Induction of *p*-Coumaroyldopamine and Feruloyldopamine, Two Novel Metabolites, in Tomato by the Bacterial Pathogen *Pseudomonas syringae*

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Submitted 4 May 2007. Accepted 5 July 2007.

Inoculation of tomato plants (*Solanum lycopersicum* cv. Rutgers) with *Pseudomonas syringae* pv. *tomato* led to the production of a hypersensitive-like response in this pathovar of tomato. Accumulation of hydroxycinnamic acid amides (HCAA) of tyramine (*p*-coumaroyltyramine and feruloyltyramine) and dopamine (*p*-coumaroyldopamine and feruloyldopamine) was detected after bacterial infection. Two of them, *p*-coumaroyldopamine and feruloyldopamine, are described for the first time. The accumulation of HCAA was preceded by an increment of hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyl transferase (*THT*) gene expression. HCAA also accumulated in transgenic NahG tomato plants overexpressing a bacterial salicylic hydroxylase. However, treatment of plants with the ethylene biosynthesis inhibitor, aminoethoxyvinylglycine, led to a reduction in the accumulation of *THT* transcripts and HCAA. Together, the results suggest that pathogen-induced induction of ethylene is essential for HCAA synthesis, whereas salicylic acid is not required for this response. In addition, notable antibacterial and antioxidant activities were found for the new HCAA, thus indicating that they could play a role in the defense of tomato plants against bacterial infection.

Higher plants are continuously exposed to a large range of biotic (viroids, viruses, bacteria, or fungi) and abiotic environmental challenges. They cannot escape from these potentially stressing agents and have developed a staggering and sophisticated battery of defense mechanisms; consequently, most plants are resistant to many pathogens of plants (Dangl and Jones 2001; Dixon et al. 1994). These mechanisms of defense are induced upon infection by pathogens leading to the so-called acquired resistance; however, a preexisting defense in the form of physical and chemical barriers also exists. When a pathogen encounters a plant, two kinds of interactions can be produced between them. In the first case, a gene-for-gene-type interaction is accompanied by cell death, producing a necrotizing reaction or hypersensitive response of the plant. The infection then is restricted to small areas immediately surrounding the initially infected cells and, in turn, signals the activation of defensive reactions in uninfected parts of the plant. These distal sites then become more resistant to subsequent infections. This response is the so-called systemic acquired resistance

(Sticher et al. 1997). Sometimes, the defensive response also can be elicited in compatible, non-necrotizing interactions by different pathogens in the absence of gene-for-gene resistance (Conejero et al. 1990; Dixon et al. 1994). Some common responses in both incompatible and compatible interactions include the induction of phenolic salicylic acid (SA; 2-hydroxybenzoic acid) (Dempsey et al. 1999; Métraux et al. 1990), the synthesis of pathogenesis-related proteins (Van Kan et al. 1992; Van Loon et al. 2006b), and natural antimicrobial products (phytoalexins) (Dixon 2001; Hammerschmidt 1999). The role in plant disease resistance of some of these metabolites has been well established in mutant and transgenic plants with altered expression of phenylpropanoid genes or modified levels of phenylpropanoid metabolites (Dixon et al. 2002; Niggeweg et al. 2004; Thomma et al. 1999; Verberne et al. 2000). Cinnamic acid (phenylpropanoid acid) is the product of deamination of phenylalanine catalyzed by phenylalanine ammonia-lyase (PAL; L-phenylalanine ammonia-lyase, EC 4.3.1.5), the first regulatory enzyme of the phenylpropanoid pathway. In higher plants, hydroxycinnamic acids such as *p*-coumaric (4-hydroxycinnamic acid) and ferulic (4-hydroxy-3-methoxycinnamic) acids can occur conjugated to the β -phenylethylamine-alkaloids tyramine (2-[4-hydroxyphenyl] ethylamine) and octopamine [2-hydroxy-2-[4-hydroxyphenyl] ethylamine), forming the corresponding *N*-hydroxycinnamic acid amides (HCAA) (Facchini et al. 2002; Strack 1997). These compounds are thought to play a defensive role in plants (Hahlbrock and Scheel 1989) and their synthesis is induced upon pathogen infection and in response to various stresses, including wounding or elicitor treatments in different plants tissues (Keller et al. 1996; Negrel and Martin 1984; Newman et al. 2001; Pearce et al. 1998; Schmidt et al. 1998). The pivotal regulatory enzyme responsible for the synthesis of HCAA is the enzyme hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyl transferase (*THT*; EC 2.3.1.110) (Facchini et al. 2002; Negrel and Javelle 1997; Negrel and Martin 1984). Recently, Von Roepenack-Lahaye and associates (2003) have found that the tomato cv. Rio Grande, challenged with the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, synthesized *p*-coumaroyloctopamine and the novel plant compound, *p*-coumaroylnoradrenaline, and that the elevated levels of these metabolites were concomitant with an increased expression of a gene encoding the *THT* isoenzyme.

Tomato plants infected with citrus exocortis viroid, which produces a systemic infection (compatible interaction), strongly accumulated gentisic acid (2,5-dihydroxybenzoic acid, a SA-derivative phenolic) (Bellés et al. 1999; Fayos et al. 2006). It has been demonstrated that gentisic acid acts as a signal, in addition

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to SA, to elicit the upregulation of defense proteins in tomato and cucumber plants (Bellés et al. 1999, 2006). Interestingly, this secondary metabolite did not accumulate when tomato plants were infiltrated with the necrotizing pathogen *P. syringae* pv. *tomato*, which elicits a hypersensitive-like reaction in tomato leaves (incompatible interaction) (Bellés et al. 1999). In an attempt to identify additional compounds from the phenylpropanoid pathway that might have some role in tomato plant defense, we have studied the synthesis of amides upon challenging tomato leaves with *P. syringae* pv. *tomato*. We show here the identification of two metabolites for plants: *N-p*-coumaroyldopamine (CD), and *N*-feruloyldopamine (FD), which, to our knowledge are novel compounds. We also observed induction of the well-known *N-p*-coumaroyltyramine (CT) and *N*-feruloyltyramine (FT), and the expression of the *THT* gene in *P. syringae*-infected tomato leaves. The antimicrobial and antioxidant activities of these compounds also were studied. All of them presented a positive antioxidant activity, and the new HCAA of dopamine had a notable bactericidal action. To gain information on the possible signals implicated in the activation of HCAA synthesis after bacterial challenging, we also have explored the implication of ethylene and SA on HCAA accumulation. Interestingly, inhibition experiments of *P. syringae*-induced ethylene with aminoethoxyvinylglycine (AVG) strongly suggest that ethylene is essential to elicit HCAA synthesis. However, exogenous application SA had no effect on HCAA levels in tomato leaves, and NahG tomato plants accumulated HCAA upon bacterial challenge, thus further supporting the notion that SA does not affect the pathogen-induced induction of HCAA synthesis.

Fig. 1. Representative absorbance chromatograms (recorded at 280 nm) obtained after reversed-phase high-performance liquid chromatography (HPLC) separation from soluble methanol leaf extracts of control and infected Rutgers tomato leaves. Plants were mock-inoculated or challenged with *Pseudomonas syringae* pv. *tomato* at 10^7 CFU/ml (*Pst*-infected). Samples were collected at 24 h postinoculation. Absorbance was monitored by a photodiode array detector with a range of maxplot between 240 and 400 nm and a spectral resolution of 1.2 nm. Peaks 1, 2, 3, and 4 represent the unknown phenolic compounds with retention times of 12.5, 12.7, 13.2, and 13.6 min, respectively.

RESULTS AND DISCUSSION

Characterization of HCAA

in Rutgers tomato challenged with *P. syringae*.

Rutgers tomato leaves inoculated with the bacterial pathogen *P. syringae* pv. *tomato* developed a hypersensitive-like response, manifested as a bacterial concentration-dependent necrotic reaction. In this article, we applied a bacterial concentration of 10^7 CFU/ml. The method employed to inoculate the bacteria first produced water soaking in the leaves, which disappeared within approximately 1 h. The observation of the hypersensitive reaction began with the glossy appearance of the inoculated leaf surface area (approximately 6 mm in diameter) and was evident as early as 12 h after bacterial infiltration. The pathogen response culminated at approximately 72 h postinoculation with the appearance of nonspreading necrotic brown spots and cellular collapse over the whole inoculated region. Also, at approximately 24 h after leaf infiltration by the bacteria, a marked epinasty of the inoculated leaves was already evident. Previously, using fluorescence detection of methanolic extracts, we had found that Rutgers tomato leaves infiltrated with *P. syringae* accumulated SA (Bellés et al. 1999), a phenolic associated with the hypersensitive response of plants to pathogens (Dempsey et al. 1999; Métraux and Raskin 1993). Therefore, to extend the search for other metabolites induced in tomato by *P. syringae*, we decided to carry out a more general analysis of UV-absorbing methanol-soluble phenolic compounds in extracts from *P. syringae*-infected tomato leaves. High-performance liquid chromatography (HPLC) chromatograms of methanol-soluble extracts from *P. syringae*-infected Rutgers tomato leaves 24 h after the challenge are shown in Figure 1. The corresponding control mock-inoculated leaves are shown for comparison. To survey metabolites of the phenylpropanoid pathway, the 280-nm wavelength was selected upon studying the absorbance spectra recorded with the diode array detector by scanning from 240 to 400 nm. As observed, a set of four major peaks (12.5-min, 12.7-min, 13.2-min, and 13.6-min relative retention times) appeared in extracts from *P. syringae*-infected tomato leaves. These peaks did not appear in HPLC chromatograms corresponding to extracts from uninoculated control leaves. In some experiments, such as the one shown in Figure 1, an additional peak with a retention time slightly longer than peak 2 appeared. However, this peak was not observed consistently and we were not able to identify its chemical nature. It is likely to correspond to an artifact due to irregularities in the separation process.

To precisely identify these unknown compounds corresponding to peaks 1 to 4, an HPLC-mass spectrometry (MS) analysis combined with electrospray ionization (ESI) of the soluble-methanol phenolic fraction from infected tomato leaves was performed. Mass spectra fragments of peaks 1 to 4 from methanolic tomato extracts obtained by ESI in the positive ion mode are shown in Table 1. Under mild ionization conditions (cone voltage of 20 V), the compounds gave a substantial fragmentation. The mass spectra from total ion current chromatograms showed protonated $(M+H)^+$ fragment ions of a mass-to-charge ratio (m/z) equal to 299, 329, 283, and 313. The product ion scan spectrum of peaks 1 to 4 gave common ions at m/z 147 and 177, which are daughter ions, characteristic of the *p*-coumaroyl and feruloyl moieties, respectively. Peaks 3 and 4 presented UV spectra identical to that of authentic CT and FT and cochromatographed with synthesized standards. Mass spectra of peaks 1 and 2 showed, in addition to common ions at m/z 147 and 177, a 16-Da higher $(M+H)^+$ at m/z 299 and 329, revealing a structure with one additional hydroxyl group, compared with tyramine, in the amine moiety. The presence of these additional ions is compatible with the identification of these compounds as conjugated amines, such as

octopamine (hydroxyl group is joined to carbon 7), or dopamine (hydroxyl function is joined to benzylic ring). We were able to positively identify peaks 1 and 2 as *p*-coumaroyldopamine and feruloyldopamine, respectively, based on the absence of a strong and characteristic (M+H-H₂O)⁺ ion present in the ESI spectra of amine conjugates with the hydroxyl group

joined to carbon 7 of the amine moiety (which is the case of octopamine) (Schmidt et al. 1999; Von Roepenack-Lahaye et al. 2003). In addition, these compounds cochromatographed with and have identical UV spectra to that of authentic HCAA that had been chemically synthesized. To our knowledge, this is the first evidence of the presence of HCAA of dopamine in plants.

Table 1. Electrospray ionization mass spectra comparisons^a

Parameters	Compound			
	1	2	3	4
Positive ions ^b				
[M + H] ⁺	300.2 (100)	330.2 (11)	284.3 (100)	314.3 (39)
(<i>m/z</i>)	147.0 (71)	...	147.0 (25)	...
(<i>m/z</i>)	...	177.1 (24)	...	177.1 (10)
Positive ions ^c				
[M + H] ⁺	300.1 (100)	330.1 (100)	284.1 (100)	314.1 (100)
(<i>m/z</i>)	147.0 (24)	...	147.0 (17)	...
(<i>m/z</i>)	...	177.0 (15)	...	177.0 (10)
λ _{max} ^b (nm)	290, 306	290, 318	291, 306	292, 318
λ _{max} ^c (nm)	291, 307	291, 319	292, 307	293, 319
RT ^b (min)	12.0	12.2	12.9	13.1
RT ^c (min)	12.5	12.6	13.2	13.5

^a Ion positive detection mode (*m/z* [%]), absorbance maxima (nm), and retention times.

^b Results of peaks 1, 2, 3, and 4.

^c Results of synthetic hydroxycinnamic acid amides.

Accumulation of HCAA in tomato infected with *P. syringae*.

Results of the analysis of the time course of induction of p-CD, FD, p-CT, and FT in Rutgers tomato leaves upon *P. syringae* challenge is presented in Figure 2A. The relative timing of the maximum accumulation of HCAA, approximately 24 h postinoculation, preceded the formation of necrotic brown spots, which appear after 72 h postinoculation. The accumulation profiles for FD and FT were quite similar and different from that of CD and CT, which also were similar. The levels of all HCAA increased in a time-dependent manner, reaching their maximum level at 24 h after inoculation and decreasing thereafter in the case of FD and FT, whereas the levels of CD and CT remained at this comparatively high level during the subsequent 2 days (72 h after inoculation). The maximum level of these compounds differ between amides, with the highest observed value corresponding to FD (46 nmol/g fresh weight) and the lowest to CD (1.7 nmol/g fresh weight).

In order to determine the localization of the production of HCAA, we compared the levels present in the inoculated area and the immediately surrounding tissue (approximately 1 mm) with the accumulation in the remaining noninoculated leaflet. The increase in HCAA primarily was confined to and around the inoculated region (Fig. 2B). This location was similar to that previously reported for HCAA of tyramine in tomato (Pearce et al. 1998) and tobacco (Guillet and De Luca 2005) leaves after wounding.

Fig. 2. A, Time courses for the accumulation of *p*-coumaroyldopamine (CD), feruloyldopamine (FD), *p*-coumaroyltyramine (CT), and feruloyltyramine (FT) in Rutgers tomato leaves at the specified times postinoculation (p.i.) with *Pseudomonas syringae* pv. *tomato* at 10⁷ CFU/ml. Results are the mean ± standard error from three replicates. **B,** Accumulation of CD, FD, CT, and FT at 24 h postinoculation in the inoculated area and the immediately surrounding tissues (approximately 1 mm) (gray bars) compared with the accumulation in the adjacent tissue (white bars). FW, fresh weight.

The four HCAA described above as being associated with the tomato Rutgers-*P. syringae* interaction were accumulated in a similar way in two additional tomato cultivars (Ailsa Craig and Moneymaker) upon *P. syringae* pv. *tomato* inoculation (data not shown). The fact that these compounds had not been detected in Rio Grande tomato infected with *P. syringae* (Von Roepenack-Lahaye et al. 2003) and that we have not found the HCAA described by these authors may be due to differential cultivar behavior. The time course of induction and relative timing of the accumulation of these amine conjugates in Rutgers tomato leaves challenged with *P. syringae* was comparable with that observed for FT and CT in pepper leaves infected with *Xanthomonas campestris* (Newmann et al. 2001) and for *p*-coumaroylnoradrenaline in Rio Grande tomato infected with *P. syringae*. Increased synthesis of *p*-coumaroyloctopamine and *p*-coumaroylnoradrenaline associated with *P. syringae* infection of tomato plants or treatment with elicitors from the fungus *Cladosporium fulvum* have been reported (Von Roepenack-Lahaye et al. 2003). Biosynthesis of FT and CT was induced in tomato leaves in response to wounding (Pearce et al. 1998), and FT accumulated in tobacco leaves after infection with *Tobacco mosaic virus* (Negrel and Jeandet 1987). CT and FT were associated with the resistance reactions of pepper to *X. campestris* (Newman et al. 2001) and of potato to *Phytophthora infestans* (Keller et al. 1996).

In plants, the storage form of many phenolic derivatives is conjugated to sugars (Harborne 1980). Hydrolysis of these conjugated forms could constitute a source of free HCAA as an alternative to their biosynthetic pathway. Therefore, we also have investigated the possible presence of glycosyl derivatives of the accumulated amides in tomato upon bacterial infection. After treatment of methanolic extracts from *Pseudomonas syringae*-inoculated tomato leaves with β -glucosidase and esterase, no release of any free CT, FT, CD, or FD could be detected. This result indicates that the amides were not accumulated as sugar conjugates. Similar results were obtained by Newman and associates (2001) for CT and FT in pepper leaves elicited by *X. campestris*.

Recently, Niehl and associates (2006), studying the compatible interaction between *Solanum tuberosum* and *Potato virus X*, have hypothesized that tyramine and dopamine (both highly induced upon infection) can contribute to active plant defense responses by forming hydroxycinnamic acid amides, which increases cell wall stability and fortification. However, the authors were not able to directly identify the HCAA of tyramine and dopamine in infected leaf material. In this work, we have found that these HCAA accumulated in tomato plants after

infection with a necrotizing pathogen. The results presented in this report extend to other plant systems and to other classes of HCAA the important role that these metabolites could play in plant resistance to pathogens, as previously suggested by others (Newman et al. 2001; Von Roepenack-Lahaye et al. 2003).

Induction of *THT* gene expression precedes HCAA accumulation in tomato infected with *P. syringae*.

Total RNA was isolated at different time points after the onset of bacterial infiltration and subjected to reverse-transcription polymerase chain reaction (RT-PCR) analysis to determine the expression levels of mRNA encoding the THT1-3 isoenzyme (Von Roepenack-Lahaye et al. 2003). The kinetic pattern of *THT* transcript accumulation at the specified times is shown in Figure 3A. *THT* transcripts of 724-bp size began to accumulate as early as 5 h after inoculation of the leaves. Maximal transcript levels are detected at 24 h, and decline thereafter. Comparison of the kinetics of the induction patterns of *THT* transcript levels (Fig. 3A) and HCAA accumulation (Fig. 2A) demonstrated an association between HCAA production and alterations in transcription of *THT*. As expected, the increment of the *THT* gene expression occurred earlier than that of HCAA accumulation. These results suggest that activation of the *THT* gene expression results in an increase of HCAA synthesis in a manner similar to that observed in other plant-pathogen interactions (Newman et al. 2001; Von Roepenack-Lahaye et al. 2003). Mock-inoculated tomato leaves do not show an incremental increase in the level of *THT* transcripts. The time course of the induction of *THT* transcripts observed in cv. Rutgers is in agreement with that observed in cv. Rio Grande upon *P. syringae* infection (Von Roepenack-Lahaye et al. 2003), and is comparable with that observed in potato cell cultures after *P. infestans* elicitor treatment (Schmidt et al. 1999) or pepper infected with *X. campestris* (Newman et al. 2001).

Ethylene is essential for the induction of *THT* gene expression and the accumulation of HCAA.

Initial studies on the interaction between Rutgers tomato and the bacterial pathogen *P. syringae* showed that infection of the leaves with this pathogen caused a rapid and pronounced synthesis of the plant hormone ethylene, a well-known response of plants to different pathogenic infections (Van Loon et al. 2006a). Previously, it had been thought (Boller 1990) that ethylene, generated in response to pathogens, would have a role in the induction of antimicrobial phytoalexins; although, in some plant-pathogen interactions, ethylene does not appear to be important for this response (Boller 1990; Reinhardt et al. 1991). Therefore, to establish the implication of ethylene in the tomato defense reactions studied in this work, we first examined whether application of exogenous ethylene was able to activate *THT* expression and HCAA accumulation in Rutgers tomato. Accordingly, tomato plants were treated with exogenous ethylene (50 μ l/liter), as indicated below. Ethylene treatment led to an increase in the expression levels of *THT* transcripts compared with control plants by 24 h (Fig. 4B). This result is consistent with the elevated levels of HCAA in ethylene-treated plants (Fig. 4A). The increments of *THT* expression in ethylene-treated plants were comparable with that in *P. syringae*-infected tomato leaves at the same time after the inoculation. To our knowledge, this is the first direct demonstration of ethylene-induced *THT* expression.

To explore the role of *P. syringae* induced-ethylene in eliciting *THT* expression and HCAA synthesis, the ethylene biosynthesis inhibitor AVG was used instead of tomato mutants (e.g., pTOM 13) affected in ethylene production. We chose this approach based on observations made in our laboratory that the inhibitory effect on ethylene accumulation produced by AVG

Fig. 3. Expression of tyramine hydroxycinnamoyl transferase (*THT*) transcripts in Rutgers tomato infected with *Pseudomonas syringae* pv. *tomato* (*Pst*) at 10^7 CFU/ml. **A**, *THT* cDNA. **B**, Ribosomal RNA loading control. Total RNA (10 μ g) was prepared from *P. syringae* pv. *tomato*-infected and control tomato leaves and reverse-transcribed using *THT*-specific primers. The resulting RT-PCR products were separated on 1% agarose gels. Total RNA (2 μ g) from each of the indicated samples is shown as a loading control. The gels were stained with ethidium bromide and visualized under UV light. Samples were collected at the specified times (h). Similar results were observed in two separate experiments.

treatments is much higher than that observed in pTOM 13. Accordingly, tomato leaves were sprayed with AVG (Adams and Yang 1979), and ethylene production, *THT* transcripts, and HCAA accumulation were monitored at the specific times after challenging the leaves. The time course of ethylene production in *P. syringae*-challenged tomato leaves is similar, although with an anticipated induction, to that found in tomato leaves infected with *X. campestris* (Fig. 5A) (Ciardi et al. 2000). Ethylene synthesis began to increase at 5 h after bacterial inoculation and peaked at approximately 17 h after infection, markedly declined thereafter to levels comparable with those observed at 5 h, and remained at this comparatively high level during the next hours. The ethylene production of uninoculated tomato leaves remained at a steady low level over the time period of the experiment. Exogenous application of 1 mM AVG markedly inhibited the burst of ethylene production occurring by 17 h postinoculation and also over the time period studied (Fig. 5A). In addition, AVG treatment also notably reduced *THT* expression throughout the 48-h time course (Fig. 5B). Importantly, activation of HCAA synthesis was notably blocked in the presence of AVG at 24 and 48 h after bacterial inoculation (Fig. 5C). The inhibition was higher for CT and FT, at both at 24 or 48 h, than for CD and FD. However, no blockage of HCAA accumulation was observed at 5 and 17 h after inoculation of the leaves (data not shown), probably because the effect of AVG on *THT* gene expression is still too low to reduce HCAA synthesis. These results suggest that ethylene, generated after *P. syringae* infection, is essential for the induction of *THT* gene expression and HCAA accumulation. These observations are in accordance with the conclusive evidence obtained on the crucial role of ethylene signaling in tomato to respond to pathogen infections (Díaz et al. 2002; Lund et al. 1998) and to elicit pathogenesis-related proteins (Conejero et al. 1990). Moreover, a correlation between infection or wounding and *THT* production had been previously reported (Ishihara et al. 2000; Negrel et al. 1993, 1995; Pearce et al. 1998). Similarly, it is well known that wounding stimulates the

Fig. 4. Effect of exogenous ethylene treatment of Rutgers tomato plants on **A**, hydroxycinnamic acid amides (HCAA) accumulation and **B**, tyramine hydroxycinnamoyl transferase (*THT*) gene expression. Plants were treated for 24 h with either ethylene at 50 μ l/liter or air, and then samples were processed to measure HCAA content and for RNA extraction and reverse-transcription polymerase chain reaction analysis. Measures of HCAA are the mean $\pm$ standard error of three replicates; *p*-coumaroyldopamine = CD, feruloyldopamine = FD, *p*-coumaroyltyramine = CT, feruloyltyramine = FT, and FW = fresh weight.

Fig. 5. **A**, Time course of ethylene production in Rutgers tomato leaves infected with *Pseudomonas syringae* pv. *tomato* at 10^7 CFU/ml or pre-treated with aminoethoxyvinylglycine (AVG) and then inoculated with the bacteria. Time course of ethylene production in the corresponding controls also is included. Samples were collected at the indicated times for ethylene measurements. Results are the mean $\pm$ standard error from three replicates. **B**, Effect of AVG on tyramine hydroxycinnamoyl transferase (*THT*) gene expression. Relative *THT* mRNA amounts obtained by reverse-transcription polymerase chain reaction before infection (T₀) and at the indicated times postinoculation were determined. Similar results were observed in two separate experiments. **C**, Effect of AVG on hydroxycinnamic acid amides (HCAA) accumulation in Rutgers tomato plants infected by *Ps. syringae* at 24 and 48 h postinoculation. Measures of HCAA are the mean $\pm$ standard error of three replicates; *p*-coumaroyldopamine = CD, feruloyldopamine = FD, *p*-coumaroyltyramine = CT, feruloyltyramine = FT, and FW = fresh weight.

production of ethylene. Here, we show that ethylene is necessary for the induction of the expression of *THT*. Taken together, these results suggest that ethylene production plays an important role in *THT* induction in response not only to infection but also to wounding.

SA is not necessary for activation of HCAA synthesis.

SA is a crucial signal to elicit plant defense reactions and its levels strongly increase after pathogen invasion (Dempsey et

al. 1999; Métraux and Raskin 1993). Previously, we had found that Rutgers tomato infected with *P. syringae* produced a rapid and sharp increase of SA after bacterial inoculation (Bellés et al. 1999). To study the possible role of SA in the induction of HCAA in this plant–pathogen interaction, tomato plants were treated with SA. Surprisingly, no induction of HCAA was detected (data not shown) when Rutgers plants were treated with SA at concentrations known to induce defense responses in tomato (Bellés et al. 1999; Van Kan et al. 1995). NahG tomato plants overexpressing a bacterial salicylic hydroxylase, which converts SA into catechol (Brading et al. 2000), were used to confirm that SA is not implicated in HCAA induction. To this purpose, these plants were infected with *P. syringae*. Noninfected plants were employed as controls. HCAA accumulation at 24 and 48 h postinoculation is shown in Figure 6A. No detectable levels of these compounds were found in control plants (data not shown). To be sure that SA induced upon bacterial challenge had been completely eliminated in NahG tomato plants, SA was monitored in the *P. syringae*-infected tomato leaves from these plants. No accumulation of SA was observed in infected NahG plants (Fig. 6B), contrary to what happened in control Moneymaker-infected plants (Fig. 6C). All these results further indicated that the accumulation of these HCAA is independent of SA as an intermediary signal. However, Nawrath and Métraux (1999), using the same bacterial pathovar but different host plant (NahG *Arabidopsis*) (Delaney et al. 1994), demonstrated that SA was necessary for the accumulation of camalexin, the major phytoalexin in *Arabidopsis*, in response to this bacterial infection.

Antibacterial and antioxidant activities of HCAA.

Up to now, the antimicrobial activity of FT and CT has been investigated in a few cases (McLusky et al. 1999; Newman et al. 2001). However, no reports exist on the antibacterial activity of FD or CD. In the present work, we have shown that FD and CD have a notable antibacterial activity against *P. syringae* compared with the tetracycline chlorhydrate as a control, thus suggesting that these two new HCAA of dopamine can act as direct antimicrobial agents. Surprisingly, no significant antibacterial effect was observed for HCAA of tyramine (Table 2). These results differ from those reported by Newman and associates (2001) working with other bacteria. These authors found a marked antibacterial activity of FT and CT against *X. campestris* pv. *campestris*. In this respect, other reports also have shown no conclusive evidence for antimicrobial action of FT and CT against fungi (i.e., Grandmaison and associates [1993] reported that FT reduces the development of vesicular-arbus-

Fig. 6. A, Accumulation of hydroxycinnamic acid amides (HCAA) in NahG transgenic tomato plants infected with *Pseudomonas syringae* pv. *tomato* at 10^7 CFU/ml. Samples were collected at 24 and 48 h postinoculation (hpi) with the bacteria. Results are the mean of two replicates and similar results were obtained in two separate experiments. **B,** Levels of total salicylic acid (SA) (the sum of free SA and its conjugated glucoside, SAG) in NahG transgenic tomato plants infected with *P. syringae* pv. *tomato* and in the corresponding control. Samples were collected at 24 and 40 hpi. Results are the mean $\pm$ standard error of three replicates. **C,** Levels of total SA (the sum of free SA and its conjugated glucoside, SAG) in Moneymaker tomato infected with *P. syringae* pv. *tomato* and in the corresponding control. Samples were collected at 24 and 40 hpi. Results are the mean $\pm$ standard error of three replicates; *p*-coumaroyldopamine = CD, feruloyldopamine = FD, *p*-coumaroyltyramine = CT, feruloyltyramine = FT, and FW = fresh weight.

Table 2. Antibacterial activity of hydroxycinnamic acid amides (HCAA)^a

Compound	MIC (μ g/ μ l)	ED ₅₀ (μ M) ^b
<i>p</i> -coumaroyldopamine	5	56.37 $\pm$ 0.01
Feruloyldopamine	5	77.96 $\pm$ 0.03
<i>p</i> -coumaroyltyramine	> 40	>200
Feruloyltyramine	> 40	150.15 $\pm$ 0.1
Tetracycline	1.2×10^{-3}	...
<i>p</i> -coumaric acid	...	>200
Ferulic acid	...	47.18 $\pm$ 0.01
Dopamine	...	11.30 $\pm$ 0.01
Tyramine	...	>200
BHT	...	61.13 $\pm$ 0.02

^a Measured as minimal inhibition concentration (MIC). Values are the mean of three individual experiments.

^b 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of HCAA, hydroxycinnamic acids, and β -phenylethyl-alkaloids. Values represent the concentration of product necessary to reduce to 50% the absorbance of DPPH (ED₅₀) and are the mean $\pm$ standard error of three individual experiments.

cular mycorrhizal fungi; however, McLusky and associates [1999] found that FT and CT had no antifungal effect on *Botrytis allii* and *B. cinerea*). Other class of HCAA have been shown to possess an important antimicrobial activity against bacteria or fungi as *p*-coumaroylnoradrenaline (Von Roepenack-Lahaye et al. 2003) or the β -phenylethylamine serotonin, 3-(2-aminoethyl)-5-hydroxyindol (Tanaka et al. 2003), respectively.

As far as we know, the antioxidant activity of HCAA of tyramine and dopamine has not been investigated. In this work, we present a potent CD and FD 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity comparable with the positive control butylated hydroxytoluene (BHT). These results demonstrate the notable antioxidant activity of these compounds (Table 2), similar to that observed for a series of phenolic acid derivatives (Cos et al. 2002) and dimers of serotonin (Zhang et al. 1996). However, the antioxidant activity of tyramine conjugates was significantly lower than that observed for the dopamine conjugates (Table 2). Moreover, it is interesting to note that the DPPH scavenging activity of dopamine was much stronger than that of tyramine. In agreement to what has been suggested by Cos and associates (2002), our results confirm that the presence of dopamine in an HCAA is crucial for its DPPH scavenging activity. Evidence for the beneficial antimicrobial and antioxidant properties of naturally occurring polyphenolic compounds is accumulating (Dixon 1999). In plants, the antioxidant metabolites could scavenge or prevent the accumulation of reactive oxygen species produced at high rates upon exposure to a pathogen infection (Matamoros et al. 2003). Engineered tomato plants accumulating high levels of chlorogenic acid (a widespread phenolic) show improved antioxidant capacity and resistance to infection by a bacterial pathogen (Niggeweg et al. 2004).

Recently, Niehl and associates (2006) have found that systemic *Potato virus X* infection induced *THT* gene expression and accumulation of dopamine in potato leaves. These authors suggested that dopamine could be conjugated into HCAA upon pathogen infection and then participate in the active defense of potato plants. However, they were not able to directly identify the CD and FD conjugated forms in infected tissues. To our knowledge, the presence of HCAA of dopamine in plants has not been reported thus far. This is the first report describing the possible implication of these HCAA in a plant-pathogen interaction, and it further supports the role of HCAA as integral components of plant defense responses to pathogen challenge, as has been previously hypothesized (Facchini et al. 2002). Work is in progress to engineer tomato plants with elevated levels of the THT enzyme in order to determine whether these dopamine conjugates accumulate. If successful, this would represent a useful tool to study the role of HCAA of dopamine as both direct barriers against pathogen attack and as antioxidants and bactericidal agents in tomato-pathogen interactions *in vivo*. Similar strategies have been applied in tobacco and rice and, in both cases, accumulation of similar metabolites was observed (Hagel and Facchini 2005; Jang et al. 2004). However, the role of accumulation of these related compounds in pathogen defense was not investigated.

MATERIALS AND METHODS

Plant material and inoculation procedure.

Seeds from tomato (*S. lycopersicum* cv. Rutgers) (Western Hybrid Seeds Inc., Hamilton City, CA, U.S.A.) or from transgenic tomato plants overexpressing a bacterial hydroxylase (*naHG* gene) (Brading et al. 2000) provided by J. D. G. Jones (John Innes Centre, Norwich, U.K.) were used in the experiments. The plants (one per pot) were grown in a controlled growth room at 27 and 23°C (16-h day and 8-h night, respec-

tively), relative humidity from 50 to 70%, in 15-cm-diameter pots containing a 1:1 mixture of peat (Biolan, Kauttua, Finland) and vermiculite. The pots were subirrigated with a nutrient solution as described (Naranjo et al. 2003). Infection of 5-week-old tomato plants at the five- to six-leaf stage with *P. syringae* pv. *tomato* was performed with a bacterial suspension obtained as follows: bacteria were grown overnight at 28°C in 20-ml petri dishes with C3 agar medium (Oxoid, Basington, U.K.) supplemented with 0.45 g of KH_2PO_4 per liter and 2.39 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (pH 6.8) per liter. Bacterial colonies then were resuspended in 10 mM MgSO_4 to a final concentration of optical density at 600 nm = 0.1. Dilution plating was used to determine the final inoculum concentration, which averaged 10^7 CFU/ml. The inoculation procedure was that described in detail by Collinge and associates (1987). Briefly, aliquots of 100 μl of this bacterial suspension were injected into the abaxial side of each leaflet (three to four panels per leaflet averaging 30 mm^2) of the third or fourth leaf from the base of the plant with a 1-ml sterilized plastic syringe without needle. Equivalent control leaflets were mock inoculated with 10 mM MgSO_4 .

Extraction and HPLC analysis of HCAA and SA.

Extraction of methanol-soluble HCAA and SA from tomato leaflets was done according to protocols previously published (Bellés et al. 2006; Naranjo et al. 2003). Briefly, leaflets (0.3 to 0.5 g fresh weight) of tissue were ground with a pestle in a mortar using liquid nitrogen, then homogenized in 1.5 ml of 90% methanol. The extracts were vortexed vigorously, sonicated for 15 min, and then centrifuged at $14,000 \times g$ for 15 min using 2-ml Eppendorf tubes to remove cellular debris. The supernatant (1.5 ml) was divided in two halves and dried under nitrogen at 40°C using glass tubes of 5 ml. One half was dissolved in 1 ml of 5% perchloric acid and kept for 1 h at 4°C and centrifuged at $14,000 \times g$ for 15 min to remove polymers. The supernatant was extracted with 2.5 ml of 1:1 cyclopentane/ethyl acetate using glass tubes of 5 ml. The organic upper phase was collected and dried at 40°C under a flow of nitrogen. The residue was resuspended in 200 μl of methanol and filtered through 0.45- μm Spartan 13/0.45RC filters (Schleicher & Schuell, Keene, NH, U.S.A.) nylon filters (Waters, Millford, MA, U.S.A.). For hydrolysis experiments, the other half was dissolved in 1 ml of 0.1 M sodium acetate (pH 4.5) containing 10 units of almond β -glucosidase (EC 3.2.1.21) (14.3 U/mg, Fluka, Buchs, Switzerland) and incubated at 37°C for 3 h. The reaction was stopped by adding perchloric acid (70%) until a final concentration of 5% and then maintained at 4°C for 1 h. After centrifugation at $14,000 \times g$ for 15 min, the remaining supernatant was extracted as above and the dried residue resuspended in 200 μl of methanol and filtered through 0.45- μm nylon filters. Aliquots (40 μl) were injected with a Waters 717 autosampler into a reverse-phase Symmetry 5- μm C18 (4.6 by 150 mm; Waters) column equilibrated in 1% acetic acid. Eluents were 1% acetic acid (eluent A) and methanol (eluent B). A linear gradient starting with 100% eluent A and 0% eluent B and ending with 0% eluent A and 100% eluent B was applied over 20 min at a flow rate of 1 ml/min. After washing the column with 100% methanol for 10 min, the initial conditions again were applied and the column was allowed to equilibrate with 1% acetic acid for 10 min, with a total run time of 40 min. The temperature of the oven was 30°C. HCAA were detected photometrically (λ 280 nm) with a Waters 460 tunable absorbance detector. Total SA (the sum of free SA and its conjugated glucoside, SAG) was detected with a 470 Waters fluorescence detector (λ excitation = 313 nm; λ emission = 405 nm). HCAA and SA were quantified with the Waters Millennium³² software using synthesized HCAA and authentic SA, respectively, as standards. Data were corrected for losses in the extrac-

tion procedure, and recovery of metabolites ranged between 50 and 80%.

Identification of HCAA.

To identify the structure of the major peaks from methanol-soluble extracts of *P. syringae*-infected tomato leaves, samples, extracted as indicated above, were analyzed by ESI-MS using a 1515 Waters HPLC binary pump, a 996 Waters photodiode detector (range of maxplot between 240 and 400 nm, spectral resolution of 1.2 nm), and a ZMD Waters single quadrupole mass spectrometer equipped with an electrospray ionization ion source. The source parameters of the mass spectrometer for ESI in positive mode were the following: capillary voltage 2,500 V, cone voltage 20 V, extractor 5 V, RF Lens 0.5 V, source block temperature 100°C, and desolvation gas temperature 300°C. The desolvation and cone gas used was nitrogen at a flow of 400 and 60 liters/min, respectively. Other mass spectrometer conditions were: low mass resolution 13.5, high mass resolution 13.5, ion energy 0.5, and multiplier 650. ESI data acquisition was in the conditions of a full scan range from mass-to-charge ratio (m/z) 100 to 700 at 1 s/scan. Samples (20 μ l) from methanolic extracts were injected at room temperature into a reverse-phase Symmetry 5- μ m C18 (4.6 by 150 mm; Waters) column. A 20-min linear gradient of 1% (vol/vol) acetic acid (J. T. Baker) in Milli Q water to 100% methanol (J. T. Baker, Phillipsburg, NJ, U.S.A.) at a flow rate of 1 ml/min was applied as indicated above. A post-column split delivered approximately 25% of the flow to the mass spectrometer and the rest to the Waters 996 photodiode array detector. Mass and UV-absorption spectra of the unknown and authentic standard peaks were performed using the Masslynx Waters software.

Synthesis of HCAA.

The HCAA studied here were obtained by the reaction of *p*-coumaric and ferulic acids with tyramine and dopamine (hydrochloride form) in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) as described elsewhere in detail (Tanaka et al. 1989). To a mixture of the corresponding hydroxycinnamic acid (0.5 mmol) and amine (0.65 mmol) in tetrahydrofuran (THF) (20 ml), a solution of DCC (0.8 mmol) in THF (5 ml) was added, and the reaction mixture stirred overnight at room temperature. After removal of the solvent, the reaction mixture was diluted with a large volume of H₂O and extracted with ethyl acetate. The organic layer was dried over Na₂SO₄ and evaporated to dryness to give viscous oil, which was purified by a column of flash 60 silica gel (0.040 to 0.0063 mm; Merck, Amsterdam) with a mixture of dichloromethane/ethyl acetate (1:1) as the mobile phase to obtain the resultant *trans*-amide. The identity of the product was checked by ¹H-NMR (proton nuclear magnetic resonance) spectra. All the synthetic compounds were identical to natural products as judged by comparison of their retention times in the HPLC chromatograms as well as UV and MS.

RT-PCR.

RNA from control and *P. syringae*-infected tomato plants or leaves treated with ethylene at 50 μ l/liter or 1 mM AVG were prepared using the TRIzol reagent (Gibco BRL, Gaithersburg, MD, U.S.A.). *P. syringae*-infected material (0.5 g) was collected at the specified times (h) after inoculation. Ethylene-treated leaves were collected 24 h after the treatment. Total RNA (10 μ g) was reverse-transcribed with 100 U of M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.) at 37°C for 60 min in a final volume of 50 μ l, using a 18-mer oligo (dT) as a primer. Reverse-transcribed DNA (5 μ l) was amplified by PCR using standard procedures with the following conditions: 1 min of denaturation at 94°C followed by 25 cycles of 1 min of de-

naturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C, finished with a final extension step of 5 min at 72°C. Direct (5'-ATGGCTCCTGCTCTTGAACAAG-3') and reverse (5'-CTAACAGCTCCCTTTCGCCGT-3') oligonucleotides were designed to amplify a 724-bp fragment from tomato THT1-3 mRNA (Von Roepenack-Layale et al. 2003). Aliquots of 25 μ l of the PCR products were resolved on 1% agarose gels. The gels were stained with ethidium bromide and visualized under UV light. Images were taken using gel-documentation systems and quantified by using Image Gauge V4.0 software.

Antimicrobial activity of CD, FD, CT, and FT.

The test was carried according to the method of paper disk diffusion (Cole 1994). *P. syringae*, maintained in 80% glycerol at -80°C, was reactivated in 20-ml petri dishes with a King's medium B solid medium containing 40 g of proteose peptone, 20 g of glycerol, 980 ml of H₂O, 10 ml of 10% K₂HPO₄, and 10 ml of 10% MgSO₄ and incubated for 48 h at 28°C. Then, bacterial colonies were cultured in 15 ml of Luria-Bertani medium (Pronadisa, Madrid) overnight at 28°C. A 1-ml culture containing approximately 10⁶ CFU was mixed with 15 ml of culture medium Plate Count Agar (Difco, Detroit) in a petri dish. When the medium was completely solidified, three Whatman disks (no. 113, 0.5 cm in diameter) impregnated with different concentrations of each HCAA dissolved in 10 μ l of methanol were added in the Whatman disks. The plates were incubated for 24 h at 28°C in the dark in order to avoid the *cis/trans* isomerization of these compounds (Towers 1984). Plate Count Agar plates containing only methanol were used as control plates, and a positive control with tetracycline chlorhydrate (10 μ g/cm²), was performed. Bactericidal activity was determined by measuring the inhibition zone developed around the paper disk, indicating a zone of no growth, and each assay was performed three times. Minimal inhibitory concentration was determined as the lowest concentration able to inhibit any visible microbial growth (Cos et al. 2006).

Antioxidant activity of CT, FT, CD, and FD.

The antioxidant activity of hydroxycinnamic acids, amines, and HCAA was evaluated using the assay based on the scavenging of the stable radical DPPH (Hirota et al. 1997) as described by Alfaro and associates (2003). Ethanolic solution (2 ml) containing the corresponding compounds at different concentrations was mixed with 1 ml of 0.5 mM DPPH (dissolved in ethanol) and 2 ml of 0.1 M sodium acetate (pH 5.5). After incubation of the mixture at 25°C for 30 min, the absorbance at 517 nm was measured using a JENWAY 6305 spectrophotometer. Radical scavenging activity was expressed as the concentration of product necessary to reduce to 50% the absorbance of DPPH at 517 nm. BHT (Sigma, St. Louis) was tested as a positive control. Data of the experiments represent the mean $\pm$ standard error of three individual samples.

AVG and ethylene treatments.

For AVG and ethylene treatments, 5-week-old tomato plants grown in the same conditions as stated above were used. Tomato plants were sprayed until run-off with 1 mM aqueous solution of AVG and again 10 h later. Two hours later, the AVG-treated plants were inoculated with the bacteria. Equivalent leaflets (0.3 to 0.5 g fresh weight) from nontreated, AVG-treated, and infected plants were detached at different times to determine ethylene production and RNA preparation, and for HCAA analysis. For ethylene treatments, plants were placed during 24 h into 125-liter sealed containers in a conditioned chamber at 25°C, with a photoperiod of 16 h. A mixture of ethylene in air (50 μ l/liter) (Linde, Barcelona, Spain) was passed through the containers at a constant flow rate of 15 liters/h.

Analysis of ethylene.

At the specific times, ethylene was measured by sealing a single equivalent leaflet (0.3 to 0.5 g fresh weight) from control, AVG-treated, and inoculated leaves into 18-ml serum flasks at 25°C for 2 h. A 1-ml gas sample was withdrawn from the flask with a gas-tight syringe through the rubber seal and injected into a TRB-1 TRACER column (60-m length, 0.56-mm interior diameter) connected to a flame-ionization detector in a 4890 Hewlett-Packard (Palo Alto, CA, U.S.A.) gas chromatograph equipped with a 3395 Hewlett-Packard integrator. The temperature of the oven was 60°C and retention time of ethylene was 0.98 min. Ethylene production by the samples was calculated as the mean of at least three independent incubations using an ethylene standard curve.

ACKNOWLEDGMENTS

This work was supported by Grant BMC2000-1136 from Comisión Interministerial de Ciencia y Tecnología, Spanish Ministry of Science and Technology. L. Zacarés was supported by a fellowship CTBPRB/2003/112 from Generalitat Valenciana, Conselleria de Empresa, Universitat y Ciencia, Spain. We gratefully acknowledge J. D. G. Jones (John Innes Centre, Norwich, U.K.) for the gift of seed of the transgenic NahG tomato plants and L. Yenush for critical reading of the manuscript and helpful discussions. I. Rodrigo is acknowledged for his continuous support during the course of this project.

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