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Use of transgenic plants to study the role of ethylene and polygalacturonase during infection of tomato fruit by *Colletotrichum gloeosporioides*

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The infection of tomato fruit by the postharvest pathogen *Colletotrichum gloeosporioides* did not proceed until the onset of ripening in response to ethylene. Compared with fruit from wild-type plants, infection progressed more slowly in transgenic fruit in which ethylene biosynthesis and ripening had been inhibited by an ACC oxidase (*ACO*) antisense transgene. In contrast, transgenic fruit deficient in polygalacturonase developed lesions at the same rate as the wild-type fruit. Ethylene biosynthesis increased rapidly in response to infection of ripe wild-type and *ACO* antisense fruit but was 25 times greater in the former. Fruit from the ripening mutant *ripening inhibitor* (*rin*), which are normally very resistant to infection, became infected quickly when incubated in the presence of ethylene, whereas fruit incubated in the absence of ethylene remained healthy. The *ACO* 1 mRNA accumulated to detectable levels within 24 h of inoculation of unripe wild-type fruit, prior to the development of visible symptoms, whereas there was no detectable expression in the inoculated *ACO* antisense fruit. *ACO* transcripts accumulated to maximum levels during the early stages of infection of ripe fruit, correlating with maximum ethylene biosynthesis. Northern analysis using gene-specific probes for each of the three *ACO* genes indicated that *ACO* 1 was the main gene expressed in response to infection and that there was no detectable expression of *ACO* 2 and 3.

Introduction

Postharvest diseases are responsible for major crop losses, particularly in tropical countries. Many postharvest diseases are typified by infection that involves the formation of an infection hypha and penetration of the epidermis followed by a period of quiescence during which symptoms do not develop until after the fruit ripens. Losses are therefore high in fruit crops such as mango, banana, avocado, peach and nectarine, which are routinely harvested before the onset of ripening. *Colletotrichum gloeosporioides* is, perhaps, the most notorious postharvest pathogen, infecting a number of tropical fruits including mango, papaya and guava (for reviews see Swinburne, 1983; Jefferies *et al*., 1990).

There is relatively little information available concerning the molecular and biochemical events that occur during the ripening of most tropical fruit. In contrast, the biochemistry, genetics and molecular biology of tomato, and in particular tomato fruit ripening, is very well characterized (Tucker & Grierson, 1987; Hobson & Grierson, 1993). Although *C. gleosporioides* is not its most commercially important pathogen, tomato is a natural host of this fungus. The well-characterized genetics of this plant provides a very useful model system for the molecular analysis of the fascinating, but poorly characterized, transition from resistant (unripe) to susceptible (ripe) plant tissue associated with some postharvest diseases.

The rate of ethylene biosynthesis greatly increases during ripening of climacteric fruit such as tomato and this gaseous hormone is known to play a major role in the initiation and regulation of ripening (Gane, 1934; Grierson & Tucker, 1983; Yang & Hoffman, 1984; Lincoln *et al*., 1987; Hamilton *et al*., 1990; Oeller *et al*.,

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1991; Picton *et al.*, 1993). Ethylene synthesis increases just prior to the onset of fruit ripening and there is evidence to suggest that it is the initial trigger (for a review see Yang & Hoffman, 1984; Oeller *et al.*, 1991), because inhibiting its production retards a number of ripening-associated changes such as colour development (Oeller *et al.*, 1991; Picton *et al.*, 1993). The synthesis of cell wall degrading enzymes such as polygalacturonase (PG) is closely correlated with ripening (Tucker & Grierson, 1982) although it is not clear whether it is ethylene-regulated. A number of pathogens are also known to synthesize PG and it has been widely implicated during invasion of host tissues (Cooper, 1984).

Understanding the role of ripening-associated ethylene in disease is complicated by the observations of increased ethylene biosynthesis in response to pathogen attack. Tomato leaves infected by *Phytophthora infestans* showed increased ethylene biosynthesis as early as 30 h postinoculation (Spanu & Boller, 1989). ACC synthase and ACC oxidase activities, which are required for ethylene synthesis, were also higher (Spanu & Boller, 1989). Studies concerning the expression of a number of putative defence-related genes have also implicated ethylene as a signal molecule synthesized during the early stages of infection (Abeles *et al.*, 1970; Broglie *et al.*, 1986; Esquerré-Tugayé *et al.*, 1993).

The recent cloning and identification of genes for ACC synthase and ACC oxidase, enzymes responsible for the conversion of *S*-adenosyl methionine (SAM) to ethylene, has enabled the role of ethylene to be studied in detail (Sato & Theologis, 1989; Hamilton *et al.*, 1990; Hamilton *et al.*, 1991; Oeller *et al.*, 1991). These genes were used to make antisense gene constructs which, when transformed into tomato plants, dramatically reduced ethylene synthesis and consequently retarded fruit ripening (Hamilton *et al.*, 1990; Oeller *et al.*, 1991; Picton *et al.*, 1993). Similarly, transgenic plants deficient in polygalacturonase activity allowed the analysis of fungal PG activity *in vivo* (Smith *et al.*, 1988). In contrast to naturally occurring nonspecific mutations, such as *ripening inhibitor* (*rin*) in which ethylene synthesis is inhibited, (Knapp *et al.*, 1989), antisense genes inhibit a targeted gene very specifically so that any altered compatibility between the transgenic host and the pathogen can confidently be attributed to the effects of gene disruption. We have used such transgenic fruit to study the role of ethylene and polygalacturonase in the development of susceptibility to postharvest pathogens at the molecular level.

Materials and methods

Fungal and plant material: inoculation and infection scoring

C. gloeosporioides was grown on malt extract agar at 27°C, with a 16-h photoperiod. Conidial suspensions were prepared by dislodging the conidia in sterile distilled water (SDW) and filtering through four layers of sterile muslin. The suspension was then centrifuged at $1000 \times g$ for 5 min to pellet the conidia, which were then washed in SDW and centrifuged again before resuspending and adjusting the final concentration to 5×10^5 mL^{-1}.

All untransformed, ethylene deficient antisense and polygalacturonase deficient antisense tomato plants were glasshouse-grown in John Innes no. 1 compost using automatic heating/ventilation to provide a 22°C day and 18°C night temperature. A 16-h photoperiod was maintained with supplementary high pressure sodium lamps. Fruits were harvested at the mature green stage, approximately 6 weeks post-anthesis and prior to the development of carotenoids or the onset of the respiratory climacteric. Ripe fruits were harvested 6–8 days after the visible onset of fruit ripening. Intact green and ripe fruits were spray-inoculated with a conidial suspension of *C. gloeosporioides*, until the surface of the fruit was completely wetted. After inoculation the fruits were incubated in sterile clear disposable tubs (10 cm diameter) at 27°C in an illuminated incubator. Disease was monitored daily and recorded as a percentage of the total surface area of fruit exhibiting lesions. These developed as sunken areas on the fruit surface, sometimes becoming discoloured.

For ethylene determinations mature green and yellow ripe *rin* fruits were inoculated with a conidial suspension of *C. gloeosporioides* and incubated as described for the untransformed and ethylene deficient antisense fruits (ripe *rin* fruits do not accumulate carotenoids, remaining a yellow green colour when fully ripe). The *rin* fruits were maintained in air-tight gas jars in the presence of 10 $\mu L\ L^{-1}$ ethylene, which was added exogenously, or in the presence of a commercial preparation of potassium permanganate (Ethesorb) which removes ethylene from the atmosphere.

Ethylene measurements

Fruits were stored in air-tight jars fitted with Suba seals (Fisons, Loughborough), to allow access for a hypodermic needle for gaseous sampling. They were then incubated as described above for a defined time. A 1-mL sample of the headspace gas was removed and ethylene levels were measured by using a Pye–Unicam gas chromatograph as described by Grierson & Tucker (1983). Ethylene was measured as $nL\ L^{-1}\ gfwt\ h^{-1}$.

RNA purification and northern analysis

Fruits for RNA isolation were dissected, and the pericarp was harvested and immediately frozen in liquid nitrogen. The tissue was ground to a fine powder in a coffee grinder. Total RNA was isolated using the Kirby extraction method (Kirby, 1956) and purified by a modification of a CTAB-based DNA extraction method (Hamilton *et al.*, 1990). Purified RNA (5 μg) was denatured with formamide at 65°C for

15 min prior to fractionation by electrophoresis on denaturing formaldehyde gels. The RNA was then transferred to Genescreen membranes (DuPont) by capillary blotting (Sambrooke *et al.*, 1989) and bound to the membrane by UV cross-linking using a Stratalinker (Stratagene).

The vector containing the TOM 13 cDNA insert was linearized by restriction enzyme digestion and used for [α-^{32}P]-ATP labelled ribo-probe synthesis. Pre-hybridization and hybridization was carried out at 65°C in 1 M NaCl, 0·1% SDS, 50% formamide, 10% Dextran sulphate and 40 μg ml^{-1} sheared Salmon sperm DNA. Membranes were prehybridized for 2 h prior to hybridization with the radiolabelled probe for 16 h, then washed briefly in 0·1 × SSPE, 0·1% SDS at room temperature, followed by two separate washes in 0·1% SDS, 0·1 × SSPE at 65°C.

PCR amplification and cloning of the *ACO*-gene specific regions

The cDNA, TOM13 (*ACO* 1), later found to encode ACC-oxidase (Hamilton *et al.*, 1990, 1991), was initially characterized as a fruit ripening related cDNA which was also expressed in leaves upon wounding (Smith *et al.*, 1986; Holdsworth *et al.*, 1988). Three highly homologous but distinct genomic clones, GTOM 17 (TOM13 cDNA), GTOM A and GTOM B, were later isolated using this cDNA as a probe, suggesting that it was a member of a small multigene family consisting of at least three genes (Holdsworth *et al.*, 1988) which are now known as *ACO* 1, 2 and 3, respectively.

Gene-specific oligonucleotides were synthesized and used to PCR amplify the 5′ section of the 3′ untranslated regions from each of the *ACO* sequences. Oligonucleotide sequences used for the amplification of the three gene specific regions were: AS1 and AS2 (CTC TAG AAG CTT ATT AAA AAT TAA AAA AAT TG and CTC TGA ATT CAC ATC TCA TTT TTG GG, respectively) for *ACO* 2; B33 and BSP (TTT CGA ATT CGT ACT ATA AAG TAA CTT TCA and GGG CTC GAG CTC TAG AAT TC, respectively) for *ACO* 3; and 13S and OS8 (TCT AGA GCT CAT CCC AAT TCA ATT TAA AAA A and GGG CTC GAG CTC TAG AAT TC, respectively) for *ACO* 1. The amplified regions were then ligated with $pT_7T_3\alpha-18$ and transformed into *E. coli* DH51, the identity of the PCR amplified fragments being confirmed by sequencing using the dideoxy chain termination method (Sanger *et al.*, 1977).

Results

Infection of fruit from wild-type and transgenic plants

Fruits from wild-type plants and plants transformed with either a polygalacturonase antisense gene (PG-as, less than 1% PG activity of ripe wild-type fruit) (Smith *et al.*, 1988, 1990), or an ACC-oxidase antisense gene (*ACO*-as, less than 5% ethylene production of wild-type fruit) (Hamilton *et al.*, 1990), were tagged at the first visible sign of carotenoid production and harvested 6–8 days later. Fruits were inoculated with *C. gloeosporioides* and infection was monitored daily for 5 days. Results (Fig. 1a) represent the mean of scores from all the infected fruit. By day 5 postinoculation, infection of the *ACO*-as fruit was delayed relative to the wild-type fruit with average infection score of 15·8% and 44·8%, respectively. Little difference was observed between PG-as and wild-type fruit (51·9% and 44·8% infection, respectively). These reported scores are untransformed values.

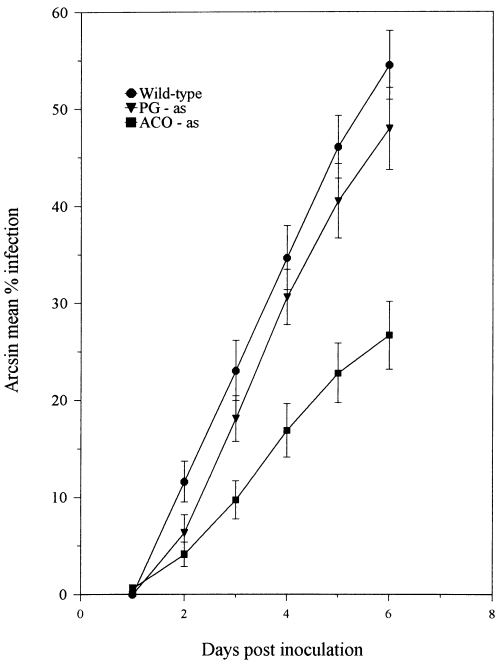
In a comparison of wild-type fruit, inoculated when mature green or red ripe, the former showed little visible sign of infection until after the onset of ripening (Fig. 1b) but then became infected at the same rate as those inoculated after ripening on the plant.

Ethylene biosynthesis in response to infection

Wild-type and *ACO*-as fruit were inoculated with *C. gloeosporioides* and incubated in gas jars. Measurements of ethylene biosynthesis in response to infection are presented in Fig. 2(a,b) (the scales for these graphs are different). Ethylene biosynthesis in ripe wild-type fruit began to increase early after inoculation, rising from 6·9 nL gfwt^{-1} h^{-1} in the uninoculated controls to a maximum of 34·4 nL gfwt^{-1} h^{-1} by 20% infection (Fig. 2a). Although the rate of ethylene biosynthesis resulting from infection of the ripe *ACO*-as fruit was greatly reduced, there was an increase from an average of 0·22 nL^{-1} gfwt^{-1} h^{-1}, to a maximum of 1·23 nL gfwt^{-1} h^{-1} in fruit exhibiting 5% visible lesion development (Fig. 2b). In both the wild-type and the *ACO*-as fruit the rate of biosynthesis peaked during the early stages of infection before falling, by 40%, to levels of infection observed in the uninoculated control fruit. Ethylene biosynthesis in the inoculated mature green wild-type and *ACO*-as fruit was also measured but was too low for any significant rise in response to inoculation with *C. gloeosporioides* to be detected.

The influence of ethylene upon infection of unripe and ripe *rin* fruit

Fruits of the *rin* phenotype do not ripen normally, but are partly yellow 14 days after the onset of colour change. In addition, they do not produce climacteric ethylene and are normally very resistant to infection. The effect of exogenous ethylene applications on infection of *rin* fruit was analysed. Fruits incubated in the presence of exogenous ethylene began to show lesion development as early as 9 days postinoculation (Fig. 3); disease progressed rapidly and they were fully infected by 14 days postinoculation. In contrast, yellow fruits incubated in an ethylene-free atmosphere were extremely resistant to infection. Mature green *rin* fruits incubated in an ethylene-enriched atmosphere developed lesions at



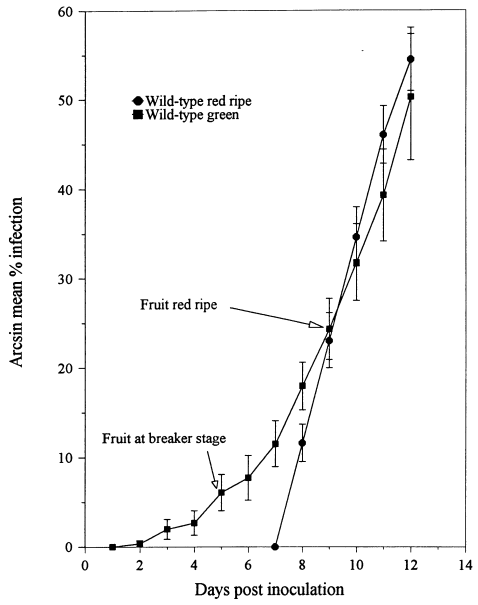


Figure 1 Infection of transgenic tomatoes with low PG and ethylene. (a) The rate of infection was compared between ripe wild-type (●), *ACO*-as (■) and PG-as (▼) tomato fruit inoculated with a spore suspension of *C. gloeosporioides* (43 fruit representing nine experiments for the *ACO*-as and wild-type fruit, and 23 fruit representing five experiments for the PG-as fruit). Fruit were maintained at 27°C, 16-h photoperiod and assessed daily for disease development. (b) Comparison of the rates of infection between green (■) and ripe wild-type fruit (●). Bars indicate the standard error of the mean.

approximately the same rate as the yellow *rin* fruits that had been treated with ethylene (data not shown).

Expression of the genes encoding ACC oxidase in response to infection

Northern analysis, with the full length *ACO* 1 cDNA (TOM13) as a probe, was used to study expression of ACC oxidase mRNA (Fig. 4). This probe hybridizes with transcripts from all three genes comprising the small multigene family known to encode ACC oxidase in tomato (Holdsworth *et al.*, 1988). The accumulation of homologous RNA began before the development of visible symptoms following inoculation with *C. gloeosporioides*. The level was high in ripe wild-type fruit samples exhibiting only 5% lesion development, peaked at approximately 10% and decreased slowly thereafter (Fig. 4a(I)). Infection scores of 10% are achieved at 1–2 days after inoculation of ripe wild-type fruit. Although low levels of ethylene biosynthesis could be detected in the ripe *ACO*-as fruit in response to infection (Fig. 2b) transcript accumulation could not be detected, probably because of the limitations of sensitivity of northern analysis.

Since green fruits are resistant to infection and ethylene has been implicated in resistance signalling, expression of the ACC oxidase genes in response to inoculation of unripe wild-type fruit was analysed. RNA was purified from inoculated and control fruit harvested at regular intervals up to 30 h. Northern analysis using the full length *ACO* 1 cDNA probe revealed induction of the transcript in the 24, 28 and 30 h postinoculation samples. Expression was not detected in any of the earlier infected or uninoculated control samples (Fig. 4b)

In order to determine the pattern of expression of *ACO* 1, 2 and 3 in response to infection, gene-specific probes were constructed. Comparisons of the 3 inch untranslated regions of each gene using the MACAW sequence analysis program (Schuler *et al.*, 1991), showed a high degree of sequence divergence. These were amplified by PCR from either cDNA or genomic DNA and ligated with the transcription vector pT_7T_3 −18. When excised from their host vectors, purified and labelled, these sequences did not cross-hybridize with one another (Fig. 4c). However, when probes were synthesized from PCR amplified products, *ACO* 1 and 3 showed a small degree of cross-hybridization (Bouzayen *et al.*, 1993). This may be the result of nonspecific hybridization with partially synthesized fragments generated during PCR amplification. For the experiments described in this paper probes were synthesized as radioactively labelled RNA transcribed *in vitro* from the cloned PCR generated fragments. The cloned gene-specific regions were named pSP13 (*ACO* 1), pSPA (*ACO* 2) and pSPB (*ACO* 3), respectively.

Northern analysis, using the *ACO* 1 gene-specific probe pSP13, revealed a pattern of expression identical to that observed using the full length *ACO* 1 cDNA as a

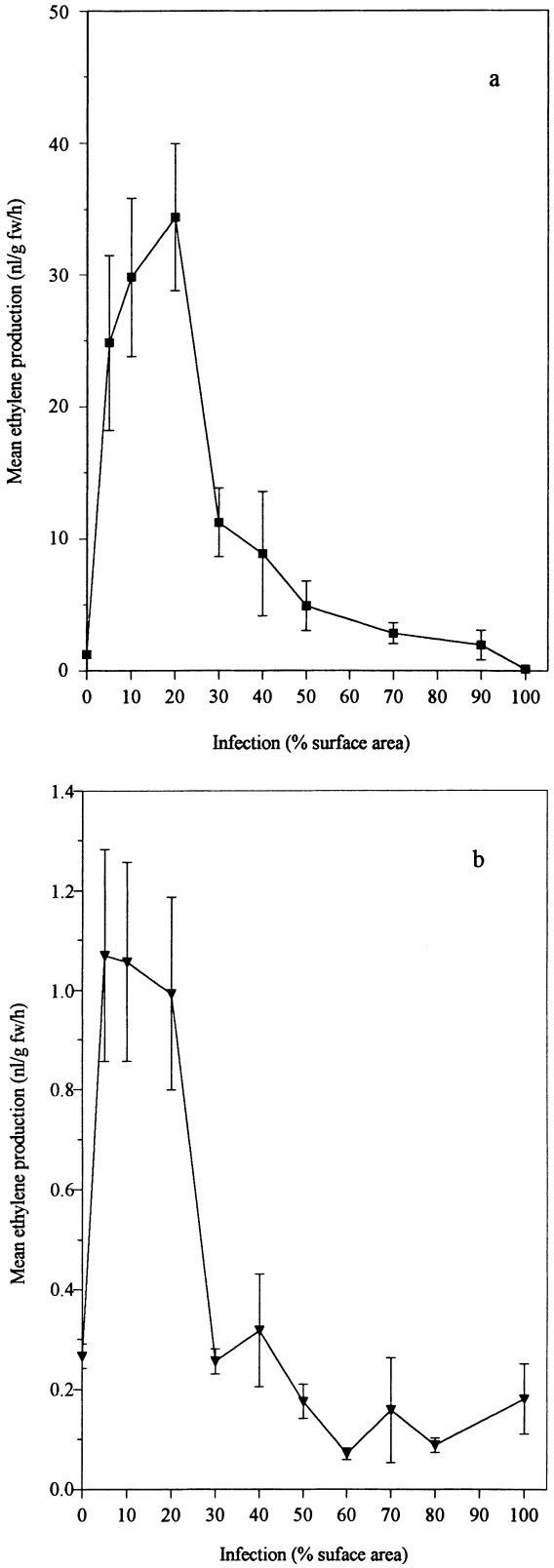


Figure 2 Ethylene biosynthesis in fruit inoculated with *C. gloeosporioides*. (a) In ripe wild-type fruit (■) and (b) ripe *ACO*-as fruit (▼). Results are nL g fwt^{-1} h^{-1} in relation to symptom severity. Bars indicate the standard error of the mean.

probe (Fig. 4a(I,II)). Northern analysis using the gene specific probes for *ACO* 2 and *ACO* 3 revealed no detectable expression during infection of ripening fruit, suggesting that *ACO* 1 is the gene predominantly expressed during infection.

Discussion

Results presented in this paper illustrate the value of the tomato fruit as a model system in which physiological changes associated with ripening can be studied in relation to infection by a postharvest pathogen. Many postharvest infections are characterized by the pathogen's inability to colonize unripe fruit. With the onset of ripening, however, infection becomes aggressive and visible lesions develop. Unripe tomato fruit infected with *C. gloeosporioides* did not develop visible symptoms until the onset of ripening, whereas ripe fruit developed visible lesions as early as 24 h postinoculation. This is typical of the biology of infection described for this pathogen (Swinburne, 1983; Jefferies *et al.*, 1990). It was also observed that disease progresses as fast in ripe fruit, inoculated when green, as in fruit inoculated when ripe, indicating the importance of the ripening process. When picked from the plant at the mature green stage, *ACO*-as (ethylene deficient antisense) fruit display an exaggerated delay in ripening compared with the same type of fruit left on the plant. It has been suggested that this is caused by an exportable inhibitor or an importable promoter of ripening, the effects of which are most obvious when ethylene synthesis is very low (Picton *et al.*, 1993). Therefore, to circumvent this nonethylene factor, the experiments were performed with fruit that had been ripened on the plant prior to inoculation.

The role of PG

A number of changes occur during fruit ripening, including the synthesis of cell-wall-degrading enzymes such as PG, colour change and an increase in ethylene biosynthesis, which is known to be intimately involved in the regulation of fruit ripening (Tucker & Grierson, 1987; Oeller *et al.*, 1991; Picton *et al.*, 1993). A transgenic tomato line (PG-as) in which polygalacturonase activity had been almost abolished by the activity of an antisense gene (Smith *et al.*, 1988) showed susceptibility to *C. gloeosporioides* infection no different from that of wild-type fruit (Fig. 1a). This is in contrast to reports suggesting that the PG-as fruit possessed some resistance to infection by other fungal pathogens (Kramer *et al.*, 1992). Our results suggest that one of the best-studied aspects of fruit ripening, the depolymerization of pectic substances, has little bearing on the progress of this postharvest disease. However, PG assays of infected PG-as fruit indicated an increase in PG activity during the late stages of infection (results not shown). This could only be attributed to the pathogen as the indigenous PG activity in these fruits is less than 0·1% of wild-type levels (Smith *et*

Figure 3 Effect of ethylene on infection of tomatoes. Mature ripe *rin* fruit were inoculated with *C. gloeosporioides* and incubated in gas jars at 27°C, 16-h photoperiod. Fruit were either incubated in an ethylene-free environment (left), or in the presence of supplemented ethylene ($10\,\mu L\,L^{-1}$ daily) (right).

al., 1988). If PG is involved in tissue susceptibility it is possible that its synthesis by the pathogen would surmount any potential effects of the inhibition of endogenous PG in these antisense fruits. When the PG-as fruit was used to study fungal PG activity in the absence of any host PG activity, pathogen-encoded PG did not appear to play a significant role during the earlier stages of infection, but it may be important in the later stages during tissue maceration. Other cell wall degrading enzymes, such as xylanase, which is synthesized during the early stages of infection of monocot plants (Cooper *et al.*, 1988), may also be important in the early stages of infection of tomato.

The role of ethylene

Transgenic fruit with much reduced ethylene biosynthesis resulting from an ACC oxidase antisense gene, had a slow-ripening phenotype with reduced over-ripening (Hamilton *et al.*, 1990; Picton *et al.*, 1993). These were used to analyse the effect of low ethylene upon postharvest infection and it was found that ripe *ACO*-as fruit developed infection symptoms more slowly than did the wild-type fruit although, eventually, the *ACO*-as fruit became fully infected (Fig. 1a). The normal infection pattern observed for the PG-as fruit also suggests that the higher resistance of the *ACO*-as fruit was related to the *ACO* antisense gene and not to transformation or the binary vector sequences transferred with the antisense gene.

As well as its involvement in ripening, ethylene is involved in the induction of genes thought to be active in defence, and ethylene synthesis has often been observed associated with infection (Barkai-Golan & Kopeliovitch, 1983; Elad, 1990; Spanu & Boller, 1989; Rickauer *et al.*, 1990; Esquerré-Tugayé *et al.*, 1993). Our results show that in the colonization of tomato fruit by *C. gloeosporioides*, the ripening-promoting effects of ethylene outweigh any defence response it may also induce. This is supported by the observation that ethylene-treated *rin* fruit rapidly become infected by *C. gloeosporioides*, whereas untreated *rin* fruit remain essentially disease-free (Fig. 3). Ethylene treatment of *rin* fruit only partially restores ripening, indicating that other factors as well as ethylene are necessary (Herner & Sink, 1973; Mizrah *et al.*, 1975). However, the rapid infection of ethylene-treated *rin* fruit by *C. gloeosporioides* suggests that it is either ethylene itself, or those ripening-associated changes induced by ethylene that confer susceptibility to this pathogen. These do not include PG (Knapp *et al.*, 1989). The recent findings by Flaishman & Kolattukudy (1994) that *C. gloeosporioides* responds directly to ethylene is consistent with this observation.

Ethylene production by the infected *ACO*-as fruit was reduced to 4% of that produced by infected wild-type fruit. This is similar to the reduction observed during fruit ripening in the antisense fruit (Hamilton *et al.*, 1991; Picton *et al.*, 1993), where residual ethylene production is a result of incomplete suppression of *ACO* gene expression by the antisense gene (Picton *et al.*, 1993). This suggests that most of the ethylene synthesized during infection is produced by the fruit. However, since a number of fungi are known to synthesize ethylene, although the biosynthetic pathway may not always be the same as in higher plants (Chalutz *et al.*,

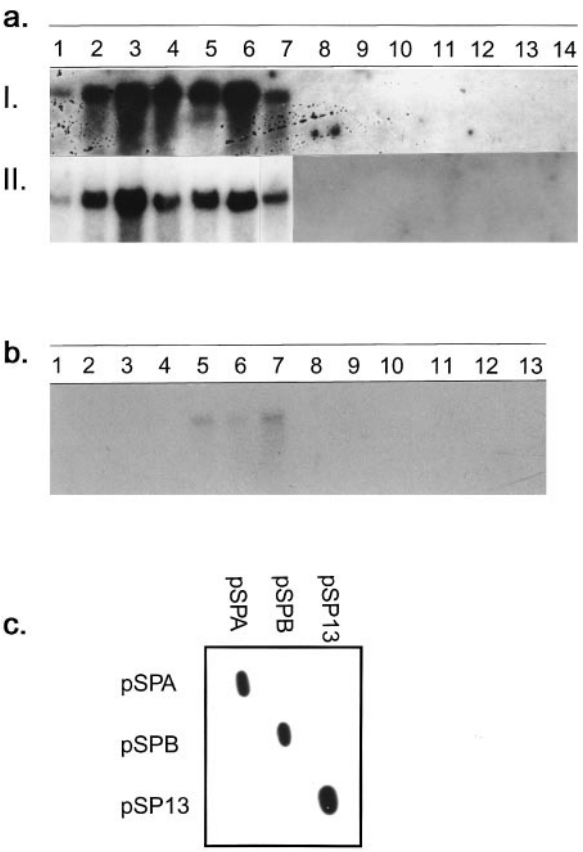


Figure 4 Expression of *ACO* 1 mRNA in response to infection. (a) (I) RNA was purified from infected ripe wild-type and *ACO*-as fruit and transferred onto hybridization membranes before hybridization with a full length *ACO* 1 ribo-probe. Samples (lanes 1–7) represent wild-type fruit (control, and 5% 10%, 20%, 30% 40%, 70% infection) and lanes 8–14 represent the *ACO*-as fruit (control uninfected, 5%, 10%, 20%, 30% 40%,70% infection). (II) Expression of the *ACO* 1 gene during infection of wild-type and *ACO*-as fruit was determined using a pSP13 gene-specific ribo-probe. (b) Expression of ACC oxidase after inoculation of mature green untransformed fruit. Mature green wild-type fruit were harvested and spray inoculated with a spore suspension of *C. gloeosporioides*. Control fruit were sprayed with distilled water. RNA was purified from fruit harvested at 0, 2, 4, 10, 24, 28 and 30 h postinoculation (lanes 1–7). Control samples were harvested 4 h, 6 h, 10 h, 12 h, 24 h, and 30 h post spraying (lanes 8–13). Total RNA (5 μg) was purified and fractionated by gel-electrophoresis before transfer to hybridization membrane. The membrane was hybridized to a radiolabelled *ACO* 1 ribo-probe. (c) Samples (approximately 10 ng) of the PCR amplified and cloned 3′ untranslated regions representing each of the three *ACO* genes were denatured and blotted onto hybridization membrane. Ribo-probes were generated using each of the cloned 3′ untranslated regions and hybridized for 16 h at 65°C before washing in 0·2 × SSPE, 0·1% SDS twice at 55°C, for 20 min.

1977; Su & Hodges, 1989), the possibility that part of this residual ethylene was produced by the fungus cannot be ruled out.

Kacperska & Kubacka-Zebalska (1985) suggested that infection-associated ethylene may be evolved via an alternative biosynthetic pathway involving lipid peroxidation as a result of membrane damage during fungal infection of plants. However, the low level of ethylene biosynthesis measured in the *ACO*-as fruit in response to infection suggests that lipid peroxidation is not responsible for most of the ethylene evolution during infection. The results of the northern analysis of ACC oxidase gene expression during infection of tomato fruit complement the enzymatic analysis presented by Spanu & Boller (1989). They demonstrated that infection of potato leaves by *Phytophthora infestans* resulted in increased activity of both ACC synthase and ACC oxidase in tissues either infected or immediately surrounding the infection site as early as 30 h postinoculation and prior to the development of visible lesions. The present results, in conjunction with the results of Spanu & Boller (1989), confirm that most of the infection-induced ethylene is synthesized via the conversion of SAM into ethylene by ACC synthase and ACC oxidase and not via alternative pathways or membrane damage and lipid peroxidation as suggested by Kacperska & Kubacka-Zebalska (1985).

Expression of ACC oxidase genes

Interestingly, one response of a mature green tomato fruit to infection by *C. gloeosporioides* is the accumulation of *ACO* mRNAs (Fig. 4). Although overall there is no significant (detectable) rise in ethylene synthesis, small but significant increases of ethylene biosynthesis in the immediate vicinity of the infection cannot be ruled out. It is generally assumed that ACC oxidase expression is constitutive (Yang & Hoffman, 1984). However, because of the rapid wound-related induction of ACC oxidase activity (Holdsworth *et al.*, 1988; Hamilton *et al.*, 1990) it is probable that experimental techniques used to analyse activity resulted in this being interpreted as constitutive ACC oxidase activity. It has been shown, using ACO1 promoter-Gus fusions in transgenic plants, that transcription of ACO1 is activated during ripening, senescence and infection (Blume & Grierson, 1997).

Ethylene biosynthesis in ripe wild-type fruit inoculated with *C. gloeosporioides* increased very rapidly during the early stages of infection, suggesting that this was not merely a nonspecific reaction (Fig. 2). Accumulation of the *ACO* transcript correlated well with the measurements of ethylene synthesis. Equivalent experiments using the *ACO*-as fruit indicated that, although ethylene biosynthesis increased during infection, the level was approximately 25 times less than in the wild-type fruit. The peak in ethylene biosynthesis was also slightly earlier than that observed in the wild-type fruit. No *ACO* transcript was detected in the *ACO*-as fruit by northern analysis.

The cDNA, TOM13 (*ACO* 1), which was later found to encode ACC-oxidase (Hamilton *et al.*, 1990; Hamilton *et al.*, 1991), was initially characterized as a fruit-ripening-related cDNA which was also expressed in leaves upon wounding (Smith *et al.*, 1986; Holdsworth *et al.*, 1988). Three homologous but distinct genomic clones were isolated using this cDNA as a probe,

suggesting that it was a member of a small multigene family consisting of at least three genes (Holdsworth *et al.*, 1988). GTOM 17, the genomic clone for TOM13 cDNA, and the two other clones, GTOM A and GTOMB, are representative of three loci now named *ACO* 1, 2 and 3, respectively.

A cDNA described by Spanu *et al.* (1991), which was identical to the deduced coding sequence of the *ACO* 3 (Bouzayen *et al.*, 1993) gene, indicated that this gene was expressed and therefore was unlikely to be a pseudogene. Since this cDNA was isolated from a library constructed from an elicitor-treated tomato cell culture it was important to investigate its expression in response to fungal attack. We were interested to find out whether the increased level of TOM 13 homologous RNA was an enhancement of ongoing ripening-related gene expression or if some other previously inactive gene, such as *ACO* 3, was now responding to the new signals.

Since the TOM 13 cDNA demonstrably hybridizes with each of the *ACO* mRNAs the construction of gene-specific probes corresponding to the 3′ untranslated regions of each of the *ACO* genes was necessary for the determination of individual gene expression during infection. Figure 4(a(II)) shows that the *ACO* 1 transcript accumulated rapidly upon infection and reached its peak by 10% infection. The pattern of expression was identical to that observed during northern analysis using the full length *ACO* 1 cDNA probe (Fig. 4a(I)). No expression of *ACO* 2 or 3 was detected, indicating that the signals produced by fungal invasion act upon the expression of the same gene, which is active during ripening. Although no *ACO* 2 or 3 transcript was detected in response to infection, this does not rule out expression. Both *ACO* 2 and 3 may still be expressed, perhaps very early during infection or at levels below the sensitivity of northern analysis. Further analysis of the significance of *ACO* 2 and 3 during infection, using more sensitive means of detection such as ribonuclease protection assays, may indicate interplay between these three ACC oxidase genes.

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References

Abeles FB, Bosshart RP, Forrence LE, Habig WH, 1970. Preparation and purification of glucanase and chitinase from bean leaves. *Plant Physiology,* **47,** 129–34.

Barkai-Golan R, Kopeliovitch E, 1983. Induced ethylene evolution and climacteric-like respiration in *Rhizopus*-infected *rin* and *nor* tomato mutants. *Physiological Plant Pathology,* **2,** 357–62.

Blume B, Grierson D, 1997. Expression of ACC oxidase promoter-GUS fusions in tomato and *Nicotiana plumbaginifolia* regulated by developmental and environmental stimuli. *Plant Journal,* **12,** 731–746.

Bouzayen M, Cooper W, Barry C, Zegzouti H, Hamilton AJ, Grierson D, 1993. EFE multi-gene family in tomato plants: Expression and Characterisation. In: Pech JC, Latché A, Balagué C, eds *Cellular and Molecular Aspects of the Plant Hormone Ethylene.* Dordrecht, the Netherlands: Kluwer Academic Publishers, 76–82.

Broglie KE, Gaynor JJ, Broglie RM, 1986. Ethylene regulated gene expression: Molecular cloning of the genes encoding endochitinase from *Phaseolus vulgaris. Proceedings of the National Academy of Sciences USA,* **83,** 6820–4.

Chalutz E, Liberman M, Sisler HD, 1977. Methionine induced ethylene production by *Penicillium digitatum. Plant Physiology,* **60,** 402–6.

Cooper RM, 1984. The role of cell wall degrading enzymes in infection and damage. In: Wood RKS, Jellis GJ, eds *Plant Disease: Infection, Damage and Loss.* Oxford: Blackwell Scientific Publications, 13–27.

Cooper RM, Longman D, Campbell A, Henry M, Lees PE, 1988. Enzymic adaptation of cereal pathogens to the monocotyledonous primary wall. *Physiological and Molecular Plant Pathology,* **32,** 33–47.

Elad Y, 1990. Production of ethylene by tissues of tomato, pepper, French-bean and cucumber in response to infection by *Botrytis cinerea. Physiological and Molecular Plant Pathology,* **36,** 277–87.

Esquerré-Tugayé MT, Bottin A, Rickauer M, Sancan JP, Fournier J, Pouenat ML, 1993. Ethylene in early signalling phenomena at the plant–micro–organism interface. In: Pech JC, Latché A, Balagué C, eds *Cellular and Molecular Aspects of the Plant Hormone Ethylene.* Dordrecht, the Netherlands: Kluwer Academic Press, 217–22.

Flaishman MA, Kolattukudy PE, 1994. Timing of fungal invasion using host's ripening hormone as a signal. *Proceedings of the National Academy of Sciences USA,* **91,** 6579–83.

Gane R, 1934. The production of ethylene by some ripening fruit. *Nature,* **134,** 1008.

Grierson D, Tucker GA, 1983. Timing of ethylene and polygalacturonase synthesis in relation to the control of tomato fruit ripening. *Planta,* **157,** 174–9.

Hamilton AJ, Bouzayen M, Grierson D, 1991. Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. *Proceedings of the National Academy of Sciences USA,* **88,** 7434–7.

Hamilton AJ, Lycett GW, Grierson D, 1990. Antisense gene that inhibits synthesis of ethylene in transgenic plants. *Nature,* **346,** 284–7.

Herner RC, Sink KC, 1973. Ethylene production and respiratory behaviour or the *rin* tomato mutant. *Plant Physiology,* **52,** 38–42.

Hobson GE, Grierson D, 1993. Tomato. In: Seymour G, Taylor JE, Tucker G, eds *Biochemistry of Fruit Ripening,* London: Chapman & Hall, 405–42.

Holdsworth MJ, Schuch W, Grierson D, 1988. Organisation and expression of a wound/ripening-related small multigene family from tomato. *Plant Molecular Biology,* **11,** 81–8.

Jefferies P, Dodd JC, Jeger MJ, Plumbley RA, 1990. The biology and control of *Colletotrichum* species on tropical fruit crops. *Plant Pathology,* **39,** 343–66.

Kacperska A, Kubacka-Zebalska M, 1985. Is lipoxygenase

involved in the formation of ethylene from ACC? *Physiologia Plantarum,* **64,** 333–63.

Kirby KS, 1956. A new method for the isolation of nucleic acids from mammalian tissues. *Biochemical Journal,* **64,** 405–8.

Knapp J, Moureau P, Schuch W, Grierson D, 1989. Organisation and expression of polygalacturonase and other ripening related genes in Ailsa Craig 'Neverripe' and 'ripening inhibitor' tomato mutants. *Plant Molecular Biology,* **12,** 105–16.

Kramer M, Sanders R, Bolkan H, Waters C, Sheehy RE, Hiatt WR, 1992. Post harvest evaluation of transgenic tomatoes with reduced levels of polygalacturonase: processing, firmness and disease resistance. *Post-harvest Biology and Technology,* **1,** 241–55.

Lincoln JE, Cordes S, Read E, Fischer RL, 1987. Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit development. *Proceedings of the National Academy of Sciences USA,* **84,** 2793–7.

Mizrah Y, Dostal HC, Cherry JH, 1975. Ethylene induced ripening in attached *rin* fruit, a non-ripening mutant of tomato. *Horticultural Science,* **10,** 414–5.

Oeller PW, Min-Wong Lu Taylor LP, Pike DA, Theologis A, 1991. Reversible inhibition of tomato fruit senescence by antisense RNA. *Science,* **254,** 437–9.

Picton S, Barton SL, Bouzayen M, Hamilton AJ, Grierson D, 1993. Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. *Plant Journal,* **3,** 469–81.

Rickauer M, Fournier J, Pouénat M, Berthalon É, Bottin A, Esquerré-Tugayé M, 1990. Early changes in ethylene synthesis and lipoxygenase activity during defense induction in tobacco cells. *Plant Physiology and Biochemistry,* **28** (5), 647–53.

Sambrooke J, Fitsch EF, Maniatis T, 1989. *Molecular Cloning: a Laboratory Manual*, 2nd edn. New York, NY, USA: Cold Spring Harbor Laboratory Press.

Sanger F, Miklen S, Coulsen AR, 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA,* **74,** 5463–7.

Sato T, Theologis A, 1989. Cloning the mRNA encoding 1-aminocyclopropane-carboxylate synthase, the key enzyme for ethylene biosynthesis in plants. *Proceedings of the National Academy of Sciences USA,* **86,** 6621–5.

Schuler GD, Altschul SF, Lipman DJ, 1991. A Workbench for Multiple alignment construction analysis. *Proteins: Structure, Function, and Genetics,* **9,** 180–90.

Smith CJS, Slater A, Grierson D, 1986. Rapid appearance of an mRNA correlated with ethylene synthesis encoding a protein of molecular weight 35,000. *Planta,* **168,** 94–100.

Smith CJS, Watson CF, Morris PC, Bird CR, Seymour GB, Gray JE, Arnold CE, Tucker GA, Schuch W, Harding S, Grierson D, 1990. Inheritance and effect on ripening of antisense polygalacturonase genes in transgenic tomatoes. *Plant Molecular Biology,* **14,** 369–79.

Smith CJS, Watson CF, Ray J, Bird CR, Morris PC, Schuch W, Grierson D, 1988. Antisense RNA inhibition of polygalacturonase gene expression in transgenic tomatoes. *Nature,* **334,** 724–6.

Spanu P, Boller T, 1989. Ethylene biosynthesis in tomato plants infected by *Phytophthora infestans. Journal of Plant Physiology,* **134,** 533–7.

Spanu P, Reinhardt D, Boller T, 1991. Analysis and cloning of the ethylene-forming enzyme from tomato by functional expression of its mRNA in *Xenopus laevis* oocytes. *EMBO Journal,* **10,** 2007–13.

Su Mei-Yu Hodges CF, 1989. Ethylene production by *Bipolaris sorokiniana* and *Curvularia geniculata* on methionine and inorganic salts. *Plant Disease,* **73,** 398–401.

Swinburne TR, 1983. Quiescent infections in post harvest diseases. In: Dennis C, ed. *Post Harvest Pathology of Fruits and Vegetables.* London: Academic Press, 1–21.

Tucker GA, Grierson D, 1982. Synthesis of polygalacturonase during tomato fruit ripening. *Planta,* **155,** 64–7.

Tucker GA, Grierson D, 1987. Fruit ripening. In: Davies DD, ed. *The Biochemistry of Plants, a Comprehensive Treatise 12.* London: Academic Press, 265–318.

Yang SF, Hoffman NE, 1984. Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology,* **35,** 155–89.