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Research Note

Virus-Induced Gene Expression for Enzymes of Ethylene Biosynthesis in Hypersensitively Reacting Tobacco

Marga Knoester, John F. Bol, Leendert C. van Loon,[1] and Huub J.M. Linthorst

Institute of Molecular Plant Sciences, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands; [1]Department of Plant Ecology and Evolutionary Biology, Utrecht University, P.O. Box 80084, 3508 TB Utrecht, The Netherlands

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A full-length cDNA clone (cEFE-26) encoding ethylene-forming enzyme (EFE) was isolated from a cDNA library, prepared from leaves of tobacco mosaic virus (TMV)-infected tobacco cultivar Samsun NN. The cDNA clone encodes a protein with 90% amino acid sequence similarity to established EFEs of tomato and other plants. By using cEFE-26 cDNA and the insert from cDNA clone pACC13 (B. A. Bailey, A. Avni, N. Li, A. K. Mattoo, and J. D. Anderson, Plant Physiol. 100:1615-1616, 1992) encoding tobacco 1-aminocyclopropane-1-carboxylic acid synthase as probes, it was established that tobacco contains small gene families for these proteins. Furthermore, RNA blot analyses indicated that transcript levels in leaves for the two ethylene pathway genes were elevated after infection with TMV. The results are discussed in relation to a possible signalling role of ethylene in induced resistance and gene expression for pathogenesis-related proteins.

Additional keywords: ACC synthase, ethylene-forming enzyme, pathogenesis-related proteins, PR proteins, resistance, signal transduction.

Ethylene controls many physiological and developmental processes in plants. These include fruit-ripening, leaf abscission, and flower senescence (for reviews see Yang and Hoffmann 1984; Kende 1993). Ethylene is also involved in the response of plants to wounding, pathogen attack, and other environmental stresses. In tobacco hypersensitively reacting to infection with tobacco mosaic virus (TMV), ethylene production sharply rises to levels 10-fold that of nonstressed plants at 48 hr after inoculation, coinciding with the appearance of necrotic local lesions (De Laat and Van Loon 1982). This hypersensitive reaction is accompanied by the development of acquired resistance against infection by various types of pathogens and associated with the induction of several classes of acidic, as well as basic pathogenesis-related (PR) proteins (Van Loon 1989). The subset of the basic PR proteins from tobacco accumulates in the vacuole. Several of these PR proteins, notably the basic β-1,3-glucanases (PR-2), chitinases (PR-3), PR-4 proteins, and osmotins (PR-5), have been shown to possess antifungal activity *in vitro* (Woloshuk *et al.* 1991; Ponstein *et al.* 1993) and *in vivo* (Sela-Buurlage *et al.* 1993), while the stress-inducible type I and type II serine proteinase inhibitors (PI; Linthorst *et al.* 1993; T. Balandin, C. Van der Does, J. M. Bellés Albert, J. F. Bol, and H. J. M. Linthorst, manuscript submitted) are believed to be involved in resistance to bacterial and insect attack (Ryan 1990). The genes encoding these proteins are highly responsive to exogenous ethylene (Brederode *et al.* 1991) and it is likely that their induced expression is the result of the TMV-induced rise in endogenous ethylene.

The sharp peak in ethylene production near the time of lesion appearance is preceded by high transitory increases in 1-aminocyclopropane-1-carboxylate (ACC) synthase activity and ACC content. Thereafter, the activity of ethylene-forming enzyme (EFE), which converts ACC into ethylene, also increases, resulting in an enhanced capacity for ethylene production throughout the subsequent period in which the expansion of the necrotic lesions ceases and PR proteins accumulate to high levels (De Laat and Van Loon 1982; Van Loon 1989). Moreover, EFE activity is increased systemically, in conjunction with the development of acquired resistance in noninoculated leaves (De Laat and Van Loon 1983).

In recent years significant progress has been achieved in modulating endogenous ethylene production. By expression of antisense or sense EFE and ACC synthase genes in transgenic tomato plants, it was shown that ethylene-dependent fruit-ripening processes could be successfully manipulated (for review see Fray and Grierson 1993). The present work focuses on the tobacco genes involved in ethylene biosynthesis. As a first step in the study of the role of ethylene in the induction of resistance mechanisms to pathogen attack, we have isolated cDNA clones encoding EFE and characterized EFE and ACC synthase gene expression in tobacco upon TMV infection.

Corresponding author: H. J. M. Linthorst, Institute of Molecular Plant Sciences, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands.

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The construction of the lambda ZAP cDNA library from TMV-infected tobacco (*Nicotiana tabacum* 'Samsun NN') leaf was as described (Linthorst *et al.* 1990). Recombinant phages were screened at low stringency, using a ^{32}P-labeled insert from EFE cDNA clone pRC13 from tomato (Hamilton *et al.* 1991), resulting in 11 positive clones. Restriction analysis indicated that the clones belonged to two different groups. The longest clone of each group was further characterized by sequence analyses. Figure 1 shows the complete nucleotide sequence of tobacco cDNA clone cEFE-26. The cDNA insert of cEFE-26 is 1,215-bp long, excluding a poly(A) tail of 16 residues and contains the complete open reading frame for a protein of 319 amino acids (Fig. 1). This 36-kDa protein is highly similar to EFEs from other plant species, including tomato (88.5% identity, Holdsworth *et al.* 1987) and *Petunia hybrida* (90.9% identity, Wang and Woodson 1992). Clone cEFE-27, belonging to the other group, was only partially sequenced and appeared >90% identical to cEFE-26 (results not shown). These results indicate that tobacco contains at least two active genes for EFE.

The left panel of Figure 2 shows the results of a DNA blot analysis with *Eco*RI- and *Hin*dIII-digested genomic DNA hybridized to the radioactively labeled cDNA insert from cEFE-26. In both digests, approximately four intense and four less intense bands were distinguished. The right panel of Figure 2 contains the outcome of hybridization of a similar genomic DNA blot with the labeled insert of the tobacco ACC synthase cDNA clone pACC13 (Bailey *et al.* 1992). Both *Eco*RI- and *Hin*dIII-digested genomic DNA contained four hybridizing fragments of which two gave a strong hybridization signal and the others gave weaker signals. Thus it appears that both EFE and ACC synthase are encoded by low copy number genes in tobacco.

In tomato, ACC synthase and EFE activity are induced upon elicitor treatment and infection with *Phytophthora infestans* (Spanu and Boller 1989) and for EFE it was shown that the induction was at least partly based on enhanced gene expression (Spanu *et al.* 1991). To determine whether the increased ethylene production in tobacco upon infection with TMV is also the result of induced gene expression, the cDNA inserts of clones cEFE-26 and pACC13 were used as probes in RNA blot hybridizations. Total RNA from leaves of TMV-infected tobacco cv. Samsun NN was isolated as described (Linthorst *et al.* 1993). Figure 3A demonstrates that the level at which ACC synthase mRNA (top panel, left), as well as EFE mRNA (bottom panel, left) accumulated in the inoculated leaf was elevated by TMV infection (local). It is evident that the amounts of both ACC synthase and EFE mRNA increased at 36 hr after inoculation and remained high at later stages of infection. In a similar time course experiment it was found that EFE mRNA accumulation remained at this elevated level at least until 6 days after inoculation, when ethylene-induced leaf senescence had developed to the stage that RNA could no longer be isolated (results not shown). These results indicate that the increased production of ethylene during the hypersensitive response of tobacco to infection with TMV is the result of induced expression of the genes for the

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                                                                  M
   1  TCTATCTATTATTCACACACTATAGCTCAAAAACACAGTCTTTATTCTACAAGAAAGAT
      E  N  F  P  I  I  N  L  E  K  L  N  G  S  E  K  A  A  T  M
  61  GGAGAACTTCCCAATTATCAACTTGGAAAAACTGAATGGTTCTGAAAAAGCTGCTACCAT
      E  M  I  K  D  A  C  E  N  W  G  F  F  E  L  V  N  H  G  I
 121  GGAAATGATTAAGGATGCTTGTGAAAACTGGGGCTTCTTTGAGTTGGTGAACCATGGAAT
      P  H  E  V  M  D  T  V  E  K  L  T  K  G  H  Y  K  K  C  M
 181  CCCCCATGAAGTAATGGACACTGTAGAGAAATTAACAAAGGGCATTACAAGAAATGCAT
      E  Q  R  F  K  E  L  V  A  S  K  G  L  E  G  V  Q  A  E  V
 241  GGAACAGAGGTTTAAGGAATTGGTGGCCAGCAAAGGTCTTGAAGGCGTACAAGCTGAGGT
      T  D  M  D  W  V  C  T  F  F  L  R  H  L  P  V  S  N  I  S
 301  TACTGATATGGATTGGGTTTGCACTTTCTTCTTGCGCCATCTTCCTGTTTCTAACATTTC
      E  V  F  D  L  D  D  Q  Y  R  E  V  M  R  D  F  A  K  R  L
 361  TGAAGTCCCTGATCTTGATGATCAATACAGGGAGGTTATGAGAGATTTTGCTAAAAGATT
      E  N  L  A  E  E  L  L  Y  L  L  C  E  N  L  G  L  E  K  G
 421  AGAGAATCTAGCAGAGGAGCTGTTGTATTTGCTCTGTGAAAATCTTGGTCTAGAAAAGGG
      Y  L  K  N  V  F  Y  G  S  K  G  P  N  F  G  T  K  V  S  N
 481  ATACTTGAAAAATGTATTTTATGGATCTAAAGGTCCAAACTTTGGAACTAAAGTGAGCAA
      Y  S  P  C  P  K  P  D  L  I  K  G  L  R  A  H  T  D  A  G
 541  TTATTCACCATGCCCAAAACCAGATTTGATTAAGGGACTGCGCGCCCATACGGACGCTGG
      G  I  I  L  L  F  Q  D  D  K  V  S  G  L  Q  L  L  K  D  G
 601  TGGCATAATCCTTCTCTTCCAAGATGACAAAGTAAGCGGCCTACAACTCCTCAAAGACGG
      Q  W  I  D  V  P  P  M  R  H  S  I  V  V  N  L  G  D  Q  L
 661  CCAATGGATCGATGTTCCTCCTATGCGCCACTCCATCGTCGTTAACCTTGGCGATCAACT
      E  V  I  T  N  G  K  Y  K  S  V  M  H  R  V  V  A  Q  K  D
 721  TGAGGTGATCACAAATGGGAAGTACAAGAGTGTGATGCACAGAGTGGTAGCGCAAAAAGA
      G  T  P  M  S  L  A  S  F  Y  N  P  G  S  D  A  V  I  Y  P
 781  CGGGACTCGGATGTCATTAGCTTCTTTCTATAATCCAGGAAGTGATGCAGTGATTTATCC
      A  P  A  L  V  E  K  E  A  A  E  S  K  Q  V  Y  P  K  F  V
 841  AGCACCAGCTCTTGTTGAGAAAGAGGCAGCGGAGAGCAAACAAGTTTATCCCAAATTTGT
      F  D  D  Y  M  K  L  Y  A  G  L  K  F  Q  A  K  E  P  R  F
 901  GTTTGATGATTACATGAAGTTATACGCCGGACTAAAGTTTCAGGCCAAGGAGCCAAGGTT
      E  A  M  K  S  I  E  S  D  V  K  M  D  P  I  V  T  A  *
 961  TGAAGCCATGAAATCTATTGAATCTGATGTCAAGATGGATCCAATTGTAACTGCATAGAT
1021  CCAAATTCCAGACTACTAAAGATGAAAATTGAAAGAAAATGATTTGTTATTGAAGTAGTA
1081  CAAGACGAGTGACATACATTATTTGCTTGTTTTGGTATAGTGGATTAATATATTTACAAA
1141  AGTGTGTGTCCCTAGTACATATGTAGTCAACAGGCTTAAAGTTTGTATGTTATAATAAAC
1201  TGATAAAGTTTCCTT[A]n
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of tobacco EFE cDNA clone cEFE-26. The polyadenylation signal (underlined AATAAA) and poly(A) tail ([A]$_n$) are indicated

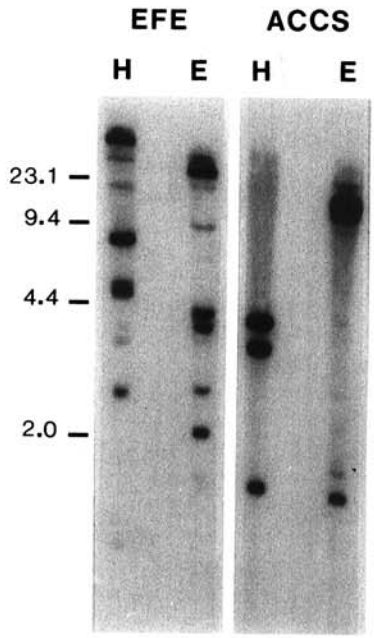

Fig. 2. Complexity of ethylene pathway genes in tobacco genomic DNA. Genomic DNA was completely digested by *Hin*dIII (H) or *Eco*RI (E), electrophoresed, blotted, and hybridized with ^{32}P-labeled cDNA inserts of clones cEFE-26 (EFE) and pACC13 (ACCS). After hybridization the blots were washed in 1.2 M NaCl, 0.12 M Na-citrate at 55° C. The sizes (kb) of coelectrophoresed marker DNAs are indicated.

two enzymes involved in ethylene synthesis. The high expression of EFE and ACC synthase genes at 36 hr after inoculation, as measured at the transcript level, corresponds well with the increased ACC and ethylene production (De Laat and Van Loon 1982) and is slightly ahead of local lesion appearance.

The noninoculated (systemic) leaves, directly above the TMV-infected leaves, were also sampled to study the accumulation of ACC synthase and EFE mRNAs. The top panel at the right of Figure 3A shows that—even after long exposure—ACC synthase mRNA was not detectable in the systemic leaves at any time from 2 to 12 days after inoculation. However, as shown in the bottom panel at the right, EFE mRNA transiently accumulated to slightly higher levels in these leaves at 4 days after inoculation. This correlates with the enhanced EFE activity in the systemic leaves, as was found by De Laat and Van Loon (1983). The fact that induced EFE gene expression and EFE enzyme activity in the systemic leaves did not result in increased ethylene production (De Laat and Van Loon 1983), is in accordance with the fact that conversion of *S*-adenosyl-methionine by ACC synthase is the rate-limiting step in the pathway (De Laat *et al.* 1981).

PR proteins can be divided in groups of extracellular and intracellular proteins. The genes encoding intracellular PR proteins in different plants are highly responsive to ethylene. In tobacco, only the genes for the intracellular PR proteins and the vacuolar PI-I and PI-II are also highly expressed upon treatment with the ethylene precursor ethephon (Brederode *et al.* 1991; Linthorst *et al.* 1993; T. Balandin, C. Van der Does, J. M. Bellés Albert, J. F. Bol, and H. J. M. Linthorst, manuscript submitted). Although ethephon may have additional ethylene-independent effects in *Arabidopsis*, where it also induces the genes for extracellular PR proteins (Lawton *et al.* 1994), this is apparently not the case in tobacco (Brederode *et al.* 1990). The absence of TMV-induced expression of the ACC synthase genes in the noninoculated, systemic leaves corresponds with the absence of TMV-induced gene expression for vacuolar PR and PI proteins in these leaves (Brederode *et al.* 1991; Linthorst *et al.* 1993; T. Balandin, C. Van der Does, J. M. Bellés Albert, J. F. Bol, and H. J. M. Linthorst, manuscript submitted). Together, these results support the concept that the expression of the subgroup of the basic, vacuolar PR proteins and of the PI proteins in tobacco is, at least partly, controlled by endogenous ethylene. Future experiments with transgenic tobacco plants having altered levels of ethylene synthesis by expression of sense or antisense EFE or ACC synthase genes are expected to provide a more conclusive evidence for a direct involvement of ethylene in PR gene expression.

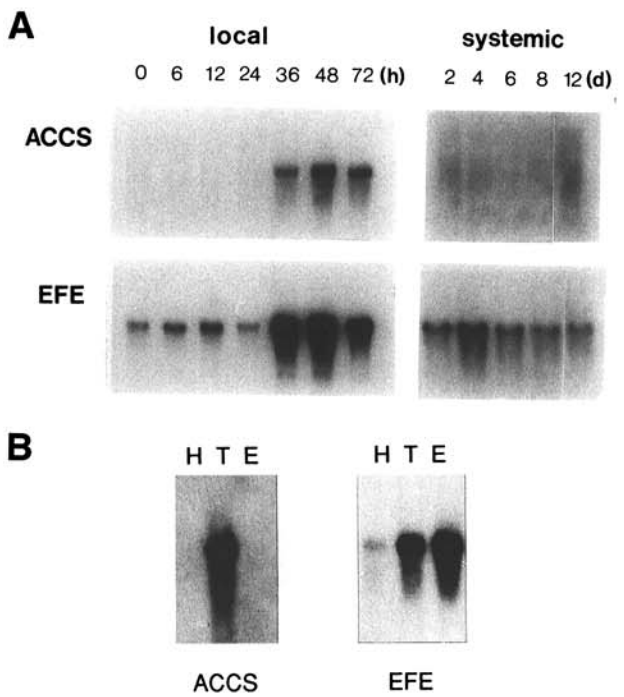

Fig. 3. Induced accumulation of mRNAs encoding ethylene pathway enzymes upon TMV infection and ethephon treatment. **A,** Total RNA was isolated from tobacco leaves at different times (h or d, as indicated) after inoculation. In the panels at the left the samples were from the inoculated leaf (local) and at the right the samples were from the noninoculated leaf directly above the infected leaf (systemic). The exposure time of the autoradiographs at the right was four times longer than that of the blots at left. **B,** Total RNA was from tobacco leaves which were noninfected (H), inoculated with TMV 3 days earlier (T), or treated with 10 mM ethephon (E). The RNA was denatured, electrophoresed, blotted, hybridized to ^{32}P-labeled cDNA inserts from clones cEFE-26 (EFE) or pACC13 (ACCS), and autoradiographed.

It has been recognized previously that ethylene is a positive regulator of gene expression leading to its own synthesis in senescing *Dianthus caryophyllus* flowers (Woodson *et al.* 1992) and ripening tomato fruits (Picton *et al.* 1993). Figure 3B shows that in tobacco leaf only EFE gene expression is induced by ethephon, while ACC synthase transcript levels are not elevated by this treatment. Since ACC production is the rate-limiting step in ethylene biosynthesis, this result suggests that in tobacco leaf ethylene cannot positively regulate its own synthesis.

By the same technique we have identified enhanced EFE and ACC synthase gene expression in pollinated ovaries during later stages of development, as well as relatively high EFE gene expression in senescing flower petals (results not shown).

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