

RESEARCH PAPER

# The role of ethylene during the infection of *Nicotiana tabacum* by *Colletotrichum destructivum*

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## Abstract

Two periods of increased ethylene production were observed after inoculation of *Nicotiana tabacum* by *Colletotrichum destructivum*. This pathogen exhibits an intracellular hemibiotrophic infection process, with a biotrophic phase followed by a necrotrophic phase. Ethylene production first increased during the biotrophic phase with a peak at 24 h before the necrotrophic phase. A second increase in ethylene occurred late in the necrotrophic phase when the lesions were expanding. Two different 1-aminocyclopropane-1-carboxylic acid synthase genes showed increased expression after the first ethylene peak with a maximum at 24 h before the second ethylene increase. Expression of an 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) gene increased during the first ethylene peak and then declined at the beginning of the second ethylene increase. A second ACO gene showed relatively little change in expression during infection with slightly higher expression at 24 h before the second ethylene increase, and a third ACO gene showed a progressive decline in expression with a major decrease occurring before the second ethylene increase. Inoculation of ethylene-insensitive tobacco with *C. destructivum* revealed that it was more susceptible than the wild type. The changes in ethylene production and associated gene expression as well as the increased disease susceptibility of ethylene-insensitive tobacco indicate that ethylene plays a role in this interaction, perhaps as a signalling molecule to trigger defense mechanisms.

Key words: 1-aminocyclopropane-1-carboxylic acid oxidase,

1-aminocyclopropane-1-carboxylic acid synthase, *Colletotrichum destructivum*, ethylene, *Nicotiana tabacum*.

## Introduction

Ethylene is a plant hormone that has been associated with the response of plants to wounding, pathogen attack, and other stresses (Arshad and Frankenberger, 2002). Ethylene produced after pathogen attack may be a stimulus for defence responses by regulating a wide range of defence-related genes, including those encoding pathogenesis-related (PR) proteins, such as chitinase and osmotin (Deikman, 1997). However, ethylene may also play a role in disease symptom development. There is a strong correlation between the timing of increased ethylene evolution following infection and the development of chlorotic, necrotic or wilt symptoms (Boller, 1991; Elad, 1990; Goto *et al.*, 1980; Stall and Hall, 1984).

Recent studies using ethylene-insensitive or ethylene-overproducing mutants have revealed the complexity of the role of ethylene in diseased plants. Ethylene appears to be able to promote either disease resistance or susceptibility depending on the particular plant–pathogen combination. For example, ethylene-insensitive tobacco showed an increased susceptibility to infection by several soil-borne pathogens, including *Pythium sylvaticum* which is not normally a pathogen of tobacco, but retained the hypersensitive reaction (HR) to tobacco mosaic virus (TMV) as wild-type plants (Knoester *et al.*, 1998; Geraats *et al.*, 2002). Ethylene-insensitive soybean mutants produced less severe chlorotic symptoms when challenged with *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae*, whereas *Septoria glycines* and

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; EIX, ethylene biosynthesis-inducing xylanase; GFP, green fluorescent protein; HPI, hours post-inoculation; HR, hypersensitive reaction; PR, pathogenesis-related proteins; TMV, tobacco mosaic virus.

*Rhizoctonia solani* caused similar or more severe symptoms on these mutants (Hoffman *et al.*, 1999).

In higher plants, the rate-limiting reaction of ethylene biosynthesis is the conversion of *S*-adenosylmethionine into 1-aminocyclopropane-1-carboxylic acid (ACC), which is catalysed by ACC synthase (ACS). ACC is then converted into ethylene by ACC oxidase (ACO), previously called the ethylene-forming enzyme (EFE) (Adams and Yang, 1979; Kende, 1993). Both enzymes play a role in the regulation of ethylene biosynthesis and are encoded by gene families. In *N. tabacum*, nine ACS genes have been identified thus far: *NT-ACS1* to *NT-ACS5* (Ge *et al.*, 2000), and *NT-ACS6* to *NT-ACS9* (GenBank accession numbers AF393206, AF393207, AF392978, and AF392979). Five ACO genes have also been found in *N. tabacum*: *EFE-26*, *EFE-27*, *DS321* (which is 98.9% identical to *EFE-27*), *NT-IAIC*, and *NT-ACO1* (GenBank accession numbers X83229 and X98493; Herbers *et al.*, 1995; Knoester *et al.*, 1995; Ohtsubo *et al.*, 1999).

Plant stresses associated with increased ethylene production have often been related to increased ACS and ACO gene expression. In tobacco, for example, *NT-ACS1* was induced during the HR to TMV as well as by an ethylene-inducing xylanase, and *NT-ACS2* was induced by multiple environmental stresses, such as wounding, chilling and sunlight (Avni *et al.*, 1994; Ge *et al.*, 2000; Knoester *et al.*, 1995). *NT-ACS3*, *NT-ACS4* and *NT-ACS5* were all induced by wounding (Ge *et al.*, 2000). The tobacco ACO genes, *EFE-26*, *EFE-27* and *DS321*, all showed increased expression during the HR to TMV (Knoester *et al.*, 1995; Ohtsubo *et al.*, 1999). However, thus far, no tobacco ACS or ACO genes have been examined for their responses to fungal attack.

*Colletotrichum destructivum* causes anthracnose disease of tobacco, as well as a number of other important crops (Shen *et al.*, 2001). Its infection strategy is described as intracellular hemibiotrophy, in which the pathogen is able to colonize its host without causing disease symptoms during an initial biotrophic phase of infection (Bailey *et al.*, 1992). At approximately 72–96 h post-inoculation (HPI), the biotrophic phase switches to a necrotrophic phase when disease symptoms of water-soaked lesions appear (Shen *et al.*, 2001).

For diseases caused by *Colletotrichum* spp., increased ethylene production was observed in melon plants, bean plants and bean cell cultures in response to infection by *C. lagenarium* or exposure to elicitors (i.e. chitin oligosaccharides or cell wall fragments) derived from this fungus (Esquerré-Tugayé *et al.*, 1993; Roby *et al.*, 1985, 1986, 1991). Increased ethylene production has also been observed in tomato, mango and apple fruits inoculated with *C. gloeosporioides*, and was correlated with the development of rot symptoms (Cooper *et al.*, 1998; Schiffmann-Nadel *et al.*, 1985).

To understand more fully the possible role of ethylene in the plant response to pathogen attack, an investigation was undertaken on the induction of ethylene production and the expression of several members of the ACS and ACO gene families following infection of *N. tabacum* by *C. destructivum*. Ethylene-insensitive transgenic Tetr1 tobacco plants expressing the mutant arabidopsis *etr1-1* gene (Knoester *et al.*, 1998) were also studied to determine the effect of ethylene perception on disease development.

## Materials and methods

### Fungal and plant materials

*C. destructivum* N150P3, which is a transgenic strain expressing green fluorescent protein (Chen *et al.*, 2002), was cultured on sodium chloride–yeast extract–sucrose agar medium (Mandanhar *et al.*, 1986). Cultures were incubated under continuous fluorescent lighting ( $60 \mu\text{mol s}^{-1} \text{m}^{-2}$ ) at 25 °C. Conidia were obtained from 7–10-d-old cultures and suspended in sterile distilled H<sub>2</sub>O for plant inoculations.

Seeds of transgenic Tetr1 *N. tabacum* cv. Samsun NN (kindly provided by Dr M Knoester, Utrecht University, The Netherlands) (Knoester *et al.*, 1998), were germinated on Murashige and Skoog medium containing  $100 \mu\text{g ml}^{-1}$  kanamycin. Plantlets were transferred to PRO-MIX 'BX' soil. Wild-type *N. tabacum* cv. Samsun NN, and Tetr1 plants were grown with 16 h light at  $200 \mu\text{mol s}^{-1} \text{m}^{-2}$  with 25/17 °C day/night temperatures.

### Inoculation and disease analysis

Wild-type or Tetr1 *N. tabacum* plants that had been habituated in a plastic-lined container for 12 h were inoculated with  $2 \times 10^5$  conidia  $\text{ml}^{-1}$  for lesion number and GFP measurements or  $2 \times 10^6$  conidia  $\text{ml}^{-1}$  for ethylene and RT-PCR analysis. Non-inoculated control plants were sprayed with sterile distilled water. The sprayed plants were then re-enclosed immediately in the plastic-lined container and incubated at 25 °C at low light ( $15 \mu\text{mol s}^{-1} \text{m}^{-2}$ ) for up to 4 d. At 96 HPI, leaves were excised, the number of lesions were counted, and the leaf area was measured with an area meter (Model 3100 Li-Cor, Lincoln, NB). To quantify fungal biomass, the samples were stored at –80 °C until fluorimetric measurement of green fluorescent protein (GFP) expressed by the fungus (Chen *et al.*, 2002). Lesion numbers, leaf area and GFP fluorescence intensity were determined using 15–27 leaves from 5–9 plants, and the experiment was repeated. Statistical comparisons were made between Tetr1 and wild-type plants using PROC GLM in SAS ver. 6.12 (SAS Institute, Cary, NC).

### Ethylene analysis

Two fully expanded leaves without petiole were detached from a plant at the 5-leaf stage after the inoculation, weighed immediately and then enclosed in a 30 ml vial with a rubber septum. Following a 3 h incubation at 23 °C under light ( $30 \mu\text{mol s}^{-1} \text{m}^{-2}$ ), 1 ml gas samples were withdrawn through the rubber seal with a gas-tight syringe, and the ethylene concentration was measured using a gas chromatograph (CP-3800, Varian, Walnut Creek, CA) equipped with a fused silica capillary column (Model PQ 1277–06, 30 m C 0.53 mm ID, Supelco, Oakville, ON) and a flame-ionization detector. Standard curves generated with pure ethylene (Aldrich, Milwaukee, WI) were created by measuring area counts from known concentrations of ethylene. The data were obtained using 4–6 leaves from 2–3 plants. The experiment was done twice. Data were analysed using PROC GLM in SAS ver. 6.12.

### Reverse transcription-polymerase chain reaction (RT-PCR) analysis

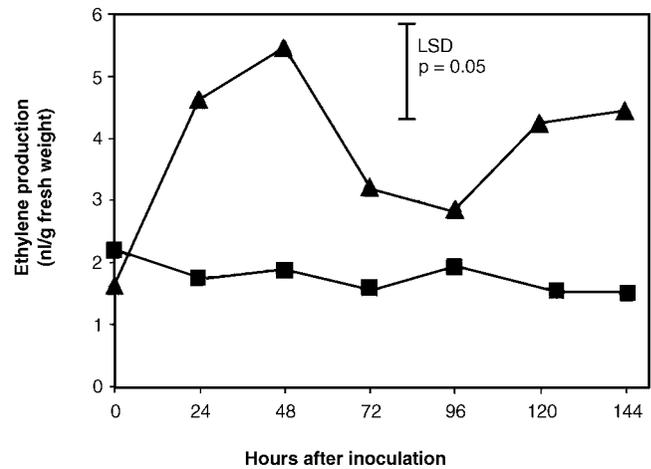
Total RNA was extracted from inoculated tobacco plants following Chen *et al.* (2002). First-strand cDNA was synthesized using SuperScriptII Reverse Transcriptase (Invitrogen Life Technologies; Burlington, ON) and oligo (dT) primers. An RNA sample lacking the reverse transcription step was used to check for the absence of genomic DNA contamination in the RNA samples. Specific primers were designed based on the sequences of *NT-ACS1*, *NT-ACS2*, *EFE-26*, *DS321*, *NT-1A1C* (GenBank accession numbers X65982, AJ005002, Z29529, AB012857, and X83229) and *NT-TEF1- $\alpha$* , a *N. tabacum* translation elongation factor 1 $\alpha$  gene (GenBank Accession No. AF120093, Table 1). Specificity of the primers was checked by excising the RT-PCR products after electrophoresis and sequencing. Relative RT-PCR was done by co-amplifying portions of each of the ACS and ACO genes with *NT-TEF1- $\alpha$*  (Dean *et al.*, 2002). The 30  $\mu$ l PCR reactions contained 3  $\mu$ l of cDNA with 2.5 U *Tsg* polymerase (Biobasic, Toronto, ON), 1 $\times$  *Tsg* buffer (50 mM KCl, 10 mM TRIS-HCl, pH 9.0), 2 mM dNTPs, 2.5 mM Mg<sup>2+</sup>, 0.5 mM NTEF-1 $\alpha$  primers, and 2 mM of the different NT-ACS or NT-ACO primers (Table 1). The PCR amplification conditions were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, an annealing temperature (Table 1) for 1 min, and 72 °C for 1 min, and then one cycle of 72 °C for 7 min. The experiment was repeated at least three times.

The relative transcript amounts were quantified by scanning the bands from the images of agarose gels after electrophoresis, and then quantifying the bands with the National Institute of Health Scion Image Program (Scion Corporation, Frederick, MD). Relative transcript levels of the genes of interest were calculated from band intensity of the ACS or ACO PCR products as a percentage of the amount of the corresponding band intensity of the translation elongation factor PCR product in the same lane.

## Results

### Ethylene production of tobacco induced by *C. destructivum*

In non-inoculated *N. tabacum* plants, ethylene production remained at a consistently low level (Fig. 1). In inoculated plants, however, a significant increase in ethylene was



**Fig. 1.** Ethylene production in inoculated (triangles) or non-inoculated (squares) leaves. Data presented are overall means from two separate experiments, and 4–6 leaves from 2–3 plants were measured at each time point. The error bar represents LSD (least significant difference) at  $P=0.05$  calculated using PROC GLM of SAS® ver. 6.12.

detected by 24 HPI that reached its highest level at 48 HPI during the biotrophic phase before any symptoms were visible. Ethylene production then began to decrease, and symptoms of small (2–3 mm) water-soaked lesions first appeared at 72–96 HPI. At 120 and 144 HPI, there was a second increase in ethylene production. At 120 HPI, the lesions were expanding, and almost all of the leaf tissue was damaged or dead by 144 HPI.

### Differential expression of ethylene biosynthesis genes in tobacco infected by *C. destructivum*

In inoculated plants, relative expression of *NT-ACS1* increased only slightly up to 48 HPI, when the plants were nearing the end of the biotrophic phase, but then showed a greater rate of increase to 72 HPI when

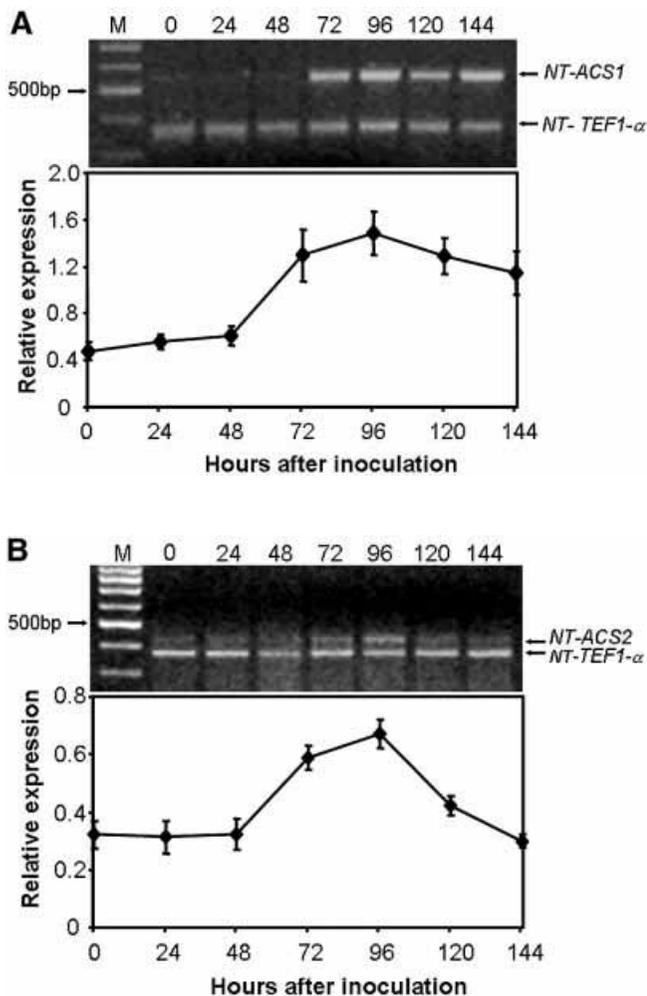
**Table 1.** Sequences, names and annealing temperatures of the primers used for RT-PCR analysis, their PCR fragment sizes, and the corresponding genes and GenBank accession numbers used to design the primers

Primer name	Sequence (5'–3')	Gene	Accession number	Annealing temperature (°C)	Fragment size (bp)
S-TOBEF	CTCCAAGGCTAGGTATGATG	<i>NT-TEF1-<math>\alpha</math></i>	AF120093	45–60 <sup>a</sup>	370
A-TOBEF	CTTCGTGGTGCATCTCAAC				
S-EFE26	AGAGAATCTAGCAGAGGAGC	<i>NT-EFE26</i>	Z29529	48	677
A-EFE26	GTATGTCACTCGTCTTGTACT				
S-DS321	ATTGGCAGAGGAGTTACTGG	<i>DS321</i>	AB012857	48	616
A-DS321	CCATCCTTCCTCTCGAATTG				
S-1A1C	TCCTCTTAGACTACCAAGACA	<i>NT-1A1C</i>	X83229	48	416
A-1A1C	GGTGATTGCTCAGCCTGAT				
ACS1-1	GACCTTACCAACCAAC	<i>NT-ACS1</i>	X65982	59	553
ACS1-2	ATCCTGGCTCTTGACAATC				
ACS2-1	GCACAACCTGAAGGAGCTG	<i>NT-ACS2</i>	AJ005002	55	434
ACS2-2	GTGTGTCACTGTCAAGAAGA				

<sup>a</sup>Annealing temperatures for *NT-TEF1- $\alpha$*  primers were varied to be compatible with the other primers used in co-amplification. For example, when used with *NT-ACS1* primers, the annealing temperature was set to 59 °C.

symptoms began to appear (Fig. 2A). After increasing to its highest level at 96 HPI, expression of *NT-ACS1* decreased slowly to 144 HPI. Expression of *NT-ACS2* was similar to *NT-ACS1*, which also remained low up to 48 HPI, then increasing to a peak at 96 HPI (Fig. 2B). *NT-ACS2* expression decreased considerably by 144 HPI, which was not observed for *NT-ACS1* expression (Fig. 2). These peaks in ACS gene expression preceded the second increase of ethylene production at 120 and 144 HPI (Fig. 1).

Expression of the ACO gene, *EFE-26*, first increased significantly at 48 HPI (Fig. 3A), which was during the biotrophic phase. Expression continued to increase slightly to a maximum at 96 HPI and then decreased (Fig. 3A).



**Fig. 2.** Relative RT-PCR analysis of *NT-ACS1* (A) and *NT-ACS2* (B) gene expression in *N. tabacum* cv. Samsun NN plants inoculated with *C. destructivum*. For each gene, the upper figure is the RT-PCR gel, and the lower figure is the quantity of *NT-ACS1* (A) or *NT-ACS2* (B) mRNA relative to the amount of a constitutively expressed translation elongation factor 1 $\alpha$  gene, *NT-TEF1- $\alpha$* . Lane M is a 100 bp size marker, and lanes numbered 0 to 144 are cDNA from leaf samples at 0, 24, 48, 72, 96, 120, and 144 h post-inoculation, respectively. Means with standard error bars were calculated from three replications.

Expression of the ACO gene, *DS321*, also increased significantly by 48 HPI and reached a maximum at 96 HPI before gradually decreasing (Fig. 3B). Although the absolute difference in relative expression units between 0 and 96 HPI were similar between *EFE-26* and *DS321*, the changes in *DS321* expression were proportionally much smaller than those of *EFE-26* or any of the other genes examined in this study. Expression of the ACO gene, *NT-IAIC*, was significantly highest at 0 and 24 HPI (Fig. 3C), and then progressively declined at a relatively slow rate until 72 HPI, after which there was a sharper decrease corresponding with the appearance of the necrotrophic phase at 96 HPI. From 96 to 144 HPI, the expression remained at a consistently low level.

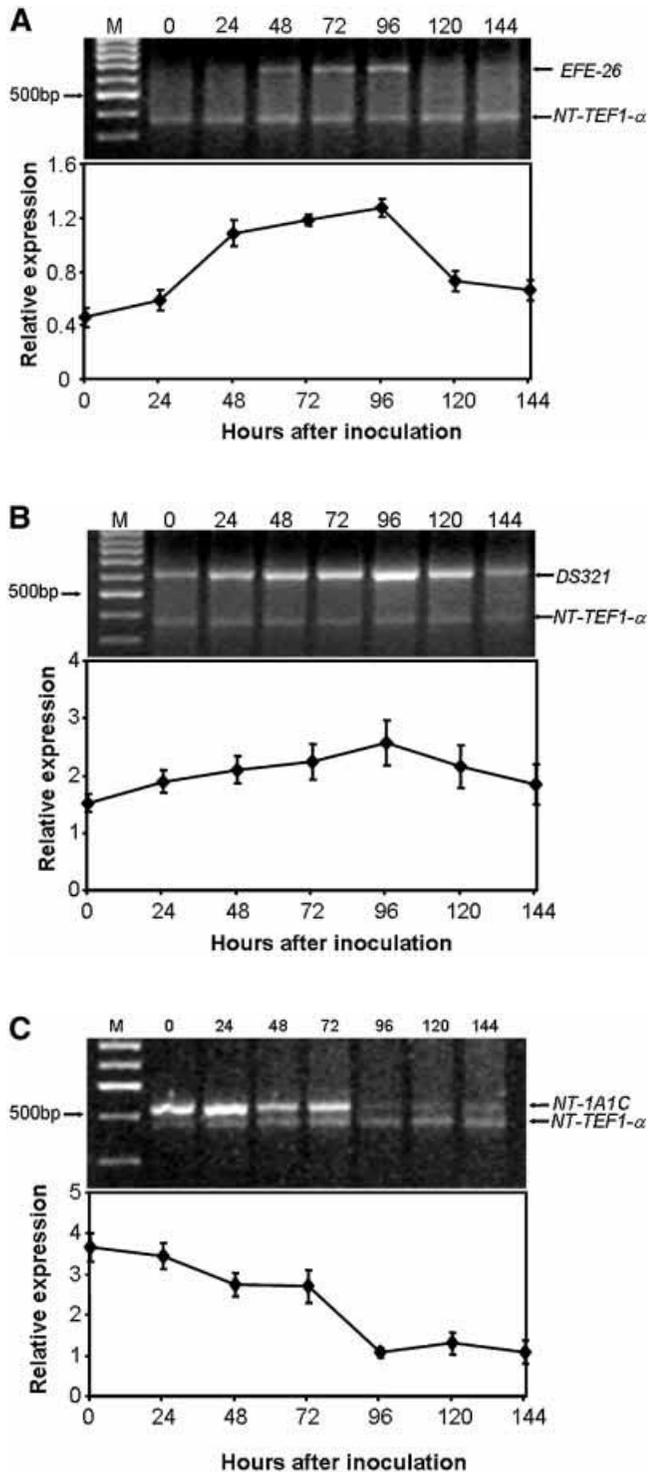
To verify the identity of the RT-PCR products, they were sequenced and compared with a *N. tabacum* ACS or ACO sequence reported in GenBank. The similarity between the nucleotide sequences of the RT-PCR products against the genes for which the primers were designed were 95% with *NT-ACS1*, 100% with *NT-ACS2*, 98% with *NT-EFE26*, 96% with both *DS321* and *NT-EFE27*, and 99% with *NT-IAIC*. This indicated that the RT-PCR products were the same or very similar to the known *N. tabacum* ACS or ACO genes.

#### *Response to C. destructivum* infection in *Tetr1* transgenic and wild-type plants

At 96 HPI, ethylene-insensitive *Tetr1* tobacco plants had approximately twice the number of water-soaked lesions compared with control, wild-type plants (Table 2). Quantification of the fungal biomass by measuring GFP fluorescence intensity of *C. destructivum* N150P3 also revealed up to twice as much fungal biomass in *Tetr1* plants compared to the wild type (Table 2) at 96 HPI. Although the disease was more severe on the *Tetr1* plants, the timing of symptom development at 72–96 HPI remained unchanged. The smaller number of lesions on wild-type plants slowed the progression of the disease, but eventually the lesions on both wild-type and *Tetr1* plants spread, killing the plants.

#### Discussion

For tobacco infected with *C. destructivum*, two increases in ethylene production were observed during disease development. The first increase in ethylene production occurred during the biotrophic phase peaking at approximately 24 h before necrotic lesion symptoms were visible. This corresponds well with the peak of ethylene that occurred approximately 24 h before the appearance of lesions on melon seedlings infected by *C. lagenarium* (Esquerré-Tugayé *et al.*, 1993), which has a similar intracellular hemibiotrophic infection process as *C. destructivum*. However, only one ethylene peak was found in the infected melon seedlings (Esquerré-Tugayé *et al.*, 1993),



**Fig. 3.** Relative RT-PCR analysis of *EFE-26* (A), *DS321* (B) and *NT-IA1C* (C) gene expression in *N. tabacum* cv. Samsun NN plants inoculated with *C. destructivum*. For each gene, the upper figure is the RT-PCR gel, and the lower figure is the quantity of *EFE-26* (A), *DS321* (B) or *NT-IA1C* (C) mRNA relative to the amount of a constitutively expressed translation elongation factor 1 $\alpha$  gene, *NT-TEF1- $\alpha$* . Lane M is a 100 bp size marker, and lanes numbered 0 to 144 are cDNA from leaf samples at 0, 24, 48, 72, 96, 120, and 144 h post-inoculation, respectively. Means with standard error bars were calculated from three replications.

**Table 2.** Disease development on 5-week-old ethylene-insensitive *Tetr1* and wild-type *N. tabacum* cv. Samsun NN inoculated with *C. destructivum* and assessed at 96 h post-inoculation

Type	Lesions cm <sup>-2a</sup>	Fungal biomass (RFUs) <sup>b</sup>	
		Experiment 1	Experiment 2
<i>Tetr1</i>	0.98 a <sup>c</sup>	1.5 $\times$ 10 <sup>5</sup> a	6.9 $\times$ 10 <sup>4</sup> a
Wild type	0.51 b	7.5 $\times$ 10 <sup>4</sup> b	4.3 $\times$ 10 <sup>4</sup> b

<sup>a</sup>Lesion number cm<sup>-2</sup> were means pooled from two separate experiments with a total of 42 leaves.

<sup>b</sup>Fungal biomass indicated by GFP relative fluorescence units (RFUs) were calculated from 15 replicate leaves for experiment 1 and 27 replicate leaves for experiment 2. The means of the two experiments are shown separately because there was a significant treatment by experiment interaction.

<sup>c</sup>Different letters in each column indicate statistically significant differences ( $P=0.05$ ).

whereas there was a second increase in ethylene from the tobacco infected with *C. destructivum*. This second increase occurred late in the necrotrophic phase when the lesions were expanding and was still increasing at the end of the experiment.

Two peaks in ethylene production have been reported a number of times for stress caused by fungal infections (Arshad and Frakenberger, 2002). For instance, detached bean leaves and hypocotyls infected with *Uromyces phaseoli* first produced an ethylene peak at the time that the infection pegs penetrated the stomata and a second peak during the appearance of necrotic spots (Montalbini and Elstner, 1977; Paradies *et al.*, 1979). The first peak in ethylene production in fungal-infected plants was associated with an increase in ACO, but not ACS, activity, indicating that ethylene production is probably regulated at different stages in the ethylene biosynthetic pathway (Paradies *et al.*, 1979).

During infection of tobacco by *C. destructivum*, the two ACS genes, *NT-ACS1* and *NT-ACS2*, showed relatively similar expression patterns. *NT-ACS1* has previously been shown to be induced by necrosis caused by an ethylene biosynthesis-inducing xylanase (EIX), Nep1 protein and TMV-induced HR (Avni *et al.*, 1994; Jennings *et al.*, 2001; Knoester *et al.*, 1995). EIX from *Trichoderma viride* acts as an elicitor causing enhanced ethylene production and a plant response similar to the HR (Avni *et al.*, 1994). EIX induced both ACS activity and *NT-ACS1* transcripts coordinately with ethylene production, but ethylene production declined with necrosis, whereas *NT-ACS1* mRNA continued to increase (Avni *et al.*, 1994). The Nep1 protein from *Fusarium oxysporum* induced necrosis and ethylene production in leaves of many dicot plant species, including tobacco, and induced *NT-ACS1* gene expression at the time of the ethylene peak, which was prior to necrosis (Jennings *et al.*, 2001). For the HR-associated necrosis caused by

TMV, *NT-ACS1* expression was also induced at the time of ethylene production before the appearance of necrosis (Knoester *et al.*, 1995; Ohtsubo *et al.*, 1999). Although *NT-ACS1* expression also increased at or just prior to the appearance of necrosis in the tobacco-*C. destructivum* interaction, this increase occurred after the first peak in ethylene. *NT-ACS2* expression patterns have not yet been examined in its response to disease or microbial elicitors, but *NT-ACS2* was called a multiple environmental stress-responsive gene in tobacco since it could be induced by wounding, chilling and, especially, sunlight (Ge *et al.*, 2000).

During the HR induced by TMV, Knoester *et al.* (1995) found that expression of the ACO gene, *EFE-26*, increased at the same time as that of *NT-ACS1* and remained elevated for at least 6 d after the induction of the HR until RNA could no longer be detected due to leaf senescence. However, in the tobacco-*C. destructivum* interaction, *EFE-26* expression increased approximately 24 h prior to that of *NT-ACS1*, during the time of the first peak of ethylene production, and this increase could have resulted from a feed-forward response to the early increase in ethylene production. Expression of *EFE-26* remained elevated for only 3 d, even though mRNA was still detectable and *NT-ACS1* expression remained elevated for 6 d.

Although expression of the ACO gene, *DS321*, proportionally changed much less than any of the other genes examined in this study, it also started to show increased expression during the first peak of ethylene production, like *EFE-26*, with the greatest expression level occurring just before the second increase in ethylene production. *DS321* has 98.9% homology to *EFE-27* (Ohtsubo *et al.*, 1999). The primers chosen to amplify *DS321* in this study matched both these genes, and the sequence of the single RT-PCR product amplified by those primers was highly similar to both *EFE-27* and *DC321*. Although *EFE-27* was found with *EFE-26* in a cDNA library of tobacco made during the HR to TMV, it was not further examined for its expression (Knoester *et al.*, 1995). Expression of *DS321* in tobacco leaves during the HR to TMV increased just before ethylene production, which preceded the HR necrosis, and this was several hours before increased *NT-ACS1* expression (Ohtsubo *et al.*, 1999). Therefore, induction of *DS321* expression in the tobacco-*C. destructivum* interaction was both different in relation to ethylene production and similar in relation to *NT-ACS1* expression compared with the HR to TMV in tobacco.

No information is available about the effects of disease on the expression of the tobacco ACO gene, *NT-IA1C*. *NT-IA1C* was isolated from a cDNA library enriched (i.e. having higher expression) for transcripts in leaves of transgenic tobacco plants that accumulated high levels of soluble sugars due to expression of an *Escherichia coli* pyrophosphatase gene (Herbers *et al.*, 1995). The high

levels of soluble sugars had multiple effects on the plants, including decreased sucrose synthesis, increased starch accumulation, increased turgor pressure, and enhanced expression of certain PR proteins. *NT-IA1C* was unique among the tobacco ACS and ACO genes examined in this study as it was the only gene to be down-regulated by *C. destructivum* infection. Considering that a pathogen absorbs plant nutrients as it grows in its host, it was perhaps not unexpected that the form of stress induced by this disease was significantly different from that resulting from high soluble sugar accumulation. Not all ACO genes have shown induced expression related to disease. Like *NT-IA1C*, one of the ACO genes of *N. glutinosa* was also suppressed following inoculation with a pathogen, which was TMV inducing the HR (Kim *et al.*, 1998).

A comparison of the expression patterns of the five genes included in this study indicates that the first peak in ethylene may be more related to ACO rather than ACS gene expression, although none of the gene expression patterns exactly matched ethylene production. In many cases, ACO activity has been found to increase prior to ACS activity, but ACS was still believed to regulate ethylene production as ACS is rapidly inactivated and ethylene production correlates with free ACC content (Imaseki, 1999). However, Ohtsubo *et al.* (1999) found that ACC content continued to increase as ethylene production declined and overexpression of an ACO gene with an ACC supplement enhanced HR lesion formation in tobacco leaves. It was concluded that ethylene production during HR lesion formation was dependent on ACO rather than ACS.

Although the expression of five different tobacco ACS and ACO genes was examined in this study, tobacco has additional ACS and ACO genes. Southern blots probed with *EFE-26* or *NT-ACS1* indicated that multiple copies of both ACS and ACO genes were present in tobacco (Knoester *et al.*, 1995). Ge *et al.* (2000) also described three additional ACS genes in tobacco, *NT-ACS3*, *AT-ACS4* and *NT-ACS5*. However, attempts in this work to obtain single RT-PCR products of *NT-ACS3*, *AT-ACS4* and *NT-ACS5* were unsuccessful, perhaps due to the limited amount of sequence information available to design specific primers for these genes. Given that no two genes examined in this study showed the same pattern of expression, it is likely that additional tobacco ACO or ACS genes will have different expression patterns in relation to the changes in ethylene production.

To assess the role of ethylene during the HR of tobacco to TMV, tobacco has been transformed with antisense constructs for ACS and ACO genes (Knoester *et al.*, 1997, 2001). Although ethylene production was lowered, it was not sufficiently reduced to impair the HR, possibly due to the existence of a multigene family for both ACS and ACO genes. However, using ethylene-insensitive Tetr1 tobacco, it was shown that although ethylene insensitivity did not

eliminate the HR, it did reduce HR lesion expansion (Knoester *et al.*, 2001).

A number of genes, such as *etr1*, *etr2*, *ctr1*, *ein2*, and *ein3* have been found to be involved in the perception of ethylene in plants (Bleecker and Kende, 2000). Tetr1 tobacco plants are ethylene insensitive because they have been transformed with a mutant form of the ethylene receptor gene, *etr1*, from *A. thaliana*. This mutation is dominant, and the ethylene-insensitive plants showed a lack of known ethylene responses, including loss of the triple response of etiolated seedlings to ethylene, retarded leaf senescence and increased flower longevity (Knoester *et al.*, 1998).

Infection of Tetr1 transgenic tobacco with *C. destructivum* resulted in both greater lesion numbers and fungal biomass, as measured by GFP, compared to the wild-type tobacco. This suggests that eliminating the perception of ethylene reduced the ability of the plant to defend itself. Ethylene production early after inoculation is believed to be associated with disease resistance because ethylene can induce the accumulation of PR-1,  $\beta$ -1,3-glucanase, chitinase, osmotin, proteinase inhibitors, phytoalexin biosynthetic enzymes, hydroxyproline-rich glycoproteins, and other defence-associated proteins (Arshad and Frankenberger, 2002). Knoester *et al.* (1998) noted that induction of basic PR gene expression was strongly reduced in Tetr1 tobacco. Therefore, one possible explanation for the increased susceptibility of Tetr1 tobacco to *C. destructivum* is that the lack of perception of ethylene has resulted in the loss of the induction of one or more host defences that could inhibit *C. destructivum*. Knoester *et al.* (1998) and Geraats *et al.* (2002) also found that Tetr1 tobacco plants were more susceptible than wild-type plants to several fungi, including a normally nonpathogenic soil-borne *Pythium* spp. and the necrotrophic fungus, *Chalara elegans*.

Other reports of ethylene-insensitive plants infected by pathogens include the ethylene-insensitive arabidopsis mutant *ein2-1*, which was more susceptible than wild-type plants to infection by the necrotrophic pathogen, *Botrytis cinerea* (Thomma *et al.*, 1999), but less susceptible to the biotrophic pathogen, sugar beet cyst nematode (Wubben *et al.*, 2001). Similarly, soybean mutants with reduced ethylene sensitivity had more severe symptoms compared with control plants when challenged with the necrotrophic pathogens, *S. glycines* and *R. solani*, whereas less severe chlorotic symptoms were observed with the initially biotrophic pathogens, *P. syringae* pv. *glycines* and *P. sojae* (Hoffman *et al.*, 1999). For ethylene-insensitive tomato, less severe disease also occurred when leaves were inoculated with *P. syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* (Lund *et al.*, 1998).

One possible explanation for the variation in the response of ethylene-insensitive plants to different pathogens was given by Thomma *et al.* (1999). Their hypothesis

is that ethylene acts as a signal for defence against necrotrophic pathogens, whereas ethylene promotes damage due to biotrophic pathogens. If this hypothesis is correct, then *C. destructivum* acts more like a necrotroph during the infection of tobacco, and ethylene is acting as a signal to induce host defences. Also if this were true, then it would appear more likely that the first ethylene peak was related to signalling defence responses. The second increase in ethylene production occurs much later in the interaction during lesion expansion and is therefore more likely be involved in pathogenesis (i.e. affecting the expansion of necrosis). However, further work is needed to determine if the two increases in ethylene following infection play similar or different roles in the interaction.

Although much remains to be learned about the role of ethylene in the *N. tabacum*–*C. destructivum* interaction, these results clearly demonstrate that ethylene is important as a signalling molecule and that this interaction has unique features not previously associated with ethylene in diseased plants.

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