulation by SA (Shirasu et al., 1997; Mur et al., 2000); hence, an important priority was to establish how far SA also influenced biphasic ethylene production. SH-expressing transgenic plants have been used to show that ozone and *X. campestris* pv *campestris*-elicited ethylene production was influenced by SA (Rao et al., 2002; O'Donnell et al., 2003). Our experiments using SH transgenic plants demonstrated that *Psp*-elicited C₂H₄-II was influenced by SA (Fig. 3B). The link with SA was substantiated when *Psp*-elicited C₂H₄-I was potentiated in SAR-exhibiting tissue (Fig. 3D).

Pathogen-challenge is perhaps too complicated a stimulus to allow the signal interactions influencing C₂H₄-I and C₂H₄-II to be readily deduced. Hence, the effect of adding various defense signals on ethylene production in tobacco leaves was assessed. H₂O₂ has been proposed to orchestrate plant defense (Levine et al., 1994) and could initiate the biosynthesis of ethylene. Equally, ethylene has been shown to be required for the initiation of oxidative signaling in stomata (Desikan et al., 2006) and in tomato suspension cells (de Jong et al., 2002). Such data could suggest the existence of a positive feedback loop based on H₂O₂ and ethylene that could explain the observed biphasic generation pattern. Some evidence for this was provided by Moeder et al. (2002), who noted that ozone induced biphasic expression patterns in tomato ACS gene expression, with the second increase being dependent on previous ethylene production. In this study, G:GO was injected into the apoplast to generate H₂O₂ at levels that we had previously shown to be an effective initiator of plant defense (Mur et al., 2005a). G:GO only produced a single burst of ethylene production (Supplemental Fig. S2) in marked contrast to ethylene production following injection of SNP, an NO⁺ donor that is a potent nitrosylating agent, but subsequently releases gaseous NO following electrophilic attack (Membrillo-Hernandez et al., 1998), which we have previously measured (Mur et al., 2006). Crucially, addition of SNP resulted in biphasic patterns of ethylene biosynthesis (Fig. 4A) and, just as during the HR, the second rise in ethylene was perturbed in SH transgenic tobacco plants. As SNP induced SA biosynthesis, it could be that SA alone was required to initiate the biphasic pattern, but when this was injected into plants, only a single burst of C₂H₄ biosynthesis was initiated (Fig. 3A). Given that NO generation would be unaffected by the SH transgene, we interpret these data as suggesting that NO is required for the biphasic switch, but needed SA to set its kinetics. To substantiate this hypothesis, when L-NAME was added to developing HR lesions, only

C₂H₄-II was affected (Fig. 5). Intriguingly, when plotting C₂H₄-I/ C₂H₄-II height and area ratios, infiltration with L-NAME shifted ethylene biosynthetic patterns toward interactions where there is no AVR recognition (Supplemental Fig. S3). This experiment assumed that L-NAME primarily affected plant-derived NO. Whereas no gene has been annotated as a NOS in the *Psp* strain 1448A genome, two are to be found within *Psp* B728a (P_{sy} 1024, P_{sy} 3724), which have orthologs in *Psp* (P_{sp} 1075; P_{sp} 3724; <http://pseudomonas-syringae.org>). Hence, although we have never noted NO production from *Psp* cultures, some in planta generation cannot be ruled out.

Psp-elicited C₂H₄-I appeared not to be affected by L-NAME (Fig. 5A), which was surprising given that SNP could initiate biphasic ethylene biosynthesis (Fig. 4A). It may be that C₂H₄-I is mostly regulated by H₂O₂ or linked signals (Supplemental Fig. S3) so that there is functional redundancy in an NO role in initiating C₂H₄-I. It is notable that timing (hai) if not amplitude of pathogen-elicited C₂H₄-I was similar and could be partially replicated by both SNP and G:GO (Supplemental Fig. S4). This could explain why C₂H₄-I is generated by the *Psp* *hrpL* mutant (but not C₂H₄-II) when this strain elicits negligible levels of NO (Mur et al., 2006) but a normal H₂O₂-I (Lamb and Dixon, 1997). Because AVG suppressed cell death at 6 h (Fig. 2D; i.e. prior to C₂H₄-II), this would implicate C₂H₄-I as a contributor to HR cell death. A contribution by H₂O₂ to C₂H₄-I to help drive C₂H₄-II could explain the latter's relative delay when elicited by SNP (where NO and SA are produced) compared to avirulent bacteria (where NO, SA, and ROS are produced). It seems likely that, as with the oxidative burst (Shirasu et al., 1997), an early transient rise influences the second peak in biphasic patterns of signal generation.

Our hypothesis is apparently at odds with the literature, which suggests an inverse relationship between NO and ethylene production during senescence (Leshem, 2000) and, further, in NO-deficient NO dioxygenase Arabidopsis plants. ACS6 expression was suppressed and senescence was delayed (Mishina et al., 2007). However, our data need not contradict such observations. Our measurements of NO generation from SNP show consistent production over at least a 24-h period (Mur et al., 2005). During this period, ethylene levels both increase and decrease, perhaps suggesting that NO has both initiatory and suppressive roles. It must also be acknowledged that the HR and senescence are very different phenomena. It seems likely that the outcomes of NO and ethylene interactions vary temporally and in different contexts.

A major task for future studies is to integrate PAMP/AVR elicitory events on ethylene production during a HR into a coherent regulatory pattern. Taking our data together, H₂O₂-I, SA, and possibly NO are likely to contribute to the generation of C₂H₄-I. It has been suggested that the second phase is initiated at AVR recognition (Draper, 1997); however, we alternatively suggest that this represents the triggering of a

biphasic switch, as a result of a high level of NO generation (Mur et al., 2005b, 2006). Such a model could explain the biphasic waves observed in the absence of combinations of bacterial elicitors. For example, fumigation with ozone alone proved to be sufficient to trigger a biphasic oxidative burst (Schraudner et al., 1998) and, in line with our hypothesis, ozone has been shown to elicit a rapid rise in NO (Ederli et al., 2006). Further, it is possible to extrapolate from biphasic transcription of ethylene biosynthetic genes in tomato following treatment with ozone (Moeder et al., 2002) to suggest that a similar mechanism occurs during HR. Our data (Figs. 3C and 4C) also suggest that NO/SA influences the transcription of ACS; hence, the biphasic switch could arise from defined transcriptional events. More defined expression studies are required than carried out in this study to substantiate this hypothesis. The importance of the biphasic switch lies in its vital role in reiterating events occurring during the first phase, but in a more potent manner, and hence aids in conferring resistance. Applying this model to SAR tissue, the preexistence of SA would lead to the potentiated first phase seen in Figure 3D. It is currently unclear why a potentiated C₂H₄-II should not also feature during SAR, but it may be that this is already at maximal levels. Validation of such a model undoubtedly requires testing using various Arabidopsis mutants, but such interactions seem appropriate for further data mining mathematical modeling so that greater understanding of the actions of ethylene can be obtained.

MATERIALS AND METHODS

Plant Growth and Chemicals

Tobacco (*Nicotiana tabacum* 'Samsun NN') was germinated in Levingtons Universal Compost (Levingtons Horticulture) and transferred to John Innes Number 2 compost after 2 weeks. Tobacco plants were injected with bacterial suspensions or chemicals at 5 to 6 weeks following germination. All plants were grown at 22°C under a 16-h photoperiod. Solutions of SNP (Sigma-Aldrich Company Ltd.) where NO had been exhausted (Na₂ [Fe (CN)₅ NO] + *hν* → [Fe (CN)₅]³⁻ + ·NO) were generated by incubation under light for >48 h.

Phytopathogenic Bacteria Strains

PspH race 6 strain 1448 elicits a nonhost HR on tobacco, a trait that is abolished in the *hrpL* mutant derivative (Rahme et al., 1991). *Pt* strain 11528R forms disease symptoms on tobacco (Thilmony et al., 1995). The *avrRpm1* avirulence gene, cloned on pVSP61B (Bisgrove et al., 1994), was introduced into *Pt* strain 11528R by triparental mating as described by Dangl et al. (1992).

Each pathogen was grown at 28°C in nutrient agar (Oxoid Limited). The culture was washed twice with sterile distilled water and finally diluted to 10⁶ colony-forming units mL⁻¹ based on spectrophotometric readings (Mur et al., 2000). The resulting bacterial suspensions were injected into the intercellular spaces of the entire leaf using a 5-mL syringe (Asahi Techno Glass) with a 2.5-gauge 5/8 needle (Microlance; Becton-Dickinson & Co. Ltd.).

Northern Hybridization

RNA extraction, northern blotting, and hybridization were undertaken as described in Draper et al. (1988). A 1.2-kb probe for the Arabidopsis (*Arabidopsis thaliana*) ACS6 (At4g11280) using the specific primers 5'-AAATCAACTTGATAGTCG-3' and 5'-TCTGTTTAGCTAATCCCGGC-3' had been previously generated (David Chrimes, Aberystwyth, UK) and exhibited the

highest homology (E-value 2×10^{-8}) to stress-activated NtACS2 (Lei et al., 2000), which was used to suggest tobacco ACS transcript accumulation. Each northern hybridization experiment was undertaken at least twice, yielding similar results.

Estimations of Cell Death by Electrolyte Leakage

Changes in the conductivity of the solution bathing 1-cm-diameter leaf explants were determined as stated in Mur et al. (2000). Significance testing employed ANOVA, using MiniTab version 13.

LPAD

Ethylene production was monitored in real time by LPAD, basically as described by Cristescu et al. (2002). Briefly, a line-tunable CO₂ laser emits 9- to 11-μm infrared light into a photoacoustic cell. A line with carrier gas (scrubbed air) passed through the cuvette with the infected tobacco leaves into the photoacoustic cell. The evolved gases in the air flow were detected via their absorption of rapidly chopped infrared light, which generated pressure variations, resulting in acoustic energy detected by a miniature microphone (Bijnen et al., 1996). The amplitude of the acoustic waves is directly proportional to the concentration of ethylene in the photoacoustic cell. Ethylene gas mixtures are sensitively measured by the laser-based ethylene detector due to the distinct fingerprint-like spectrum of ethylene in the CO₂ laser wavelength range (Brewer et al., 1982). A system of valves allows three cuvettes with biological samples to be measured in sequence. Each cuvette was measured for 20 min. When not being measured, the gas flow through the cuvette was maintained, but was vented into the atmosphere rather than passed into the photoacoustic chamber. All data are corrected for weight. Repeated inoculations with water or 10 mM MgCl₂ elicited trivial levels of C₂H₄ (approximately 5 nmol h⁻¹ g⁻¹ fresh weight), which was monophasic (data not shown).

Replication of LPAD Measurements

The photoacoustic system was organized to sample from one of three cuvettes for 20 min before moving on to the next. Hence, each figure gives the results from a single experiment (i.e. three traces), one from each cuvette. Each experiment was repeated at least three times, on separate days and plants, giving similar trends. Although plant/leaf age and the stage of bacterial culture used as an inoculum period was standardized between experiments, the interval between the first and second peaks of ethylene when challenging with identical strains varied between experiments (e.g. compare the results with *PspH* in Figs. 1A, 3B, and 5) when undertaken on separate days. As a result, the data from different experiments could not be pooled if the pattern of ethylene production, which was the major theme of this work, was to be clearly discerned. Other parameters describing features in the patterns of ethylene production were determined using Origin Pro 7 (Origin Lab Corporation) and are given as supplemental data.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of ethylene biosynthetic genes in Arabidopsis following pathogenic challenge or treatment with defense-associated chemicals.

Supplemental Figure S2. Ethylene production initiated by oxidative stress.

Supplemental Figure S3. The effect of suppressing NO levels on the patterns of ethylene production during a HR elicited by *PspH* in tobacco.

Supplemental Figure S4. The timing and maximal rates of ethylene production of C₂H₄-I.

Supplemental Table S1. Peak characteristics of ethylene production in tobacco in response to *P. syringae* pathovars.

Supplemental Table S2. The influence of SA on ethylene production in tobacco in response to *P. syringae*.

Supplemental Table S3. The effect of NO suppression on characteristics of ethylene production in tobacco in response to *PspH*.

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