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Involvement of ethylene in lesion development and systemic acquired resistance in tobacco during the hypersensitive reaction to tobacco mosaic virus

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Different approaches were taken to investigate the significance of ethylene in lesion development and systemic acquired resistance (SAR) in tobacco (*Nicotiana tabacum*) reacting hypersensitively to tobacco mosaic virus (TMV). Gaseous ethylene, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and the ethylene releasing compound ethephon all reduced primary lesion size when applied before or shortly after virus inoculation. Inhibiting TMV-induced ethylene production in primary infected leaves by the inhibitor of ACC-synthase 1-aminoethoxyvinylglycine, the inhibitor of ACC-oxidase cobalt chloride, or the inhibitors of ethylene action silver nitrate and 2,5-norbornadiene (NBD) also reduced lesion expansion. The results support previous findings that exposure of leaves to ethylene or ethylene-releasing compounds prior to inoculation causes an early cessation of lesion growth, whereas ethylene synthesized during lesion development contributes to continued lesion expansion. Local treatment with ethephon-induced systemic resistance, whereas treatment with NBD of a primary TMV-inoculated leaf tended to reduce the extent of SAR attained in both upper and lower leaves. Transgenic plants with modulated ethylene levels obtained through expression of sense or antisense ACC-synthase RNA did not show alterations in primary TMV lesion size or SAR, apparently because ethylene production was not altered sufficiently to affect lesion development. In contrast, the use of ethylene insensitive (Tetr) plants, transformed with a mutant *etr1-1* gene from Arabidopsis, confirmed that virus-induced ethylene promotes lesion expansion and demonstrated that the hormone contributes to the level of SAR attained. In the Tetr plants the SAR response was substantially reduced. The results indicate that in tobacco ethylene perception is involved in lesion expansion, as well as in the generation and/or release of the mobile signal that induces SAR in non-infected plant parts.

Keywords: ethylene; hypersensitive reaction; *Nicotiana tabacum* L.; systemic acquired resistance; tobacco mosaic virus; transgenic plants.

INTRODUCTION

Ethylene is an important signaling component in the reaction of plants to pathogens, but its role in pathogenesis and resistance is far from clear [*1*, *12*, *13*, *41*]. On the one hand, application of ethylene or ethylene releasing compounds can aggravate diseases and some fungi and bacteria can produce ethylene as a pathogenicity factor. On the other hand, ethylene production in infected plants has been associated with defense responses and exogenous application of ethylene or ethylene releasing compounds has been described to promote resistance against some fungal, bacterial and viral diseases. Notably, an early increase in the activity of the rate-limiting enzyme of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate (ACC)-synthase (ACS), leads to a burst of ethylene production concomitant with necrotic lesion formation [*6*, *8*, *24*, *27*, *31*, *35*]. In *N*-gene-containing tobacco the hypersensitive reaction to tobacco mosaic virus (TMV) is associated with consecutive increases in ACS and ACC oxidase (ACO) activities [*8*], as a result of transcriptional activation of the corresponding genes [*16*]. The significance of this increased ethylene production has been investigated by either mimicking the burst

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Abbreviations used in text: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC-oxidase; ACS, ACC-synthase; AVG, 1-aminoethoxyvinylglycine; INA, 2,6-dichloroisonicotinic acid; MS, Murashige and Skoog; NBD, 2,5-norbornadiene; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance; TMV, tobacco mosaic virus.

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of ethylene by exogenous application to non-infected plants, or by applying inhibitors of ethylene synthesis or action to TMV-infected plants. As reported in Pritchard and Ross [29], treating tobacco leaves with as high as 300 ppm ethylene for 40 h induced no necrosis, only moderate chlorosis (A. F. Ross, unpublished data). However, Van Loon [38] described that pricking Samsun NN tobacco leaves with needles moistened with the ethylene releasing compound 2-chloroethylphosphonic acid (ethephon) gave rise to necrotic spots resembling virus-induced local lesions. Treatment with ethephon resulted in the accumulation of pathogenesis-related proteins (PRs) and changes in peroxidase activity and isoenzyme patterns similar to those induced by TMV. Furthermore, upon challenge-inoculation with TMV, lesion expansion was reduced, comparable with the effect of systemic acquired resistance (SAR) resulting from virus infection. No such changes were apparent upon pricking leaves with solutions of the accompanying degradation products of ethephon, hydrochloric acid and phosphoric acid, indicating that ethylene production must be responsible for the effects observed. Similar results have been reported by Ye *et al*. [47]. Moreover, treatment with ethephon-induced systemic resistance in tobacco against the powdery mildew fungus *Erysiphe cichoracearum* [23] and to the late blight-causing oomycete *Phytophthora infestans* in potato [7].

In those experiments the concentration of ethephon required greatly exceeded the amounts of ethylene generated during a hypersensitive reaction. Moreover, the effects of ethylene might have been enhanced synergistically by wound responses resulting from treatments such as pin pricking. Analysis of the role of ethylene in primary TMV-inoculated leaves demonstrated that lesion development was not significantly altered by treatment with the inhibitor of ethylene synthesis, succinate-2-2′-dimethylhydrazide, or the inhibitor of ethylene action, CO_2 [29]. Similarly, the competitive inhibitor of ACS, 1-aminoethoxyvinylglycine (AVG), inhibited the large increase in ethylene production by 95 %, but did not inhibit lesion formation [11]. It could be argued that in this latter case the level of inhibition was insufficient to reduce ethylene production at the site of lesion formation to a level comparable with that in healthy leaves. Indeed, contrasting findings were described recently by Ohtsubo *et al*. [25] indicating that the inhibitors of ethylene biosynthesis, AVG and cobalt chloride, as well as the inhibitor of ethylene action, 2,5-norbornadiene (NBD), significantly suppressed lesion formation in Samsun NN tobacco after a shift from 30 to 20°C. Moreover, lesion formation was accelerated in transgenic plants overexpressing the last enzyme in the ethylene biosynthetic pathway, ACO, upon application of ACC. However, transgenic Samsun NN tobacco plants expressing the mutant ethylene receptor encoding gene *etr1-1* from Arabidopsis, are ethylene insensitive, but still developed necrotic local lesions upon infection with TMV [18].

Collectively, these results suggest that ethylene is not directly responsible for the induction of TMV lesions, but could modulate the response of the plant during a hypersensitive reaction. An enhanced localization of the virus in plants in darkness and in old leaves was associated with a sharp peak in ethylene production near the time of lesion appearance, whereas in continuous light or in young leaves lesion expansion continued and virus-induced ethylene production was delayed [10]. Thus, an early burst in ethylene production appears to be associated with the virus localizing mechanism, and it can be hypothesized that high early ethylene production may stimulate the resistance mechanism. In contrast, ethylene synthesized during lesion development appears to contribute to continued lesion growth rather than restriction of virus movement, because lesions were appreciably smaller in leaves in which the infection-stimulated ethylene was rapidly removed by reduced pressure treatments [29]. Generally, treatments that stimulated ethylene production during lesion development stimulated lesion growth, while those treatments that reduced ethylene levels had the opposite effects. Indeed, ethylene treatment of tobacco leaves prior to inoculation with TMV was reported to inhibit subsequent lesion growth, whereas treatment of inoculated leaves with ethylene during the early stages of lesion development resulted in increased lesion size [33]. Since lesion expansion is a reflection of virus multiplication and spread, any effect of ethylene on the level of resistance can be related directly to lesion size [40]. As concluded by Pritchard and Ross [29], infection-stimulated ethylene may either promote or inhibit lesion development depending upon local concentrations, timing with respect to the infection process, and its interaction with other host-directed responses to infection.

Ethylene might not only have an effect on the size of the primary lesions, but could also affect induction and/or expression of SAR. Pritchard and Ross [29] described that the TMV-induced synthesis of ethylene began earlier in tissues expressing SAR. This earlier onset appears to result from an enhanced capacity to convert ACC to ethylene in systemically-induced leaves. After challenge inoculation of induced leaves ACC did not accumulate, but was immediately converted to ethylene [9]. In contrast to the results with pin pricking reported by Van Loon [38], Brederode *et al*. [3] were unable to induce SAR by spraying leaves of Samsun NN tobacco plants with ethephon. Ethephon strongly induced the mRNAs for the basic PRs, thus indicating that basic PRs are not involved in SAR against TMV. The genes encoding the acidic PRs were also, be it moderately, inducible by ethephon spraying. Therefore, the acidic

PRs do not seem to be involved in resistance against TMV either, as also evidenced by the expression of lesions of normal size in transgenic plants [*21*].

However, ethylene and its perception are required for the expression of resistance against e.g. *Botrytis cinerea* [*36*] and for the rhizobacteria mediated induction of systemic resistance against e.g. *Pseudomonas syringae* pv. *tomato* in Arabidopsis [*28*]. SAR in tobacco against TMV is dependent on salicylic acid (SA), but cross talk between SA and ethylene may occur. SA is known to inhibit ethylene synthesis at least at the level of ACO [*15*]. Conversely, exposure of TMV-inoculated tobacco leaf disks to ethylene (10 μl l^{-1}) resulted in a reduction in SA accumulation [*34*]. However, the inhibitor of ethylene action, NBD, did not produce a significant change in SA accumulation in TMV-inoculated leaf tissue. These results suggest that ethylene is not directly involved in the signal transduction pathway that leads to SA accumulation and its export from the tissues infected with necrotizing pathogens [*34*]. In Arabidopsis, the ethylene-insensitive mutant *etr1* showed induction of SAR to *Peronospora parasitica* upon pretreatment with *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) [*20*], indicating that ethylene is not necessary for the induction or expression of SAR. Similarly, SA- and 2,6-dichloroisonicotinic acid (INA) induced SAR inhibited downy mildew disease strongly both in wild type and in the ethylene-insensitive *etr1* and *ein2* mutants [*19*]. However, ethylene stimulated the SA induced activation of the *PR-1* gene up to about 20-fold, indicating that ethylene enhanced the sensitivity of Arabidopsis plants to SA.

Since the role of ethylene in the resistance of tobacco to TMV is controversial, several approaches were taken and compared with the influence of ethylene production or action before or during infection. Ethylene generating compounds and inhibitors were applied locally to modify ethylene production at the site of primary-inoculation, whereas transgenic plants with altered ethylene production or perception were affected both locally and systemically. Effects on necrotic lesion formation and primary lesion size, as well as on lesion size after challenge inoculation of plants with SAR, were determined.

MATERIALS AND METHODS

Cultivation of plants

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were grown from seed either in a greenhouse at a minimum temperature of 24 °C during the day and 21 °C at night, or in a growth chamber at 20–22 °C [*37*]. Transgenic sense and antisense ACS plants and Tetr18 plants, transformed with the mutant *etr1-1* gene of *Arabidopsis thaliana*, were constructed as described previously [*17*, *18*]. Primary transformants were allowed to self-pollinate. T_1 seed was germinated on Murashige and Skoog (MS) medium containing 100 μg ml^{-1} kanamycin, after which surviving plantlets were transferred to soil. P12 tobacco plants, transformed with the P1 and P2 genes of alfalfa mosaic virus, were used as transgenic control plants. These plants were in all respects phenotypically similar to untransformed plants, and no differences between untransformed or P12 plants were observed in any of the characteristics investigated. In experiments with Tetr18 plants, both Tetr18 and control plants were grown in two times autoclaved potting soil [*18*].

Inoculation and treatment of plants

For studying the effect of inhibitors of ethylene synthesis or action on primary TMV lesions, 9–11 week old plants were trimmed to three consecutive leaves at selected stem positions. The leaves were dusted with carborundum and inoculated with purified TMV WU1 (5 mg l^{-1}) or sterile water as a control, followed by a water rinse. Solutions of the test compounds or water were applied to both upper and lower surfaces of the leaves as a fine spray until run-off. For testing ethylene or NBD, plants were put under a glass bell with a water lock and the appropriate amount of gas was introduced with a syringe. Three to five plants per treatment were used and experiments were conducted at least three times.

Once formed by 2 days after inoculation, lesions developed by expanding for 3–5 days, reaching final size 5–7 days after infection. To compare the effects of the different treatments, lesion sizes were determined 7 days after inoculation by measuring the diameters of 10–20 lesions per leaf using a magnifying glass at a magnification of 10×. Differences were statistically analysed using Student's *t*-test. In general, differences surpassing 10 % were significant at at least the 5 % level [*37*].

To determine the effect of local application on the development of SAR, a single, just fully-grown leaf on 9–11 week old plants was inoculated with either 100 mg TMV l^{-1} or water and immersed overnight in a beaker containing 0·1 mM AVG, 0·1 mM ACC, or water. Alternatively, a conical flask was placed over the leaf and the opening was plugged with cotton wool around the petiole and covered tightly with aluminum foil. NBD or ethylene was injected into the flask to a final gas concentration of 8 ml l^{-1} and 10 μl^{-1}, respectively. The flask was left for 7 days and the gas atmosphere was replaced once at the end of the 4th day after inoculation. Seven days after the primary-inoculation, the third leaf above (upper leaf) and the third leaf below (lower leaf) the treated leaf were challenge inoculated with TMV and lesion sizes were measured 7 days later.

In experiments with transgenic ACS plants and Tetr18 plants, 7–9 week old plants were used. Three leaves per

plant were inoculated with virus solution or water. Challenge inoculations were performed 7 or 10 days later on upper, non-infected leaves, and lesion sizes were determined 4 or 7 days later. Three ACS plants, and four or five Tetr18 plants per treatment were used in triplicate. Data were statistically analysed using analysis of variance followed by Fisher's test for LSDs at $\alpha = 0{\cdot}05$.

For the triple response assay, sterilized seeds were germinated on MS medium pH 5·8, containing 100 μg ml^{-1} kanamycin and 0·7 % bactoagar in the presence or absence of 20 μM ACC. Seedlings were grown in the dark at 25 °C for 8 days.

Ethylene analysis

Since stimulation of ethylene production by TMV is directly related to the number of lesions formed [*29*] but this number is difficult to control [*46*], virus-inducible ethylene production was mimicked by application of α-aminobutyric acid (αAB) [*17*, *22*]. Six leaf discs were floated on either 10 ml water containing 0·01 % Tween-20 or 10 ml l mM αAB, 0·01 % Tween-20 in 30 ml vials. After incubation for 3 days in the light, ethylene was determined by gas chromatography as described previously [*8*].

RESULTS

Effects of increased ethylene levels on TMV lesion size in primary-inoculated leaves

Three compounds were used to increase the level of ethylene in treated tobacco leaves: gaseous ethylene, the ethylene precursor ACC, and the ethylene-releasing compound, ethephon. At various concentrations tested, none of these compounds affected the appearance of local lesions by 2 days after TMV-inoculation. Enclosing leaves of intact Samsun NN plants in an atmosphere containing 10 or 50 μl l^{-1} ethylene from the time of inoculation with TMV onwards, reduced lesion expansion, final lesion size being 16 and 39 % smaller, respectively, compared with leaves held in an atmosphere of ambient air. However, the ethylene-treated leaves yellowed prematurely, indicative of ethylene-promoted senescence. Since TMV lesion size decreases with increasing leaf age [*37*], the ethylene-induced reduction in lesion expansion might be due to accelerated ageing of the treated leaves. Spraying leaves on trimmed plants 12 h after inoculation with TMV with a concentration range of ACC did not lead to leaf yellowing, and up to 0·3 mM did not influence lesion size compared with leaves treated with water. However, at 1 and 3 mM ACC significantly reduced lesion size by 19 and 38 %, respectively. At these concentrations, ethylene production in tobacco leaves was increased over 100-fold (data not shown).

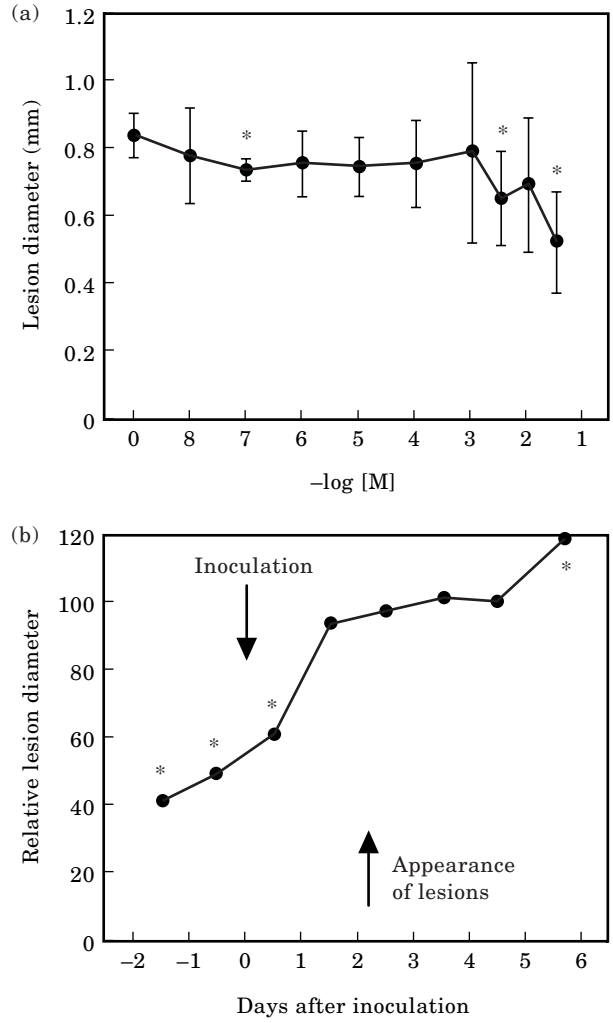

FIG. 1. (a) Average final lesion diameter ± S.D. on leaves of trimmed tobacco plants sprayed with different concentrations of ethephon between 6 and 12 h after TMV inoculation; 0 = water control. (b) Effect of a single spray of 3 mM ethephon, at different times with respect to TMV inoculation, on final lesion size relative to water-sprayed controls (100). Asterisks indicate statistically significant differences (Student's *t*-test, $P < 0{\cdot}05$) from water-sprayed controls.

In accordance with previous results [*38*], treatment with ethephon by either spraying or rubbing between 6 and 12 h after inoculation reduced TMV lesion size in a concentration dependent manner [Fig. 1(a)]. No significant effect was noticeable at concentrations up to 0·1 mM, but higher concentrations of ethephon progressively reduced lesion size up to 34 % at 30 mM. No yellowing of the leaves as a result of the application of ethephon was seen. However, at concentrations exceeding 3 mM plants developed visible necrosis as a result of a toxic action of ethephon. Control experiments in which leaves were treated with mixtures of the degradation products that accompany the release of ethephon, hydrochloric acid and phosphoric acid, showed that the

acids caused similar leaf damage but did not reduce TMV lesion size. Hence, it must be concluded that the ethylene released from ethephon, rather than the necrosis *per se*, was responsible for the reduction in lesion size. Collectively, the results from the treatments with ethylene, ACC and ethephon demonstrate that increased levels of ethylene can reduce TMV lesion expansion.

To determine in how far time of application of ethephon with respect to TMV-inoculation influences lesion development, plants were sprayed with 3 mM ethephon, or water as a control, at different times before or after inoculation. As shown in Fig. 1(b), ethephon reduced final lesion size only when applied well before the appearance of local lesions. The reduction was almost 60 % when the compound was sprayed on the plants 40 h before inoculation, and decreased over the following 3 days to insignificant levels. Application of ethephon later than 3 days after inoculation tended to slightly stimulate lesion expansion. These results indicate that the effect of ethylene on lesion confinement is strongly time dependent.

Effects of inhibitors of ethylene synthesis or action on TMV lesion size in primary-inoculated leaves

TMV lesion formation is accompanied by a burst of ethylene production around the time of lesion appearance. To investigate how far inhibition of this TMV-induced ethylene production or interference with its action has an influence on final lesion size, several inhibitors were applied to TMV-inoculated plants. The inhibitor of ACS, AVG, effectively inhibits TMV-induced ethylene production in tobacco, at 0·1 mM reducing ethylene emanation from inoculated leaves by 90–95 % [*11*]. Spraying leaves on trimmed plants 9–12 h after inoculation with AVG reduced final lesion size by 16–36 % over a concentration range from 10 μM to 10 mM. Similarly, when a TMV-inoculated leaf on an intact plant was immersed for 16 h in a 0·1 mM AVG solution, lesions remained considerably smaller than on a leaf of a plant that had been immersed in water. Moreover, the AVG-treated leaf remained dark green, whereas the water-treated leaf yellowed and senesced (Fig. 2). However, the leaves that developed after the treatment with AVG had taken place displayed severe yellowing, particularly along the margins. Moreover, further plant growth was severely reduced, resulting in stunting of the upper part of the plant. No virus was recovered from these newly emerged leaves, and similar treatment with AVG of plants that were never infected caused identical effects. These symptoms resembled known toxic effects of the natural AVG analog rhizobitoxin [*26*], and suggested that AVG taken up by the treated leaf was transported to young growing tissues.

Spraying trimmed plants with the inhibitor of the last step of ethylene biosynthesis, $CoCl_2$, which inhibits the conversion of ACC into ethylene, likewise inhibited lesion expansion. At 0·1 mM, $CoCl_2$ reduced TMV-induced ethylene production by about 60 %. A reduction of up to 18 % in lesion size was recorded, but concentrations of $CoCl_2$ higher than 0·1 mM caused leaf damage. Similar damage was also apparent after spraying with the inhibitor of ethylene action, silver nitrate. When sprayed within 12 h after TMV-inoculation, 0·1 mM $AgNO_3$ reduced final lesion size by up to 50 %, but not when applied by 3·5 days after inoculation. When Ag^+ was applied complexed with the thiosulphate ion [*44*], 0·1 mM $Ag_2S_2O_3$ did not cause leaf damage but inhibited lesion expansion by only about 20 %. Comparatively little virus could be recovered from leaves sprayed with $AgNO_3$, suggesting that the free Ag^+ ion may reduce lesion expansion mainly by interfering with the multiplication or spread of TMV in tobacco leaves.

Enclosing a TMV-inoculated leaf on an intact plant in an atmosphere containing 8000 ppm of the gaseous inhibitor of ethylene action, NBD, did not alter local lesion formation on the enclosed leaf.

FIG. 2. Effect of a 16 h treatment of a single, TMV-inoculated leaf by immersion in (left) 0·1 mM AVG or (right) water (control) on Samsun NN tobacco plants 7 days later. Note the yellowing of the inoculated leaf on the control plant (right) and the chlorophyll retention and lesser lesion expansion on the inoculated leaf of the AVG-treated plant (left). The yellowing of the newly emerged leaves is caused by toxicity of the inhibitor.

Effects of local interference with ethylene levels or action on expression of systemic acquired resistance

Since the hypersensitive reaction of TMV is accompanied by strongly increased ethylene production and because increased levels of ethylene prior to TMV-inoculation reduced lesion size, it could be expected that the ethylene produced upon primary-inoculation contributes to the enhanced lesion limitation upon challenge inoculation of leaves expressing SAR. Indeed, spraying, rubbing or pricking lower leaves of Samsun NN plants with high concentrations (10–300 mM) of ethephon-induced virus like lesions and SAR to the same level as TMV-inoculation [*38*]. However, the necrosis itself could have contributed to the level of induced resistance attained.

In a first set of experiments, selected leaves of tobacco plants were treated with ethylene, ACC, or ethephon at non-toxic concentrations. Seven days thereafter the effect on TMV lesion size was determined by challenge-inoculation of upper or lower leaves, compared with plants that were treated with air or water. Enclosing a single, fully developed leaf on an intact plant for 7 days in an atmosphere containing 10 μl l^{-1} ethylene significantly reduced ($P < 0{\cdot}05$) lesion size in non-treated leaves by an average of 16 %. In contrast, immersion of a single leaf for 16 h in a solution of 0·1 mM ACC did not significantly alter lesion size in challenge-inoculated upper or lower leaves. Rubbing lower leaves with 1 mM ethephon followed by inoculation of upper leaves 7 days later significantly reduced lesion size by 20 %. Treating lower leaves with 10 mM ethephon reduced lesion size in upper leaves up to 40 %. However, at this concentration, the ethephon-treated leaves developed signs of injury. The apparent induction of a SAR like state in upper leaves after treatment of lower leaves with ethephon raised the question whether ethephon treatment of SAR-expressing leaves could further reduce lesion expansion. Indeed, when SAR was induced by primary-inoculation of lower leaves with TMV and upper leaves challenge-inoculated with TMV 14 days later were themselves sprayed with ethephon, this compound reduced lesion size to a similar extent as upon treatment of primary-inoculated leaves (cf. Fig. 1). For instance, in a typical experiment final lesion sizes on SAR-expressing leaves sprayed with water, 3 mM ethephon and 30 mM ethephon were 0·49 $\pm$ 0·15, 0·40 $\pm$ 0·15 and 0·28 $\pm$ 0·07 mm, respectively.

In a second set of experiments, of several tobacco plants, a single leaf was inoculated with TMV and subsequently treated with either AVG or NBD to block TMV-induced ethylene synthesis or action, respectively. After 7 days, one upper and one lower leaf on the same plant were challenge-inoculated with TMV and lesion sizes measured 7 days later. As non-induced controls, plants were used that were primarily inoculated with water instead of TMV. Preliminary experiments indicated that treatment of a single TMV-inoculated leaf with AVG reduced the extent of SAR in upper leaves [*42*, *43*], but treatment with AVG also increased lesion size in upper and lower leaves of control plants that were not induced. Similar experiments were conducted in which the primarily TMV- or water-inoculated leaf was enclosed in a flask containing NBD, or air as a control, because of the obvious side effects of AVG. In four experiments, TMV-inoculation of the single leaf held in air induced SAR in both upper and lower leaves: upon challenge inoculation, lesion size was reduced by on average 30 % compared with plants of which the single leaf had been inoculated with water (Table 1). Incubation in NBD caused substantial variation in lesion size

TABLE 1. *Effect of treatment of a TMV-inoculated leaf with NBD on extent of SAR in an upper (H) and a lower (L) leaf*[a]

		Lesion size (mm)[b]					
		Control			NBD		
		Water	TMV	Percentage[c]	Water	TMV	Percentage[c]
Exp. 1	H	1·11	0·49*	44	1·11	0·90*	81
	L	0·86	0·42*	49	0·86	0·59*	69
Exp. 2	H	1·24	0·56*	45	2·17	0·91*	42
	L	0·73	0·70	96	0·72	0·89*	124
Exp. 3	H	1·87	1·40*	75	1·55	1·05*	68
	L	1·05	0·91*	87	0·94	0·79*	84
Exp. 4	H	0·95	0·82*	86	1·13	1·00	89
	L	0·83	0·64*	77	0·80	1·02*	128
Average				70			85

[a]A single leaf on an intact tobacco plant was enclosed in an atmosphere containing NBD. Seven days later one upper and one lower leaf were inoculated with TMV. The diameter of 20 lesions per leaf was measured 7 days thereafter and averaged.

[b]Asterisks indicate statistically significant differences (Student's *t*-test, $P < 0{\cdot}05$).

[c]Percentage = (lesion size TMV/lesion size water) × 100 %.

upon inoculation of an upper or a lower leaf of non-induced plants. Similarly, substantial variation occurred upon challenge inoculation of induced plants, with lesion sizes ranging from 42 to 128 % of those in water-treated controls. Whereas in experiment 3, SAR was as strong in NBD-treated plants as in the controls, in experiment 2 SAR was clearly not expressed in the lower leaf, and in experiments 1 and 4 SAR was substantially less as a result of NBD treatment. Thus, compared with water-treated control plants, in TMV-induced plants lesion sizes upon challenge inoculation were reduced by an average of no more than 15 % (Table 1), indicating that NBD increased lesion size in induced plants compared with treatment with air. It can be concluded, therefore, that local treatment with NBD tended to reduce the development of SAR in both upper and lower leaves.

TMV lesion size and systemic acquired resistance in transgenic ACS plants

To circumvent the use of inhibitors, transgenic tobacco plants containing either sense or antisense constructs corresponding to ACS or ACO, or both, under the control of the CaMV 35S promoter [*17*] were tested for ethylene production, TMV lesion size and development of SAR. Although efficient overexpression or gene silencing occurred at the transcript level, there was no correlation with the levels of ethylene produced by these plants. The largest effects on endogenous ethylene production were found in sense and antisense ACS lines, that also showed alterations in plant growth and leaf chlorophyll levels [*17*]. As a result of TMV infection, ACS mRNA levels were increased by 3 days after inoculation, not only in the sense but also in the antisense plants, indicating that infection-induced ACS-mRNA accumulation could, at most, be only partially inhibited. Likewise, stimulation of α-aminobutyric acid induced ethylene production, which mimics the effect of TMV-infection, was only partially reduced in antisense lines [*17*]. Whereas ethylene production differed more than three-fold in the transgenic lines and lesion size varied between 64 and 146 % of that on control plants, no relationship between ethylene levels and primary lesion size was observed [Fig. 3(a)].

Ten days after TMV-inoculation the plants were challenge-inoculated on upper, non-infected leaves. All the ACS lines displayed SAR. One ethylene overproducing line appeared to have a higher level of induced resistance than the control plants (low relative SAR lesion size), whereas one less ethylene producing line showed less induced resistance (high relative SAR lesion size). However, in spite of their varying ethylene production rates, the other transgenic lines showed an induced resistance of the same level as seen in control tobacco [Fig. 3(b)]. Thus, any relationship between ethylene

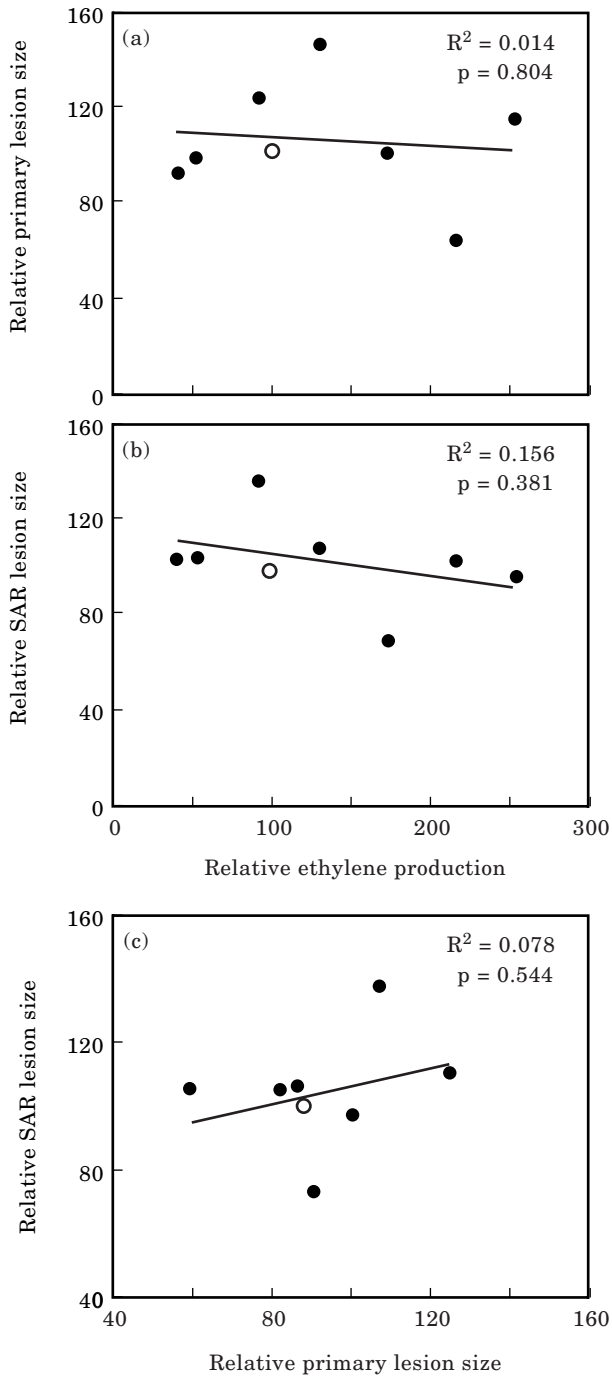

FIG. 3. Regression analysis of (a) Average lesion size on, and inducible ethylene production of, primary-inoculated leaves of transgenic sense and antisense ACS lines relative to control plants (○); (b) Relative SAR lesion size, 4 days after challenge of upper leaves infected 10 days earlier on lower leaves with TMV, and relative inducible ethylene production of primary-inoculated leaves; and (c) Relative SAR lesion size on upper leaves of induced plants and relative primary lesion size prior to the induction of SAR.

production and SAR was absent in these transformants, and neither was there any correlation between primary lesion size and the level of SAR attained [Fig. 3(c)].

TMV lesion size and systemic acquired resistance in ethylene-insensitive plants

Since tobacco plants transformed with the mutant *etr1-1* gene from Arabidopsis overproduced ethylene but lacked typical ethylene responses [*18*], the representative line Tetr18 was selected and tested further for its reaction to TMV-infection. That these transformants were truly ethylene insensitive was shown by the absence of the triple response in etiolated seedlings. In the presence of ACC, ethylene produced by control seedlings induced the typical effects, consisting of the inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and root, and exaggeration of the curvature of the apical hook. In contrast, elongation growth in Tetr18 seedlings was unaffected by ACC-induced ethylene production (Fig. 4).

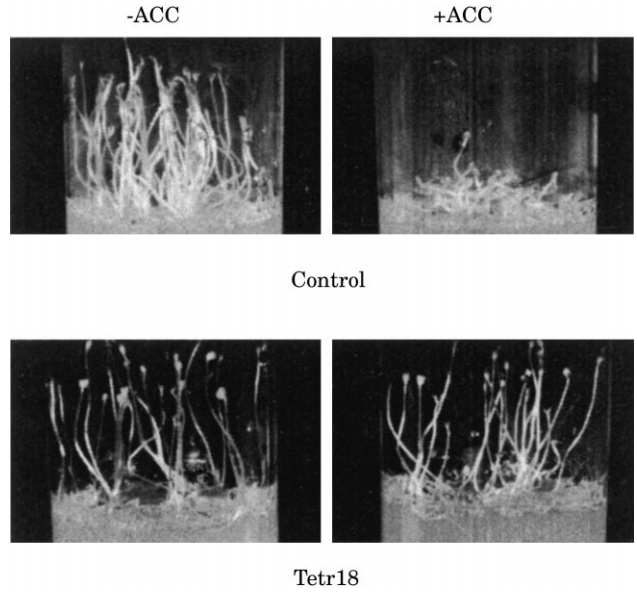

FIG. 4. Eight day old seedlings of control and Tetr18 plants. Seedlings were grown in the dark at 25 °C on Murashige and Skoog medium containing 100 μg ml^{-1} kanamycin in the presence or absence of 20 μM ACC.

Upon inoculation of Tetr18 plants with TMV, lesion formation was unimpaired. After 3 days, lesion sizes on control and Tetr18 plants were not significantly different, but after 7 days lesions on Tetr18 plants had hardly enlarged and were significantly smaller than on control plants [Fig. 5(a); cf. Table 2]. Also the appearance of the lesions on Tetr18 plants was different in that these lacked the dark brown margin characteristically observed on control plants. Furthermore, in control plants the tissue surrounding the lesions was yellow because of ethylene-induced chlorophyll breakdown, whereas in Tetr18 plants it remained green [Fig. 5(b)].

The effect of ethylene insensitivity on SAR was analysed by determining TMV lesion size upon challenge inoculation of upper leaves 7 days after primary inoculation of the plants on lower leaves (Table 2). In control plants the development of SAR in upper leaves resulted in a mean lesion diameter one third of that in non-induced plants. In Tetr18 plants, lesions on non-induced plants were smaller than on transformed controls (cf. Fig. 5), but on induced plants challenge inoculation resulted in relatively large lesions, indicative of a reduced SAR response. In this case, the lesion diameter in upper leaves of induced plants was two thirds that of non-induced plants, a SAR response only half as strong as in control plants. Thus, interference with ethylene sensitivity reduced, but did not abolish, expression of SAR against TMV.

When SAR was induced by inoculation with a concentration range of TMV, the number of lesions on

TABLE 2. *Size of TMV lesions on challenge-inoculated leaves of non-induced and TMV-induced control and Tetr18 plants* [a]

	Lesion size (mm)[b]					
	Control			Tetr18		
	Water	TMV	Percentage[c]	Water	TMV	Percentage[c]
Exp. 1	1·60 a	0·47 d	29	1·02 b	0·58 c	57
Exp. 2	1·43 a	0·56 d	39	1·05 b	0·80 c	76
Exp. 3	1·68 a	0·56 d	33	1·15 b	0·86 c	75
Average			34			69

[a]Tobacco plants were inoculated on three consecutive leaves with either water or TMV. Seven days later upper, non-inoculated leaves were inoculated with TMV. The diameter of the lesions was measured 7 days thereafter and expressed as the average size of 60 lesions on four or five plants.

[b]Within each experiment different letters indicate statistically significant differences between treatments (Fisher's LSD test, $P < 0{\cdot}05$).

[c]Percentage = (lesion size TMV/lesion size water) × 100 %.

FIG. 5. Response of control and Tetr18 plants to TMV: (a) Lesion size in control and Tetr18 tobacco, measured 3 and 7 days after inoculation; (b) Phenotype of lesions on leaves of control plants 7 days after inoculation; and (c) Phenotype of lesions on leaves of Tetr18 plants 7 days after inoculation. Bar = 1 cm.

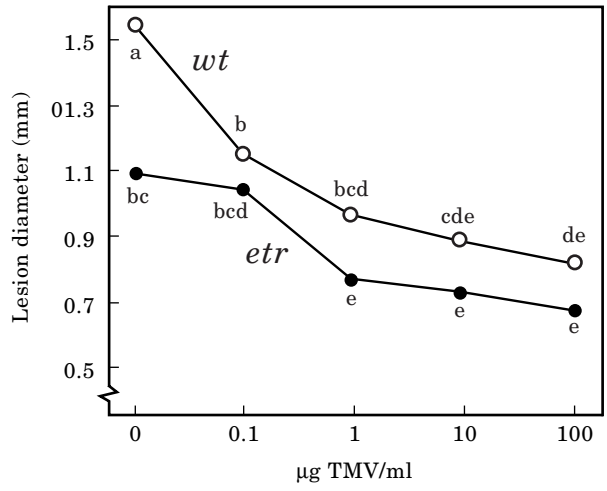

FIG. 6. Average TMV lesion diameters 7 days after inoculation of upper leaves of non-transformed (wt) and Tetr18 (etr) plants in which SAR had been induced 7 days earlier by inoculation of three lower leaves with different concentrations of TMV. Different letters indicate statistically significant differences between treatments (Fisher's LSD test, $P < 0{\cdot}05$).

the primary-inoculated leaves increased about linearly with the logarithm of the virus concentration {data not shown; cf. [*37*]} and did not differ appreciably between non-transformed and Tetr18 plants. Upon challenge inoculation of upper leaves of non-transformed control plants a 24 % reduction in lesion diameter was evident upon induction with as little as 0·1 mg TMV l^{-1}, increasing logarithmically to a 48 % reduction at 100 mg TMV l^{-1}. In contrast, in Tetr18 plants no significant SAR was induced at 0·1 mg TMV l^{-1}, but SAR did develop upon primary-inoculation with higher concentrations of TMV. However, SAR was never as strong as in non-transformed plants and the reduction reached only 36 % upon induction with 100 mg TMV l^{-1}. Collectively, these results indicate that ethylene perception is required for full expression of SAR in tobacco.

DISCUSSION

Involvement of ethylene in lesion development in primary-inoculated leaves

Ethylene has been implicated both in pathogenesis and in defense responses in infected plants [*1*, *12*, *13*, *41*] and is increased early after infection of tobacco with TMV [*11*]. To investigate the role of ethylene, we attempted to gain further insight into the relationship between virus-induced ethylene production, lesion development and SAR, on the one hand by applying ethylene generating compounds or inhibitors of ethylene synthesis or action and, on the other hand, in transgenic plants with

modulated ethylene synthesis or insensitivity to the hormone. Under all conditions tested, inoculation with TMV led to the appearance of phenotypically normal local lesions, confirming previous results [*11*, *17*, *18*] that the hypersensitive reaction of Samsun NN tobacco to TMV is not influenced by altered ethylene production or perception. These results are at variance with those described recently by Ohtsubo *et al*. [*25*] that ethylene is directly involved in the formation of necrotic lesions. However, those authors used a temperature-dependent accelerated lesion formation system in which detached tobacco leaves were shifted from 30 to 20 °C, whereas we employed intact plants maintained at about the latter temperature. TMV lesion development in detached leaves differs phenotypically from that in leaves attached to the plant [*39*] and ethylene may affect processes related to the shift in temperature.

Both ethylene or ethylene-generating compounds and inhibitors of ethylene synthesis or action were found to influence lesion expansion, resulting in differences in final lesion size as compared with plants treated with water. Gaseous ethylene, the ethylene precursor ACC and the ethylene releasing compound ethephon, when applied at high concentrations shortly after inoculation, all reduced lesion size. However, incubating plants in ethylene-accelerated leaf senescence and ethephon was most effective at concentrations that were toxic to the leaves. Applying ethephon at a non-toxic dose before inoculation strongly reduced final lesion size, supporting previous results that ethephon induces SAR in tobacco [*38*]. This effect was reduced when ethephon was applied near the time of inoculation and lost completely by the time of lesion appearance. Thereafter, ethephon tended to increase lesion size. A similar situation was apparent upon treatment of TMV-inoculated Samsun NN tobacco with the auxin indoleacetic acid at concentrations that promote ethylene production by the tissue [*39*]. These results fully agree with the conclusions of Pritchard and Ross [*29*] that exposure of leaves to ethylene or ethylene-stimulating compounds prior to inoculation causes an early cessation of lesion growth, whereas ethylene synthesized during lesion development contributes to continued lesion expansion. The latter is most obviously supported by our observation that TMV lesion size in the transgenic, ethylene insensitive Tetr18 plants was similar at 3 days after inoculation, but lesions did not increase thereafter, in contrast to a doubling in size in control plants. At 7 days after inoculation, the reduced lesion expansion in Tetr18 plants coincided with a lack of ethylene-induced leaf yellowing around the lesions. Apparently, ethylene-induced senescence of the tissue surrounding local lesions enhances the spread of the virus.

Similar effects of ethylene on resistance have been reported for other plant–pathogen combinations. Mature green Robinson tangerines showed enhanced resistance against *Colletotrichum gloeosporioides* when inoculated after treatment with ethylene, whereas when inoculation preceded treatment, ethylene was found to increase disease development [*4*, *5*]. Application of ACC or ethephon-induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato plants only when applied before inoculation [*2*]. Thus, the timing of ethylene exposure before or after infection determines whether disease development is reduced or enhanced. A requirement of ethylene perception for lesion expansion also explains the seemingly contradictory results that, similar to ethylene-generating compounds, inhibitors of ethylene synthesis or action likewise reduce lesion size in TMV-inoculated leaves. Treatment of plants shortly after inoculation with TMV with 0·1 mM AVG reduced primary lesion size by on average 19 % [*43*]. Similar results were obtained with $CoCl_2$ and silver salts. However, the strong reduction in lesion expansion observed upon treatment with $AgNO_3$ appeared to result from inhibition of the multiplication or spread of the virus by the metal ion. No significant effect was apparent upon application of NBD.

At least AVG effectively inhibited TMV-induced ethylene production from the time of lesion appearance onwards [*8*, *11*]. Only enhanced ethylene production well before lesion appearance increases resistance, but such early enhanced ethylene production is absent in TMV-inoculated leaves. Hence, the inhibitors cannot reduce resistance and increase lesion size but, rather, block ethylene synthesis or action during the phase of ethylene-stimulated lesion expansion. The normal lesion expansion on NBD-treated leaves appears at variance with the reduced lesion expansion seen in Tetr18 plants. However, the NBD-treated plants were not ethylene insensitive and the very high ethylene production in the ring of tissue surrounding the developing lesions [*9*] is likely to have competitively inhibited NBD action, allowing lesions to expand normally. At this later stage added ethylene or ACC had no effect, indicating that virus-induced ethylene production was already saturating.

In the transgenic ACS plants there was no correlation between altered ethylene production and final lesion size. Although the ethylene levels of the individual plants differed more than three-fold, these differences were probably insufficient to consistently affect primary TMV lesion size.

Involvement of ethylene in systemic acquired resistance

Previously, local application of ethephon to non-infected tobacco plants was shown to induce SAR comparable with the result of TMV-infection [*38*]. Conversely, local treatment of a TMV-inoculated leaf with either AVG or NBD reduced the level of SAR attained. The present investigation confirmed the result of Ross and Pritchard [*33*] that local gassing of tobacco plants with ethylene

reduces lesion size upon challenge inoculation of distant leaves. Treatment of a single leaf with 0·1 mM ACC was not effective. At this concentration, ACC likewise did not affect lesion size on primary-inoculated leaves and, hence, the amount taken up was apparently too low to induce systemic resistance. AVG was previously demonstrated to interfere with the establishment of SAR in opposite, non-infected leaf halves, as well as in upper and lower leaves [*43*]. However, AVG was apparently taken up and transported in the plant, leading to symptoms of toxicity in the young, developing leaves. Although these experiments were insufficient to conclude that ethylene is required for SAR to be expressed, they did point to its involvement in this phenomenon in tobacco. This hypothesis was supported by the results obtained with NBD. Ensuring maintenance of the NBD atmosphere in the flask enclosing a single tobacco leaf on an intact plant for several days proved technically demanding and may be the cause of the large variation in the results between repeated experiments. Nevertheless, NBD, local gassing which had no obvious effects on the plants, reduced the extent of SAR in distant leaves. Since NBD did not significantly affect lesion size upon primary-inoculation, the inhibitor did not appear to interfere with lesion development itself. Similar observations have been reported by Silverman *et al*. [*34*], who also established that NBD did not affect the increases in free and total SA or the accumulation of PR-1 in TMV-inoculated tobacco leaves.

To avoid non-specific side effects of the use of inhibitors, transgenic sense and antisense ACS plants with modulated ethylene levels were analysed. These plants showed alterations in growth and leaf chlorophyll levels consistent with known effects of ethylene [*17*]. Although the rates of ethylene production in those lines varied over three-fold, the level of SAR attained upon induction by TMV was not altered. Upon TMV-infection ethylene production is increased to very high levels in the tissue immediately surrounding developing local lesions [*9*, *40*], whereas in the transgenic *ACS* plants ethylene production supposedly occurs evenly over the whole leaf. Thus, it is not surprising that ethylene production did not appear to be modulated to an extent necessary for affecting SAR induction. In view of our results with plants exhibiting reduced responsiveness to ethylene, it must be concluded that the transgenic sense and antisense ACS lines are not sufficiently altered to significantly affect their expression of SAR.

Ethylene insensitive tobacco plants allowed the role of ethylene to be established most clearly. In these Tetr18 plants the SAR response was substantially reduced. On challenge-inoculated leaves of TMV-induced Tetr18 plants, the lesions expanded more than on TMV-induced control plants, in spite of the limited lesion size on primary-inoculated leaves of Tetr18 plants compared with control plants. Although SAR was substantially reduced in Tetr18 plants, it was not abolished. It might be argued that the smaller lesions on the primarily-inoculated leaves of the Tetr18 plants generated less of the SAR-inducing mobile signal than the larger lesions on the control plants and, hence, the level of SAR attained in Tetr18 plants was reduced. However, the reduction in SAR seen in untransformed plants treated with NBD, that did not affect primary lesion size, suggests that interference with ethylene perception does reduce SAR. Moreover, in the transgenic ACS plants no relationship between the level of SAR and primary lesion size was apparent. Indeed, in control plants small numbers of lesions induced by a low dose of TMV were already effective in reducing lesion size in distant leaves half maximally, whereas under the same conditions in Tetr18 plants no significant SAR was apparent. Only at higher doses of the virus, significant SAR was induced in the Tetr18 plants, but never to the level observed in control plants. Thus, the Tetr18 plants appear to be less sensitive to the induction of SAR by TMV than normal Samsun NN tobacco.

Ethylene can exert some influence on the SA signalling pathway, as was shown in Arabidopsis where ethylene enhanced the sensitivity of the tissue to the action of low concentrations of SA [*19*]. Raz and Fluhr [*30*] demonstrated that in tobacco, in the presence of the inhibitors of ethylene action, NBD or silver thiosulphate, SA-induced acidic chitinase accumulation was abolished. As it is known that SA accumulation in the inoculated leaves is instrumental in the induction of PRs and the development of SAR [*14*], a decrease in sensitivity to SA might reduce the extent of SA-induced signalling. SA is unlikely to be itself the translocated signal [*45*], and an intact ethylene response appears to be required for optimal transmission of the signal to non-infected plant parts.

Lawton *et al*. [*19*, *20*] concluded that in Arabidopsis acquired resistance signal transduction is ethylene independent. In spite of reduced *PR* gene expression, in *etr1* mutants SAR induced by inoculation with *P. syringae* pv. *tomato* appeared to be fully expressed upon challenge inoculation with *Peronospora parasitica*. Ethephon-induced SAR gene expression in both the wild type and ethylene mutants, whereas ethylene alone did not, suggesting that induction of these genes by ethephon is not due to the action of ethylene. Instead, both hydrochloric and phosphonic acid significantly induced PR-1 mRNA accumulation, and this effect was increased substantially in the presence of ethylene [*19*]. However, in tobacco neither PRs, nor SAR are induced by acid treatment [*38*], in agreement with the conclusion by Ross [*32*] that no SAR is induced by mechanical or chemical injury in tobacco. In Arabidopsis, the effect of ethephon treatment on subsequent infection with a challenging pathogen was not tested, whereas in tobacco treatment with either ethephon or ethylene-induced resistance against TMV.

Moreover, in ethylene-insensitive tobacco, SAR induced by inoculation with TMV was substantially reduced. It must be concluded, therefore, that in tobacco SAR is stimulated by ethylene perception, whereas in Arabidopsis this seems less apparent, suggesting that SAR is differently regulated in tobacco and Arabidopsis.

How ethylene signalling contributes to SAR in tobacco is unclear. Notably, in inoculated leaves, lack of ethylene perception reduces TMV lesion expansion, because in TMV-infected leaves of ethylene-insensitive Tetr18 plants lesions were smaller than in control plants. In contrast, upon challenge inoculation of non-infected leaves of TMV-induced, NBD-treated wild-type plants, or TMV-induced Tetr18 plants, lesions were larger than on challenge-inoculated leaves of TMV-induced control plants. If ethylene would act on the expression of SAR, one would expect that upon challenge inoculation of induced Tetr18 plants lesion size would be reduced, as it is on primary-inoculated leaves of ethylene-treated plants, as well as on ethephon-treated SAR-expressing leaves of TMV-induced plants. However, this was not the case. Therefore, ethylene does not seem to act primarily on the expression of SAR, but rather to interfere with SAR signalling. One explanation could be that the lack of ethylene perception reduces the sensitivity of the tissue to SA and, hence, decreases the level of SAR. However, in untransformed tobacco plants treated locally with NBD, challenge-inoculated leaves were not impaired in their ethylene perception and should have retained their sensitivity to SA. Yet, SAR was reduced similarly as in Tetr18 plants. Therefore, it appears that ethylene action in the primary-inoculated leaf is involved in the generation and/or release of the mobile signal, that is responsible for the systemic character of SAR in tobacco. This hypothesis is being studied further by comparative analysis of the levels of SA and *PR*-gene expression in the TMV-infected ethylene-sensitive control and ethylene-insensitive Tetr18 Samsun NN plants.

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