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Induction of Ethylene Biosynthesis in *Nicotiana tabacum* by a *Trichoderma viride* Xylanase Is Correlated to the Accumulation of 1-Aminocyclopropane-1-Carboxylic Acid (ACC) Synthase and ACC Oxidase Transcripts

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Xylanase (EIX) from the fungus *Trichoderma viride* elicits ethylene biosynthesis in leaf tissues of *Nicotiana tabacum* cv Xanthi but not in cv Hicks. The increase in ethylene biosynthesis is accompanied by an accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC), an increase in extractable ACC synthase activity, and increases in ACC synthase and ACC oxidase transcripts. Priming of leaves with ethylene (120 μL/L, 14 h) sensitizes the tissue, resulting in an enhanced response to EIX and increases in both the in vivo ACC oxidase activity and ACC oxidase transcript level. EIX and ethylene independently induce ACC oxidase. Inhibition of ethylene biosynthesis by aminoethoxyvinylglycine is not accompanied by a reduction in ACC oxidase transcript level, indicating that ethylene biosynthesis is not required. In contrast to the differential induction of ethylene biosynthesis by EIX in Xanthi versus Hicks cultivars, both cultivars respond to a chemical stress (induced by $CuSO_4$) by enhancing ethylene production. This induction is accompanied by an increase in ACC synthase transcript but not in that of ACC oxidase.

Ethylene as a plant hormone influences many aspects of plant growth and development (Mattoo and Suttle, 1991; Abeles et al., 1992) as well as the induction of some plant defense responses (Boller, 1991). The biosynthetic pathway of ethylene production in higher plants is well defined (Yang and Hoffmann, 1984). The rate-limiting steps in this pathway in most vegetative tissues are the reactions catalyzed by ACC synthase (*S*-adenosyl-L-Met methylthioadenosine-lyase, EC 4.4.1.4), which converts *S*-adenosyl-L-Met to ACC, and ACC oxidase, which converts ACC to ethylene.

An EIX produced by the fungus *Trichoderma viride* (Dean and Anderson, 1991) elicits ethylene biosynthesis and plant defense responses in *Nicotiana tabacum* leaf tissue (for a review, see Anderson et al., 1993). When EIX is applied to a cut petiole on an intact tobacco plant, it moves through the vasculature to leaves above and below the point of application, particularly into the mesophyll tissue (Sharon et al., 1992), where ethylene production is enhanced and the tissue becomes necrotic (Bailey et al., 1991). The protein itself appears to be the elicitor and not a product of xylanase activity on the cell wall because Xanthi leaf protoplasts (cells without cell walls) respond to EIX (Sharon et al., 1993). If the tobacco leaves are first primed by an exposure to ethylene, both ethylene production and necrosis are greatly enhanced in tissue treated with EIX (Bailey et al., 1990, 1991). Ethylene priming also enhances ethylene production in response to cell-wall-digesting enzymes (Chalutz et al., 1984; Anderson et al., 1985). However, not all cultivars of tobacco respond to EIX treatment (Bailey et al., 1993), suggesting a requirement for EIX-specific recognition factors in the plant. The EIX-insensitive cultivar, Hicks, however, does produce ethylene in response to other cell-wall-digesting enzymes (Sharon et al., 1993). The cultivar-specific response of EIX may be compared to the hypersensitive response in plant-pathogen interactions, where single dominant genes predominate as the controlling factors (Gabriel, 1989). Ethylene has been postulated to play a role in plant defense responses (Pegg, 1976). It is known that ACC synthase activity and ethylene production are enhanced in tobacco leaves undergoing a hypersensitive response (De Laat and van Loon, 1982).

We have used the Xanthi and Hicks cultivars of tobacco to compare the patterns of accumulation of enzyme activities and the related transcripts in response to EIX as well as to another chemical inducer of ethylene biosynthesis, $CuSO_4$. We show that a strong correlation exists between an increase in ethylene production and that in the ACC synthase transcript. Furthermore, we demonstrate that although Hicks does not respond to EIX in producing ethylene, as does Xanthi, both cultivars respond to $CuSO_4$ by enhancing ethylene production and accumulating ACC synthase transcript.

MATERIALS AND METHODS

Tissue Treatment

Fully expanded leaves (10–15 cm long) from greenhouse-grown *Nicotiana tabacum* (cv Xanthi and Hicks) plants (25–

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Abbreviations: AVG, 1-aminoethoxyvinylglycine; EIX, ethylene biosynthesis-inducing xylanase; PLP, pyridoxal 5-phosphate.

30 cm tall) were incubated in the dark for 14 h in a humid chamber under an atmosphere of 120 μL/L ethylene (ethylene priming) or an atmosphere purged of ethylene with the ethylene absorbent Purafill II[3] (air pretreatment). EIX, purified from xylan-induced *Trichoderma viride* cultures (Dean and Anderson, 1991), was applied as a hanging drop (2.5 μg/g tissue) to freshly cut petioles of detached leaves as previously described (Bailey et al., 1990). After the solution was absorbed, the leaf petioles were placed in an assay medium containing 10 mM Mes (pH 6.0) and 250 mM sorbitol. At various times after EIX treatment, the treated leaves were placed in jars (475 mL), which were sealed for 15 min after which gas samples were taken and ethylene was measured as previously described (Chalutz et al., 1984). Alternatively, EIX (1 μg/mL) was applied to leaf discs (1 cm diameter) floated on an assay medium containing various combinations of ACC (1 mM) and/or AVG (0.1 mM). Ethylene production was measured after sealing 25-mL flasks containing leaf discs (six per flask, average total weight 85 mg) for 4 h.

ACC Content and ACC Synthase Measurements

ACC content and ACC synthase activity were determined by grinding appropriately treated tobacco leaves in 100 mM Hepes (pH 8.0), 4 mM DTT, 2.5 mM PLP, and 25% polyvinylpolypyrrolidone. Samples for ACC synthase activity determination were filtered through cheesecloth, centrifuged for 10 min at 10,000*g*, and desalted over a Presto column (Pierce). The ACC synthase assay was carried out using 100 μg of protein (Bradford, 1976) in 50 mM Hepes, 200 μM *S*-adenosyl-L-Met, and 2.5 mM PLP (pH 8.0) at 37°C for 1 h (Mehta et al., 1988). The ACC content of the original extracts and that produced during the ACC synthase assay were quantified (Lizada and Yang, 1979).

Preparation of ACC Oxidase Probe

Two degenerate primers, one, GA[CT]GC[GAT]TG[CT]GA[GA]AA[CT]TGGGG[CT]TT[CT]TT[CT]GA, corresponding to residues 245 to 273 of the coding strand, and the other, CC[GA]TT[GA]GT[GA]AT[TA]AC[TC]TC[GC]AG[TC]TG[GA]TC[GA]CC, corresponding to residues 821 to 849 of the noncoding strand of the tomato ACC oxidase gene (Holdsworth et al., 1987), were used to amplify an ACC oxidase gene fragment (604 bp) from a Xanthi cDNA library (Bailey et al., 1992a) using PCR. The ACC oxidase probe shares 80% homology at the DNA level with a tomato ACC oxidase (pTom 13) (Holdsworth et al., 1987).

RNA Extraction and Detection

Total RNA was isolated from detached tobacco leaves or leaf discs (Chirgwin et al., 1979), quantified by measuring A_{260}, and then fractionated on formaldehyde gels (Sambrook et al., 1989). The RNA was transferred to Nylon membranes (Zeta-Probe GT, Bio-Rad) with 20 mM sodium acetate (pH 6.0). Blots were hybridized overnight at 58°C in 0.5 M sodium phosphate, 7% SDS, and 1 mM EDTA (pH 7.2) with the ACC synthase probe, clone pTACC-13 (Bailey et al., 1992a), or the ACC oxidase probe. DNAs were labeled by the random primer method according to the manufacturer's instructions (Amersham). Blots were washed at 58°C twice for 30 min in 40 mM sodium phosphate, 5% SDS, and 1 mM EDTA (pH 7.2), then exposed to Kodak X-Omat film. To verify equal RNA loading of lanes, the blots were stripped of the probe by boiling twice for 20 min in 0.1× SSC, 0.5% SDS (pH 7.2) and rehybridized with a 16S ribosomal RNA probe from *Xenopus* (Sollner-Webb and Reeder, 1979).

RESULTS

Induction of Ethylene Biosynthesis

Treatment of *N. tabacum* cv Xanthi leaves with EIX causes a hypersensitive-type response characterized by increased production of ethylene, induction of PR proteins, and tissue necrosis (Anderson et al., 1993). Time-course studies showed that EIX elicited ethylene biosynthesis (Fig. 1A) in both air- and ethylene-primed tissues, typically reaching a maximum within 3 to 4 h. In some experiments, the maximum was reached 1 or 2 h later. The ethylene-primed tissues treated with EIX had higher levels of ethylene production. The increase in ethylene biosynthesis was accompanied by an increase in extractable ACC synthase activity (Fig. 1B) and accumulation of ACC (Fig. 1C). ACC synthase activity paralleled ethylene biosynthesis, whereas ACC content continued to accumulate up to 6 h after extractable ACC synthase activity began to decline. The rates of ethylene biosynthesis and ACC synthase activity and ACC accumulation induced by EIX were all enhanced by ethylene pretreatment.

Induction of ACC Synthase Transcript

To ascertain if EIX-mediated stimulation of ethylene production and ACC synthase activity was regulated at the level of transcription, we analyzed the steady-state level of ACC synthase transcript under these conditions. An increased level of ACC synthase transcript was evident within 1 h of EIX treatment (Fig. 2). Pretreatment of leaves with ethylene (120 μL/L, 14 h) alone had, at best, a slightly positive effect on ACC synthase transcript level. Except for the first 2 h, EIX-induced ACC synthase transcript level in ethylene-primed leaves was 3- to 4-fold higher than in air-primed leaves. The accumulation of ACC synthase transcript in response to EIX in ethylene-primed tissue continued for 8 h even when ethylene production and ACC synthase activity had declined after 6 h (Fig. 2).

Induction of ACC Oxidase Transcript

An increase in the conversion of ACC to ethylene in ethylene-primed tobacco tissue has been observed (Chalutz

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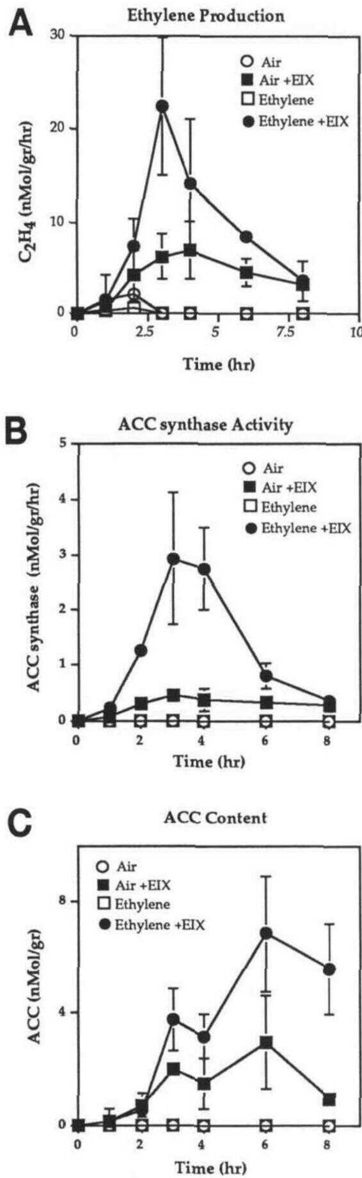

Figure 1. Modulation in the components of ethylene biosynthesis in detached leaves treated with EIX (2.5 μg/g tissue) as a hanging drop. Ethylene production (A), ACC synthase activity (B), and ACC content (C) were measured. Each time represents the average ± SD of three different experiments.

et al., 1984). A similar increase in in vivo ACC oxidase was seen in Hicks (Fig. 3). In both Xanthi and Hicks, enhanced ACC oxidase activity in vivo in ethylene-primed tissue was found associated with an enhanced accumulation of ACC oxidase transcript (Fig. 3). The addition of EIX to air-primed Xanthi leaf discs resulted in an additional enhancement in their ability to convert ACC to ethylene (Fig. 4), which was also accompanied by an increase in the level of ACC oxidase transcript (Fig. 5A). AVG, an inhibitor of ACC synthase, reduced EIX-induced ethylene biosynthesis (Fig. 4). This inhibition of EIX-induced ethylene biosynthesis by AVG, however, was not accompanied by reduction in the conversion of exogenously applied ACC to ethylene (Fig. 4) or in ACC oxidase transcript level (Fig. 5A).

Similar Response of *N. tabacum* cv Xanthi and Hicks to a Chemical Stress Induced by $CuSO_4$

Cupric ions are known to stimulate ethylene production in tobacco leaves (Mattoo et al., 1992). To determine if insensitivity of Hicks to EIX in producing ethylene was a specific response or a general inability to induce ethylene production, we treated leaves from Hicks and Xanthi with 10 mM $CuSO_4$. Results in Figure 6A show that Hicks had as good or even a better response than Xanthi in producing ethylene in response to cupric ions. Ethylene-primed leaves were more responsive in both cultivars. The induction of ethylene was accompanied by an accumulation of ACC synthase transcript (Fig. 6B). In

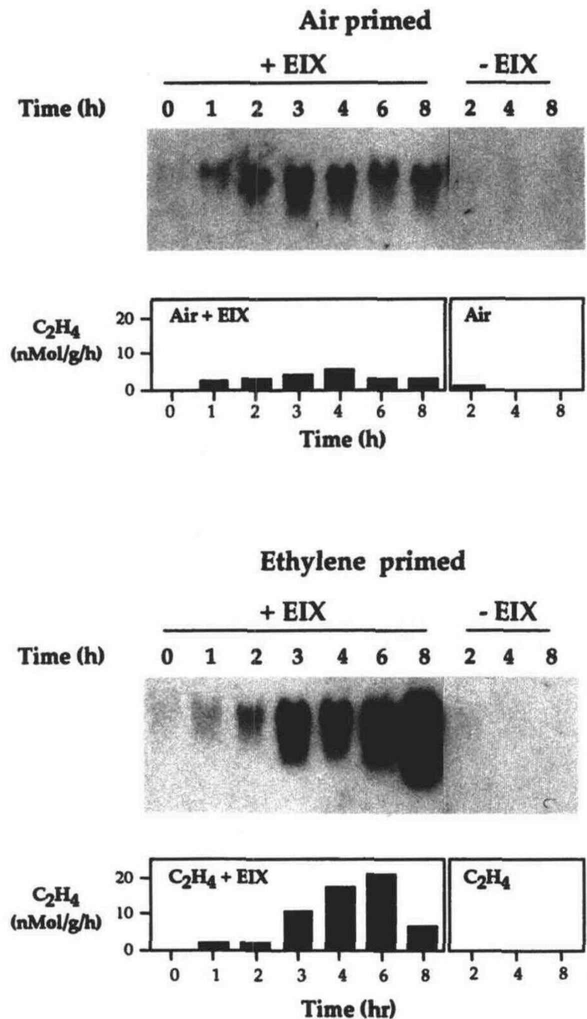

Figure 2. RNA gel blot analysis of ACC synthase transcript. Air- and ethylene-primed leaves were treated with EIX by the hanging-drop method for the indicated times and ethylene production was measured. Total RNA was isolated from each sample and 10 μg were fractionated on formaldehyde gels, transferred to Nylon membranes, and hybridized with the ACC synthase clone pTACC13.

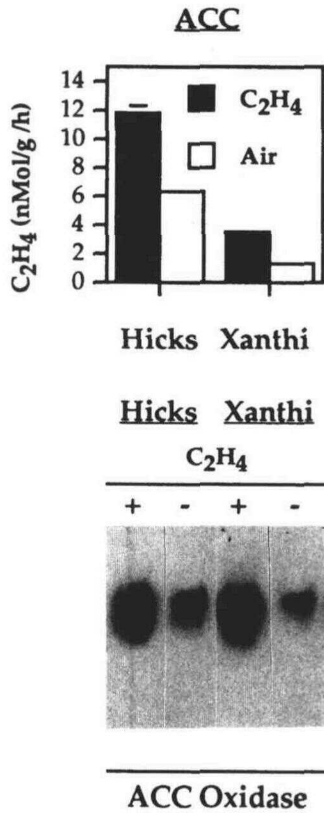

Figure 3. Effect of ethylene treatment on in vivo ACC oxidase activity. The conversion of ACC to ethylene was determined by incubating leaf discs in 1 mM ACC for 1 h (top). Two micrograms of total RNA were separated on formaldehyde gels, transferred to Nylon membranes, and hybridized with the ACC oxidase probe (bottom).

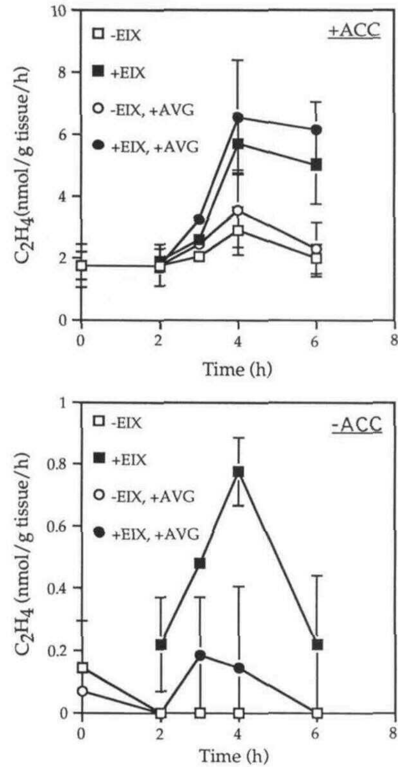

Figure 4. Induction of ACC oxidase activity by EIX in air-treated leaves. Leaf discs were treated with or without AVG (0.1 mM) 1 h prior to the addition of EIX. A, To measure in vivo ACC oxidase activity, 1 mM ACC was added to appropriate flasks and ethylene was measured after 1 h. B, The same as A, but without the addition of ACC.

contrast, only Xanthi leaf discs responded similarly to EIX (Fig. 6). The ACC oxidase transcript was constitutively present in both Hicks and Xanthi (Figs. 3 and 5B). Upon treatment with $CuSO_4$, only a slight increase was seen in the ACC oxidase transcript compared to that seen in the ACC synthase transcript (compare Figs. 5B with 6B). These data suggest that both Hicks and Xanthi respond similarly to cupric ions, whereas Hicks is unable either to accumulate ACC synthase transcript or to produce ethylene in response to EIX treatment.

DISCUSSION

The accumulation of ACC synthase transcript in Xanthi tobacco leaves in response to EIX is initially coordinated with increases in the activity of ACC synthase and ACC content in the tissue. This enhancement is further stimulated in Xanthi tobacco leaves primed with ethylene. The enhancing effect of ethylene pretreatment on ethylene production in response to various signals has been known for many years (Cronshaw and Pegg, 1976; Chalutz et al., 1984), although the mechanism of this effect is unknown at present. Once the tissue starts to necrose (within 4–7 h), ethylene production declines while extractable ACC synthase transcript continues

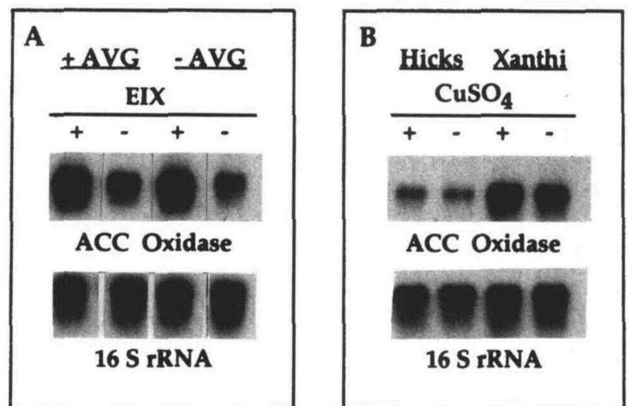

Figure 5. RNA gel blot analysis of ACC oxidase transcript. A, Discs from air-treated Xanthi leaves were incubated with or without AVG (0.1 mM) for 1 h before adding EIX (1 μg/mL). RNA was isolated from the discs after 4 h. Two micrograms of total RNA were fractionated on formaldehyde gels, transferred to Nylon membranes, and hybridized with the ACC oxidase probe and a 16S rRNA probe. B, Xanthi and Hicks leaf discs were treated for 4 h with $CuSO_4$ (10 mM), after which RNA was isolated. Two micrograms of total RNA were fractionated on formaldehyde gels, transferred to Nylon membranes, and hybridized as in A.

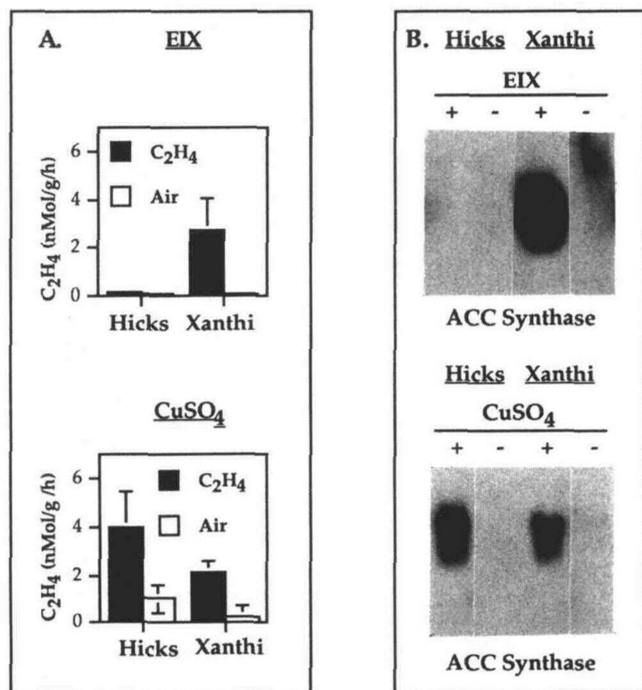

Figure 6. Accumulation of ACC synthase transcript in response to EIX and $CuSO_4$ in Hicks and Xanthi tobacco cultivars. A, Effect of ethylene priming on ethylene production by leaf discs treated for 4 h with EIX (1 μg/mL) or $CuSO_4$ (10 mM). Leaf discs were cut from leaves incubated either in an ethylene-purged atmosphere or 120 μL/L ethylene. B, Ten micrograms of total RNA isolated from each sample were fractionated on formaldehyde gels, transferred to Nylon membranes, and hybridized with the ACC synthase probe (pTACC13).

to increase, suggesting that other factors, such as membrane damage, are limiting ethylene biosynthesis. The EIX-insensitive Hicks cultivar (Bailey et al., 1993) does not show enhanced ACC synthase transcript level in response to EIX, but like the Xanthi cultivar it does respond to $CuSO_4$ treatment by increasing ethylene production and accumulating ACC synthase transcript. These results demonstrate that both EIX-sensitive (Xanthi) and -insensitive (Hicks) cultivars have functional signal-transduction pathways for the induction of ethylene biosynthesis. Although the gene that confers sensitivity to EIX does not influence sensitivity to $CuSO_4$, other components of the signal-transduction pathway leading to ethylene biosynthesis may be shared between the two systems.

Ethylene treatment has been shown to enhance the level of ACC oxidase transcript in preclimacteric apple fruit (Dong et al., 1992) and carnation flowers (Woodson et al., 1992). However, in both of these cases ethylene biosynthesis as well as ACC synthase are induced by ethylene, whereby autocatalytic ethylene production develops. In orchids, ethylene treatment induces ACC oxidase and ACC synthase in a tissue-specific manner (O'Neill et al., 1993). Our results further extend these observations by showing that in vivo ACC oxidase activity as well as ACC oxidase transcript accumulation are enhanced in tobacco leaves (Xanthi and Hicks) primed with ethylene. However, ACC synthase transcript accumulation and enzyme activity are not greatly affected by ethylene treatment. The gene conferring sensitivity to EIX does not appear to be influenced directly by ethylene. Interestingly, little induction of ACC oxidase transcript accumulation is seen in $CuSO_4$-treated tissue, whereas the accumulation of ACC synthase transcript in both EIX-sensitive and -insensitive cultivars is dramatically increased. EIX, on the other hand, induces both ACC synthase and ACC oxidase transcripts in the Xanthi cultivar. Therefore, the $CuSO_4$ effect is distinguishable from the EIX effect, further demonstrating the specific nature of the interaction between EIX and *N. tabacum* cv Xanthi.

It is well known that ethylene biosynthesis is induced during host-pathogen interactions in many plants (Boller, 1991) including incompatible interactions (van Loon, 1984). This induction does not require the presence of intact microbes because cell-free extracts (Chalutz et al., 1984) or purified proteins (Roby et al., 1986; Fuchs et al., 1989; Blein et al., 1991), as well as other elicitors (Chappell et al., 1984; Felix et al., 1991a, 1993), mimic the microbial action. Spanu et al. (1993) found that specific ACC synthase transcripts accumulated in tomato leaves infected with *Phytophthora infestans* and also in cell suspensions responding to elicitor from yeast extracts. It is not known if the transcripts accumulating in response to EIX and/or ethylene represent a single gene product or the products of several genes, since ACC synthase (Huang et al., 1991) and ACC oxidase (Bouzayen et al., 1993; Tang et al., 1993) exist as multigene families. The response to EIX appears to differ from that to most other elicitors in that the sensitivity to EIX is regulated by a dominant gene (Bailey et al., 1993). The type of plant defense responses induced by EIX (Anderson et al., 1993) and their control by a single dominant gene draw a close corollary with the hypersensitive response in plant-pathogen interactions. De Wit et al. (1993) isolated a proteinaceous elicitor that is the determining factor in the race-specific resistance to *Cladosporium fulvum* conferred by the Cf9 gene in tomato. Our understanding of the importance of proteinaceous elicitors in initiating hypersensitive responses and the signal-transduction pathways in plants is continuing to expand as more elicitors are found and the subject receives intense attention. The mechanisms by which elicitors induce plant responses may involve, among other things, recognition by binding sites in the plasma membrane of the plant cell (Cheong et al., 1993). Elicitor binding probably leads to production of signals that induce other effects such as pH changes (Bailey et al., 1992b; Felix et al., 1991b, 1993) and ion fluxes, which may be related to membrane acylation of sterol glycosides (Moreau et al., 1994), protein phosphorylation (Felix et al., 1993), or inositol phospholipid kinase activity (Chen and Boss, 1990).

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