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Ethylene Promotes the Necrotic Lesion Formation and Basic PR Gene Expression in TMV-Infected Tobacco

Norihiro Ohtsubo [1, 2, 3], Ichiro Mitsuhara [1, 2], Masaaki Koga [1, 4], Shigemi Seo [1, 2] and Yuko Ohashi [1, 2, 5]

[1] *Department of Molecular Genetics, National Institute of Agrobiological Resources, 2-1-2 Kannondai, Tsukuba, Ibaraki, 305-8602 Japan*

[2] *Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation (JST), Chiyoda-ku, Tokyo, 101-0062 Japan*

Ethylene has been thought to be an important factor for the induction of defense responses against pathogen attack, but its function in these responses has not been well defined. Here we show the direct involvement of ethylene in the formation of necrotic lesions and in basic pathogenesis-related (PR) protein gene induction in tobacco. Increase in ethylene production as well as accumulation of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) gene transcript obviously preceded the lesion appearance in tobacco mosaic virus (TMV)-infected leaves in an N gene-dependent synchronous lesion formation system. Inhibitors of ethylene biosynthesis or action significantly suppressed both lesion formation and basic PR gene expression. Induction of these genes was enhanced in 1-aminocyclopropane-1-carboxylic acid (ACC)-treated leaves especially in ACO-overexpressing transgenic tobacco plants. Further, we found that ethylene production during hypersensitive reaction is restricted at the level of ACO activity.

Key words: ACC oxidase — Basic PR genes — Ethylene — Hypersensitive reaction — Lesion formation — Transgenic tobacco plant.

Ethylene influences many aspects of plant growth and development, both as a plant hormone (Ecker and Theologis 1994) and in the induction of plant defense response (Boller 1991). In the ethylene biosynthesis pathway, ACC has been shown to serve as the immediate precursor to ethylene. The conversion of *S*-adenosyl methionine (SAM) to ACC is catalyzed by the pyridoxal phosphate-requiring enzyme ACC synthase (ACS) and represents the rate-limiting step in the ethylene biosynthetic pathway in many plant species (Kende 1989, 1993). The final step in the ethylene biosynthetic pathway is catalyzed by ACO, which is also called the ethylene-forming enzyme (EFE) (Kende 1989, 1993). This enzyme also plays a role in the regulation of ethylene biosynthesis.

Ethylene is thought to enhance plant resistance against pathogen infection by activating the enzymatic activities of chitinase, peroxidase, phenylalanine ammonia-lyase, and polyphenol oxidase, and producing defense-related materials such as phenolic compounds, lignins and suberins to suppress multiplication of pathogens in the infected and adjacent tissues (Pritchard and Ross 1975, Boller 1991, Vera et al. 1993). Incubation of tobacco mosaic virus (TMV)-infected tobacco cultivars carrying the N resistance gene below 24°C (at which the N gene is functional) leads to cell death of the infected tissue associated with formation of necrotic lesions within 48 h (Holmes 1938). Such a hypersensitive reaction (HR) (Goodman and Novacky 1994) is accompanied by an increase in ethylene production near the time of lesion appearance, and production gradually subsides during subsequent lesion development (Nakagaki et al. 1970, Pritchard and Ross 1975, De Laat et al. 1981).

It is known that ACS activity as well as ethylene production is increased in tobacco leaves undergoing HR (De Laat and Van Loon 1982). Induced ethylene production is also associated with increased ACO activity in vivo (Kende 1993). Similar to ACS, ACO activity increased in response to diverse stimuli, including ethylene (Dong et al. 1992, Tang et al. 1994), wound (Baragué et al. 1993, Hyodo 1993), elicitor treatment (Felix et al. 1991), and during the developmental process of fruit ripening and flower senescence (Woodson et al. 1992). Both ACS and ACO genes constitute multigene families, respectively. For example, at least 6 isoforms for ACS (*LE-ACS1* to *LE-ACS6*) have been found in tomato (Rottmann et al. 1991) and 3 isoforms for ACO (*CM-ACO1* to *CM-ACO3*) in melon (Lasserre et al. 1996). ACC-dependent ethylene biosynthesis pathway has not been found in animals. Some kinds of bacteria and fungi produce ethylene (Primrose and Dil-

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; AVG, L-α-(2-aminoethoxyvinyl)-glycine; EFE, ethylene-forming enzyme; HR, hypersensitive reaction; JA, jasmonic acid; NBD, 2,5-norbornadiene; NOS, nopaline synthase; PI-II, proteinase inhibitor II; PR genes/proteins, pathogenesis-related genes/proteins; SA, salicylic acid; SAM, S-adenosyl methionine; TMV, tobacco mosaic virus.

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[3] Present address: National Agriculture Research Center, 3-1-1 Kannondai, Tsukuba, Ibaraki, 305-8666 Japan.

[4] Present address: Faculty of Science, Fukuoka University, Johnan-ku, Fukuoka, 814-0180 Japan.

[5] Corresponding author: Phone, +81-(0)298-38-7440; Fax, +81-(0)298-38-7044; E-mail, yohashi@abr.affrc.go.jp

worth 1976, Billington et al. 1979) without depending on the ACC pathway.

HR in pathogen-infected tissues is accompanied by the development of local and systemic acquired resistance against the second infection of pathogens (Ross 1961a, b) and by the induction of several classes of acidic and basic pathogenesis-related (PR) proteins (Cornelissen et al. 1987, Van Loon 1989, Dore et al. 1991, Ohashi and Oshima 1992). The genes encoding the subset of the basic PR proteins as well as type I and type II serine protease inhibitors, recently grouped into PR-6 family (Van Loon et al. 1994), are highly responsive to ethylene (Margossian et al. 1988, Brederode et al. 1991, Eyal et al. 1992, 1993, Linthorst et al. 1993, Sato et al. 1996) suggesting that the induced expression is the result of increased endogenous ethylene in TMV-infected tissue. However, there is no direct experimental evidence available.

In this paper, we propose a direct contribution of ethylene to necrotic local lesion formation during HR in a TMV-resistant tobacco cultivar, *Nicotiana tabacum* cv. Samsun NN. We used a temperature-dependent synchronous lesion formation system to study the profiles of ethylene production during HR and the effect on HR. Using ACO-transgenic tobacco plants, we also show at least two kinds of basic PR protein genes, the basic PR-1 gene and proteinase inhibitor II (PI-II) gene, are positively regulated by endogenously produced ethylene.

Materials and Methods

Plant materials—Fully expanded upper leaves of 10- to 12-week-old tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN), grown in a greenhouse were used as mature tobacco leaves for the experiments. Detached tobacco leaves were inoculated with tobacco mosaic virus (TMV, 25 μg ml^{-1}) suspended in 10 mM phosphate buffer (pH 7.0), and incubated at 30°C in a humidified box under continuous light (45 μEm^{-2} s^{-1}).

Synchronized lesion formation by temperature-shifting—Detached tobacco leaves inoculated with TMV were incubated at 30°C for 24 to 46 h, allowing virus multiplication without lesion formation. Synchronized lesion formation was initiated in the infected tissue 8 h after the temperature shift to 20°C, a permissive temperature for the N gene.

Differential screening of cDNA clones—Using cDNA libraries prepared from poly(A)+ RNA of TMV-infected tobacco leaves at 3 h after the temperature shift to 20°C as described (Seo et al. 1995), seventy clones preferentially expressed 3 h after the shift were selected. Obtained cDNAs were subcloned into the *Eco*RI site of pBluescript SK+ and subjected to sequencing. Both strands of the clones were sequenced by the dideoxy nucleotide chain termination method using a DNA sequencing kit (PERKIN ELMER, Norwalk, CT, U.S.A.) and a DNA sequencer (373, PE Applied Biosystems, Foster City, CA, U.S.A.).

Southern blot analysis—Ten μg each of genomic DNA isolated from *N. sylvestris, N. tomentosiformis* and *N. tabacum* by the CTAB method (Rogers and Bendich 1985) was digested with *Eco*RI, *Hin*dIII or *Xba*I, separated on a 1% agarose gel then transferred to a NYTRAN nylon membrane filter (Schleicher & Schuell, Dassel, Germany). Blots were prehybridized at 42°C for 6 h in a solution containing 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 3×SSC, 50% deionized formamide (Wako Pure Chemicals, Osaka, Japan), 1×Denhardt's solution, 0.5% SDS and 0.1 mg ml^{-1} salmon sperm DNA, followed by overnight hybridization in the same solution supplemented with ^{32}P-labeled probe and 5 mg ml^{-1} Blocking Reagent (Boehringer Mannheim GmbH, Mannheim, Germany). Blots were washed twice for 15 min each in 2×SSC and 0.1% SDS at room temperature followed by two washes in 1×SSC and 0.1% SDS at 55°C for 15 min each. The probe template DNA was prepared by digestion of *DS321* in pBluescript SK+ with *Hpa*I and *Pst*I, corresponding to +713 to +1,236 including the 3′ portion of the protein coding region and 3′- untranslated region. This template was also used in the RNA blot analysis.

Northern analysis—Wild type or ACO-transgenic tobacco leaves were homogenized in a solution of 4 M guanidine isothiocyanate, 0.5% sodium N-lauroylsarcosine, 5 mM sodium citrate, 0.1 M 2-mercaptoethanol and 0.3% (w/v) antifoam AF and total RNA was purified by CsCl equilibrium centrifugation. Ten μg of RNA was electrophoretically separated on a 1.2% formaldehyde agarose gel then transferred to a Hybond-N nylon membrane filter (Amersham, Buckinghamshire, U.K.). Hybridization and wash were performed as Southern analysis except for a change in washing temperature in 1×SSC and 0.1% SDS to 65°C. PCR-amplified fragment from +565 to +1,015 of pACC-13 (Bailey et al. 1992) was subcloned into the *Hin*dIII and *Bam*HI sites of pUC119 and used as a probe. Probes for the detection of acidic PR-1, basic PR-1 and PI-II mRNA were described previously (Seo et al. 1995).

Treatment with ethylene inhibitors—Four h after the temperature shift, detached TMV-inoculated leaves were fumigated with 9 ml liter^{-1} 2,5-norbornadiene (NBD) in a gas-tight box. After exposure for 4 h, leaves were returned to 30°C. For treatment with $CoCl_2$ and aminoethoxy-vinylglycine (AVG), TMV-inoculated leaf pieces measuring 6 cm×4 cm or disks 19 mm in diameter were incubated with the chemicals at 30°C for 24 h, kept at 20°C for 8 h, then returned to 30°C. Intensity of necrosis 24 h after the shift to 20°C was compared.

Ethylene measurement—The concentration of ethylene was determined with a gas chromatograph (GC-8A, SHIMADZU, Kyoto, Japan), equipped with an alumina column (Porapak Q 50/80; Shinwa, Kyoto, Japan) and a flame-ionization detector.

Determination of ion leakage—Five leaf disks, 19 mm in diameter, were incubated in 20 ml of deionized water in a 100-ml flask for 60 min with shaking at 90 r.p.m. Electric conductivity of the solution was measured with a conductivity detector (CDD-6A; SHIMADZU).

ACO enzyme assays—The ACO assay was performed as described by Mekhedov and Kende (1996). Approximately 1 g FW of wild type or ACO-transgenic tobacco leaf tissues was used as the starting material. Measurement of the ethylene produced was performed as described above. The protein concentration in extracts was determined with Bradford reagent (Protein Assay, BIO-RAD, Richmond, CA, U.S.A.) according to the manufacturer's instructions.

Determination of ACC concentration—Mock- or TMV-infected leaf materials with a fresh weight of 1.3 to 2.0 g were frozen in liquid nitrogen at appropriate times after the temperature shift. The ACC concentration was measured by chemical conversion into ethylene basically according to the method of Lizada and Yang (1979) with modifications by De Laat and Van Loon (1983).

Generation of transgenic tobacco plants harboring ACO cDNA—For the transformation of tobacco, we prepared a binary

vector pEl2Ω-ACO, which contains *DS321* cDNA under the control of the high efficiency promoter El2Ω (Mitsuhara et al. 1996). We first made a modified binary vector pEL2Ω-MCS which has a multicloning sequence (MCS) downstream of the promoter to facilitate subcloning of the ACO cDNA. A *Bam*HI-*Sac*I fragment of pBE2113-GUS (El2Ω-GUS) corresponding to the β-glucuronidase coding sequence was replaced with a synthetic DNA fragment; 5′-GATCCGTTAACCTCGAGGAATTCGGTACCACTAGTGAGCT-3′ and 5′-CACTAGTGGTACCGAATTCCTCGAGGTTAACG-3′ which contains the recognition sites of *Bam*HI, *Hpa*I, *Xho*I, *Eco*RI, *Kpn*I, *Spe*I and *Sac*I. An *Eco*RI site downstream to the termination sequence of the nopaline synthase (NOS) gene in pBE2113-GUS was fixed by treatment with Klenow fragment after *Eco*RI digestion followed by re-ligation. The *Eco*RI fragment of *DS321* was subcloned into the *Eco*RI site of MCS in both directions to obtain pEl2Ω-ACO (transcribes sense ACO mRNA) and pEl2Ω-ACOr (antisense). These constructs were introduced into tobacco by *Agrobacterium*-mediated gene transfer as described previously (Ohshima et al. 1990).

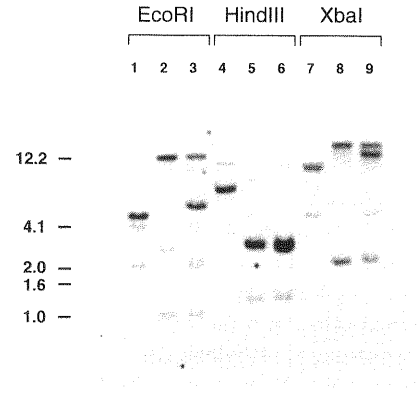

Fig. 1 Genomic organization of tobacco ACO cDNA clone *DS321*. DNA blot analysis of genomic DNAs isolated from *Nicotiana sylvestris, N. tomentosiformis* and *N. tabacum*. Ten micrograms each of genomic DNA prepared from *N. sylvestris* (lanes 1, 4 and 7), *N. tomentosiformis* (lanes 2, 5 and 8) and *N. Tabacum* (3, 6 and 9) was digested with *Eco*RI (lanes 1–3), *Hin*dIII (lanes 4–6) or *Xba*I (lanes 7–9). The blot was hybridized with random-prime-labeled ACO cDNA (*DS321*) from *N. tabacum*.

Results

Cloning and analyses of a cDNA DS321 encoding putative ACO—To identify genes involved in the early stage of necrotic local lesion formation in TMV-infected Samsun NN tobacco leaves, we first performed differential screening using a synchronized lesion formation system which has been shown to be controlled by the temperature-dependent switch via the N gene (Shimomura 1971, Weststeijn 1981, De Laat and Van Loon 1983, Whitham et al. 1996). Seventy cDNAs were obtained as clones preferentially expressed 3 h after temperature shift from 30°C to 20°C. In these clones, we found a putative ACO cDNA *DS321*, of which mRNA showed transient and significant accumulation between 3 and 6 h.

The cDNA insert of *DS321* is 1,236-bp long, 81.5% and 98.9% homologous to tobacco cDNA clones cEFE-26 and cEFE-27 respectively, which have been cloned by a homology-dependent screening procedure (Knoester et al. 1995, EMBL accession no. Z29529 and Z46349). *DS321* has longer 5′ and 3′ terminal sequences than cEFE-27 by 10 bp and 7 bp respectively, contains a complete open reading frame for a protein of 319 amino acids and an authentic polyadenylation signal AATAAA at +1,204 to +1,209. This deduced 36-kDa protein has a homology to ACOs from other plant species including tomato and petunia, up to 90% at the amino acid level.

DNA blot analysis of *N. tabacum* and its parent cultivars, *N. sylvestris* and *N. tomentosiformis* (Fig. 1) showed that *Nicotiana tabacum* cv. Samsun NN contains at least two copies of ACO genes originated from each parent, *N. sylvestris* and *N. tomentosiformis*. This agreed with the observation of Knoester et al. (1995) that ACO is encoded by low copy number genes in tobacco.

Ethylene production precedes necrotic local lesion formation—Increased ethylene production during local lesion formation was first shown by Balázs et al. (Balázs et al. 1969) and detailed examinations were performed thereafter (Nakagaki et al. 1970, Pritchard and Ross 1975, De Laat and Van Loon 1981, 1982, 1983). They have shown that the increase in ethylene production occurs slightly before necrotic lesions become visible (Pritchard and Ross 1975, De Laat and Van Loon 1982), and accumulation of ACC at the site where the virus is actually localized (De Laat and Van Loon 1983). In those reports, the authors concluded that the lesion formation is not the cause of increased ethylene production, being inconsistent with our results that *DS321* was isolated as the cDNA clone expressed 5 h before lesion formation. If the induced expression of ACO gene by the temperature shift actually precedes the lesion formation, ethylene production could contribute to lesion formation. Then we first examined the time-course of ethylene production after the temperature shift. TMV-inoculated leaf disks were incubated at 30°C for 40 h, at which virus multiplication occurs without lesion formation. When these disks were transferred to 20°C at which the N gene is functional, lesions became visible 8 h after the shift, then gradually became clearer in TMV-infected tissue. As shown in Fig. 2a, a considerable level of ethylene production was found only in TMV-infected and temperature-shifted leaves, suggesting that neither TMV-infection nor the temperature shift itself induces ethylene production. The increase was observed 4 h after the shift and continued up to 32 h. Thus, ethylene induction apparently preceded lesion formation in this system.

We next determined the duration of 20°C treatment required to trigger HR, in other words the "point of no-return". One h of 20°C treatment triggered a detectable

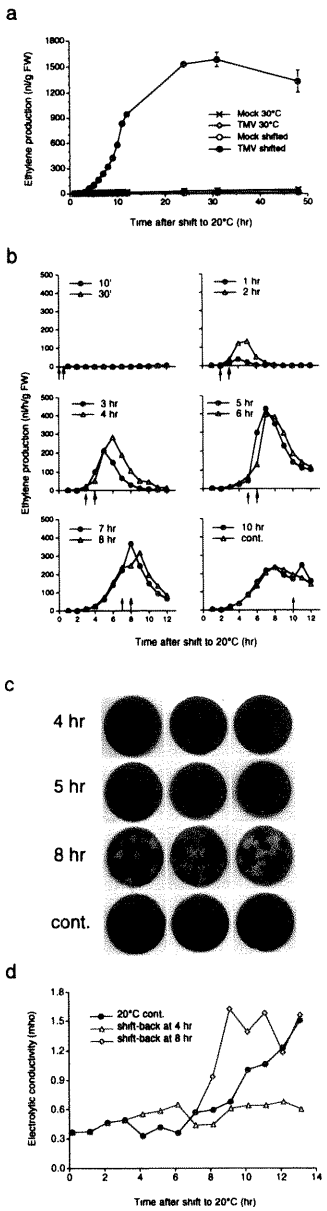

Fig. 2 Ethylene production, lesion formation and ion leakage after the temperature shift. (a) Ethylene production under continuous 20°C after the temperature shift. Fifteen disks measuring 13 mm diameter from mock- or TMV-infected tobacco leaves, which had been incubated for 40 h at 30°C, were carefully transferred to a 50-ml flask. Total ethylene production after the temperature shift to 20°C under continuous light (45 $\mu Em^{-2} s^{-1}$) was measured at the indicated time. (b) Ethylene production under limited 20°C treatment after the temperature shift. TMV-infected tobacco leaf disks after the shift were incubated for the indicated time, then returned to 30°C. Ethylene production was measured every 1 h after the shift. Air in flasks were replaced with fresh air after each measurement. Arrows below each graph indicate the shift-back points from 20°C to 30°C. (c) Leaf disks in the experiment of (b) at 24 h after the shift. (d) Ion leakage from leaf disks after the shift to 20°C. TMV-infected leaf disks of 19 mm diameter were incubated at 20°C, then appropriate numbers were returned to 30°C at 4 h and 8 h respectively. Five disks were picked up every 1 h and conductivity of the solution was measured.

level of ethylene production (Fig. 2b, top-right panel, 1 h), and the levels increased with prolonged 20°C treatment. At least 5 h of 20°C treatment was required for the induction of necrotic lesions in this system (Fig. 2c, 4 h and 5 h). Interestingly, treatment for 6 to 8 h caused more severe necrosis than continuous 20°C treatment (Fig. 2c, 8 h and cont.). The amount of ethylene produced seems to tightly correlate with the severity of visible necrosis observed 24 h after the shift (Fig. 2c). This phenomenon may be explained by accelerated ethylene biosynthesis at 30°C compared to 20°C.

As a marker of HR, ion leakage from TMV-infected leaf disks was examined (Fig. 2d). When the leaf disks were transferred from 30°C to 20°C, ion leakage was detected around 8 h after the shift, simultaneously with the lesion appearance. This is consistent with our previous observation using *N. glutinosa*, which also carries the N gene (Ohashi and Shimomura 1976). Accelerated ion leakage was observed when the leaf disks were returned to 30°C after the 8 h incubation at 20°C, but was not observed after 4 h incubation, indicating that ethylene would be a determinant of the severity of necrosis as observed in Fig. 2c.

Induced expression of ethylene-related genes during HR—Increased ethylene production after the shift would be the result of induced expression of genes involved in ethylene biosynthesis. Then, we performed RNA blot analysis (Fig. 3). Accumulation of ACO mRNA was ob-

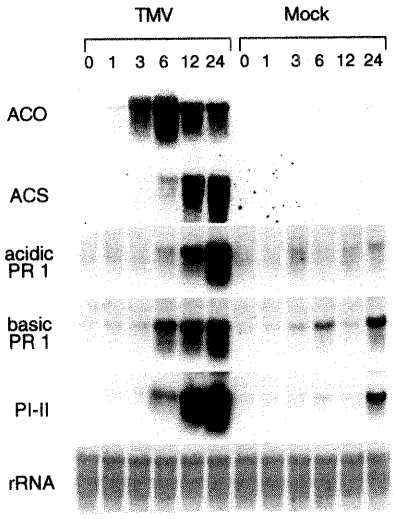

Fig. 3 Expression of ethylene biosynthesis-related genes and PR genes after the temperature shift. RNA blot hybridizations were performed using total RNA from mock- or TMV-infected tobacco leaves harvested at 0, 1, 2, 3, 6, 12 and 24 h after the temperature shift to 20°C. RNA blots were successively hybridized with ^{32}P-labeled probes specific for ACO, ACS, acidic PR-1, basic PR-1 and PI-II. Hybridization with each probe was performed on the same blot. To confirm equable loading of RNA, blots were stained with 0.004% Methylene Blue-0.1×SSC, then destained with H_2O to detect rRNA.

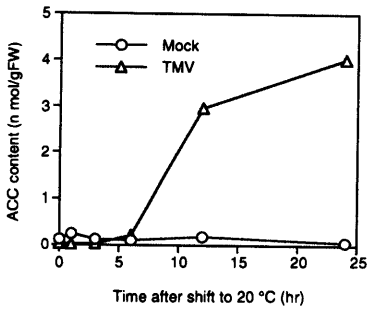

Fig. 4 Accumulation of ACC after the temperature shift. Mock- or TMV-infected tobacco leaves at 0, 1, 3, 6, 12 and 24 h after the temperature shift were harvested and frozen in liquid nitrogen, then subjected to the ACC assay. A mixture of leaf pieces from three separate plants was used.

served 3 h after the shift, preceding the lesion appearance. However, accumulation of ACS mRNA was detected at 6 h and continued to increase even 24 h or more after the shift, similar to that in some PR genes tested. Induction of both ACS and ACO genes was observed in the TMV-infected leaves but not in the mock-infected leaves, indicating that induction of both genes is associated with lesion formation.

Control of HR-dependent ethylene production by ACO—ACS has generally been shown to be a rate-limiting enzyme in the ACC-dependent ethylene biosynthesis pathway (Kende 1993). However, the mode of ethylene production in Fig. 2b seems to reflect the increased expression of ACO gene rather than ACS gene. ACC content in TMV-infected tobacco leaves increased rapidly between 6 h and 12 h after the shift, and slowly up to 24 h (Fig. 4). This profile reflects the mode of accumulation of ACS transcripts. However, actual ethylene production peaked 8 h after the shift and gradually reduced thereafter (Fig. 2b, cont.), suggesting the amount of ethylene produced during lesion formation is determined at the level of ACO rather than ACS.

Suppression of lesion formation and basic PR gene induction by ethylene inhibitors—We next tested the effect of NBD, an inhibitor for ethylene action, on lesion formation. To make the effects of the reagent clear and efficient, we adapted the synchronous lesion formation system. NBD at 9 ml liter^{-1} as the gas phase was added 4 h after the temperature shift when the ethylene production started to increase as observed in Fig. 2a and 2b. In NBD-treated leaves, the clarity of induced lesions was significantly reduced (Fig. 5a). Four h of NBD treatment immediately after the shift did not inhibit lesion formation (data not shown), suggesting that ethylene may play an important role in lesion development at 8 h and thereafter rather than in initial signaling.

Further we tested other inhibitors of ethylene biosynthesis, $CoCl_2$ and AVG, that prevent conversion of ACC into ethylene and SAM into ACC, respectively. As shown in Fig. 5b and 5c, the clarity of necrosis 24 h after the shift was significantly reduced when the leaves were pre-treated with each reagent at 0.1 and 1 mM for 24 h, though AVG was more effective than $CoCl_2$. These results suggest that the lesion formation depends on the activity of the ethylene biosynthetic pathway.

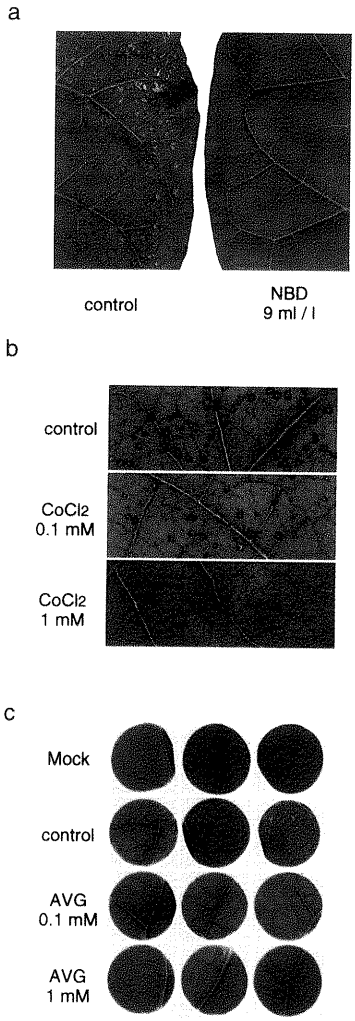

Fig. 5 Inhibition of lesion formation by ethylene inhibitors. Detailed procedures for the treatment of tobacco leaves or leaf disks (pieces) with each reagent are described in the *Materials and methods*. Lesion formation was observed 24 h after the shift to 20°C. (a) NBD treatment. (b) $CoCl_2$ treatment. (c) AVG treatment.

Basic PR-1 and PI-II genes have been shown to be induced by exogenously applied ethylene (Brederode et al. 1991, Eyal et al. 1993, O'Donnell et al. 1996). However, there is no direct evidence of the induction of these genes by ethylene produced in lesion-formed leaves. If the induction depends on the produced ethylene, the amount of

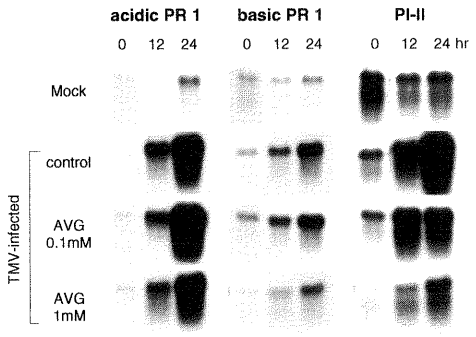

Fig. 6 Effects of AVG treatment on PR gene expression after the temperature shift. Total RNA from the leaf disks harvested at 0, 12 and 24 h after the shift in the experiment in Fig. 5c was used for blotting. Hybridization with each probe was performed on the same blot.

transcripts must be reduced by treatment with ethylene inhibitors. RNA blot analysis showed that the AVG treatment significantly suppressed the induction of basic PR genes 24 h after the shift (Fig. 6), but did not affect the induction of acidic PR-1 gene which is generally thought to be induced by SA. The weak induction of basic PR genes observed in AVG-treated samples would be caused by incomplete inhibition of ethylene biosynthesis even at 1 mM concentration. The inhibitory effect of AVG on the induction of basic PR-1 gene seems to be milder than that of PI-II gene, probably reflecting the difference in sensitivity to ethylene or its saturation level. This result suggests the expression of basic PR genes in tobacco is controlled by endogenously produced ethylene in local lesion formation.

Accelerated lesion formation in ACO-overexpressing transgenic plants—For further analysis of the role of ethylene in HR, we generated transgenic tobacco plants containing sense or antisense ACO cDNA under the control of the strong constitutive promoter El2Ω (Mitsuhara et al. 1996). After selection with kanamycin, 30 sense and 20 antisense lines were investigated to determine steady state levels of ACO mRNA and ACO activity (data not shown). Then two representative "sense" lines, 13-13 with the highest levels of ACO mRNA and enzymatic activity, and 13-X4 with the lowest level of wound-induced ethylene production in the presence of ACC (Fig. 7a) were chosen and subjected to the subsequent experiments. The reduction of ACO activity in 13-X4 is possibly caused by cosuppression because of only a slight increase in the amount of ACO mRNA and enzymatic activity after lesion formation (data not shown). Because we could not obtain antisense transgenic plants defective in ACO activity, we used 13-X4 line instead. To evaluate the effect of ACO on lesion induction, TMV-infected leaf disks were incubated with ACC for 6 h before the temperature shift (Fig. 7b). The length of 20°C exposure was set to 3.5 h, a period insufficient for lesion

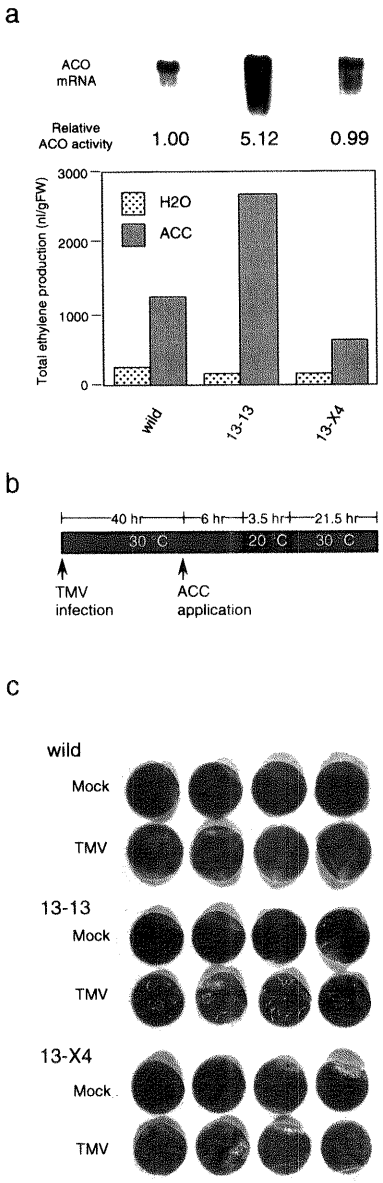

Fig. 7 Acceleration of lesion formation in ACO-overproducing plants by ACC application. (a) ACO mRNA amount, ACO activity and ethylene production in healthy ACO-transgenic tobacco plants. ACO activities are given by relative values. To confirm the actual ability to produce ethylene, a mixture of leaf disks from 3 independent plants of each line were incubated with H_2O or 1 mM ACC at 30°C for 8 h. Total accumulation of ethylene was measured. (b) Method for ACC treatment. Mock- or TMV-infected leaf disks 19 mm in diameter were incubated at 30°C for 46 h, transferred to 20°C and incubated for 3.5 h, then returned to 30°C. One mM ACC was supplied at 6 h before the shift to 20°C. Lesion formation at 24 h after the shift to 20°C was observed. (c) Leaf disks at 24 h after the shift.

induction in wild type leaves even when ACC was added. If ethylene production is a limiting factor for lesion formation, enhancement of lesion development would be ex-

pected in the ACO-overexpressing transgenic line 13-13. As shown in Fig. 7c, clear necrotic rings were observed only on TMV-infected 13-13 leaf disks that had the highest ACO activity. ACC treatment had no effect on mock-infected tobacco leaf disks. This result indicated that lesion formation is enhanced by overproduction of ethylene, resulting from elevated ACO activity and ACC supplement.

Basic PR genes are induced by endogenously produced ethylene—We next confirmed that ethylene-dependent expression of the basic PR genes using transgenic plants. RNA blot analysis revealed that the basic PR genes are induced by ACC treatment in all lines tested (Fig. 8a). Induction of the PI-II gene was significantly higher in the ACO-overexpressed 13-13 line and much lower in ACO-cosuppressed 13-X4 line than in wild type tobacco. Induction of basic PR-1 gene also depended on ACO activity while the difference in the induction rate between the lines was smaller than that of the PI-II gene. This result clearly reflected differences in ethylene production ability between the plant lines (bottom of Fig. 8a) and supports the observation in Fig. 6 that these genes are regulated by endogenously produced ethylene.

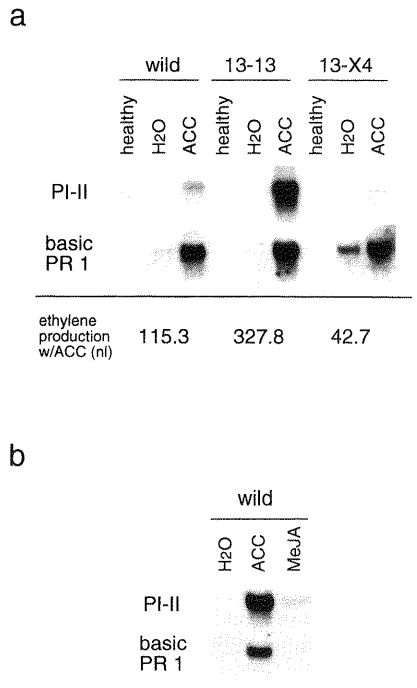

Fig. 8 Induction of basic PR genes by endogenously produced ethylene. (a) Induction of gene expression in leaf disks. Leaf disks were incubated with H_2O or 1 mM ACC at 30°C for 8 h, then subjected to Northern analysis. Total ethylene production in ACC-treated disks (bottom of the panel) was also measured. (b) Induction of gene expression in intact plants. One mM of ACC or 50 μM MeJA was supplied through the roots of unwounded 30-day-old wild type tobacco plants grown under a 16 h light/8 h dark cycle at 28°C. Total RNA was prepared from whole shoots harvested 24 h after application of the reagents and subjected to Northern analysis.

To exclude the possibility of wound-induced expression of the basic PR genes, we performed ACC treatment using 30-day-old intact plants grown in pots at 28°C without wounding. MeJA, which has been shown to be a mediator of wound-based signaling for PI-II or *pin2* gene expression in tomato (Farmer and Ryan 1990, Hildmann et al. 1992, O'Donnell et al. 1996) and for basic PR genes in tobacco (Seo et al. 1995, Niki et al. 1998), was also tested. As shown in Fig. 8b, PI-II gene and basic PR-1 gene were strongly induced by ACC treatment. Both the genes are also induced by MeJA, but the rate of induction was much lower than that by ACC in this system. We concluded that the basic PR genes are effectively induced by endogenously produced ethylene regardless of wound signaling.

Discussion

Using ACO cDNA *DS321* specifically induced at the early stage of HR, we examined the contribution of ethylene to necrotic local lesion formation and induction of basic PR genes. The temperature-dependent lesion induction system made it possible to observe novel and detailed profiles of ethylene biosynthesis, action, and the effect of their inhibitors on HR. In this paper we documented two findings, (a) ethylene production is essential for lesion formation, (b) induction of the basic PR genes during HR is controlled by endogenously produced ethylene. All the results indicate that ethylene is not a by-product but an indispensable factor for HR.

Ethylene directly contributes to the necrotic local lesion formation—Only 1 h exposure of TMV-infected tobacco leaves to 20°C induced the production of ethylene at detectable levels without lesion formation (Fig. 2b), suggesting that ethylene production precedes lesion formation. In addition, intensity of necrosis of induced lesions was depended on the amounts of ethylene produced (Fig. 2b, c). These results clearly indicate the direct contribution of ethylene to lesion formation. Specific inhibitors of ethylene biosynthesis or action inhibited lesion development (Fig. 5), and increased ethylene production in ACO-overproducing plants accelerated lesion appearance (Fig. 7), supporting this idea.

Knoester et al. (1997) reported that sense and antisense ACO transgenic tobacco plants with altered ethylene levels were not impaired in the ability to form local lesions upon TMV infection, and concluded that ethylene is dispensable for HR. Lesion formation is also observed in the line 13-X4 when incubated under continuous 20°C after TMV infection (data not shown). Because ethylene production ability in the line had been lowered but was still sufficient for lesion formation, we probably could not see a clear difference between wild type plant and the transgenic line under continuous 20°C. Only the specific condition used in Fig. 7

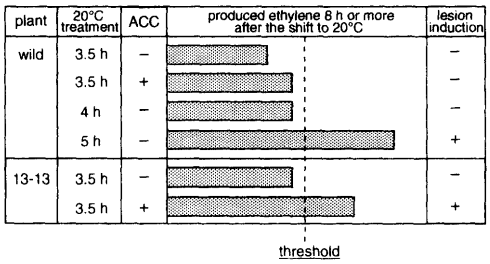

Fig. 9 The "threshold" model for ethylene-dependent local lesion formation in the synchronous lesion formation system.

facilitated observation of the difference in lesion formation ability. This may be also the case in transgenic tobacco harboring the mutant *etr1-1* gene from *Arabidopsis* (Knoester et al. 1998), in which ethylene sensitivity has been reduced but not completely eliminated.

At least 5 h exposure to 20°C was necessary for lesion induction in mature tobacco leaves (Fig. 2c). The most striking difference between the 4 h and 5 h exposure was found at the level of ethylene production around 8 h after the temperature shift, when lesions become visible. Therefore, we speculate that the amount of ethylene over a certain threshold at lesion appearance and thereafter is essential for subsequent lesion development. Fig. 9 represents a model for the relationship between ethylene production and lesion formation. In the wild type plant without ACC addition, over 5 h incubation at 20°C is necessary to produce ethylene over the threshold, while the ACC-treated 13-13 plants produce sufficient ethylene after 3.5 h exposure to 20°C by increasing the basal ethylene level through its increased ACO activity. This threshold model represents dual profiles of ethylene action as proposed by Picton et al. (1993). They suggested that ethylene acts as a switch and also a rheostat for ripening; the small amount of ethylene produced by the ACO-antisense tomato fruit is sufficient to stimulate certain aspects of ripening but a larger amount is required for complete ripening to occur. In our case, the large amount of ethylene produced in the 5 h-treated leaves must be necessary to induce necrosis while the amount below the threshold would be sufficient to trigger other ethylene-dependent events, such as basic PR gene induction.

Bufler et al. (1980) and Mayak et al. (1981) showed that the ACC level is kept high even after the rate of ethylene production lowers (Bufler et al. 1980), and found this ACS-independent control of ethylene production is caused by the reduction of ACO activity (Mayak et al. 1981). These results strongly support our idea of ACO-dependent control of ethylene biosynthesis.

Basic PR gene expression during HR is induced by endogenously produced ethylene—Induction of the basic PR-1 and PI-II genes after the temperature shift was diminished by AVG treatment while that of acidic PR-1 gene was not affected (Fig. 6). This is consistent with the findings that genes encoding basic PR proteins as well as stress-induced type I and type II serine proteinase inhibitors are induced by ethylene (Brederode et al. 1991, Eyal et al. 1993, Sessa et al. 1995, Shinshi et al. 1995, Sato et al. 1996). O'Donnell et al. (1996) showed that the induction of tomato proteinase-inhibitor gene (*pin2*) induction by wound, ethylene, oligogalacturonide, systemin and jasmonic acid (JA) is abolished by treatment with inhibitors of ethylene or JA and concluded that both ethylene and JA are necessary for induction. PI-II gene expression in ACC-treated leaf disks (Fig. 8a) clearly reflect the ACO activity as well as actual ethylene production in each transgenic line (Fig. 7a), indicating ethylene-dependent regulation of the gene. Regarding the H_2O-treated control in Fig. 8a as wound-induced expression, we can speculate that induction of PI-II gene expression during HR is much stronger than that by wounding. The amount of ethylene produced by lesion formation (over 400 nl h^{-1} (g FW)$^{-1}$ in Fig. 2b) is also higher than that by wounding (up to 10 nl h^{-1} (g FW)$^{-1}$), supporting this idea.

The strong induction of basic PR-1 gene by ACC in all lines tested would be explained by a higher sensitivity to ethylene, and the level of induction may almost reach the upper limit under the condition we used. This is consistent with the observation in Fig. 6, in which the basic PR-1 gene is almost fully induced in the presence of 0.1 mM AVG. Induction of basic PR-1 gene in H_2O-treated 13-X4 disks in Fig. 8a is reproducible and may be caused by an increased wound response in this line, while the mechanism involved is unknown. In contrast, ACC application enhanced the PI-II gene expression only 1.6-fold in 13-X4, excluding the possibility of ACC acting as a signal molecule as reported by Wang et al. (1996). It remains important to elucidate the difference in regulatory mechanisms between basic PR-1 and PI-II genes to understand the overall ethylene-dependent plant defense response.

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