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INDUCTION OF *p*-COUMAROYLDOPAMINE AND FERULOYLDOPAMINE, TWO NOVEL METABOLITES, IN TOMATO BY THE BACTERIAL PATHOGEN *PSEUDOMONAS SYRINGAE*

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ABSTRACT

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 amides (HCAAs) 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 HCAAs was preceded by an increment of hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyl transferase (*THT*) gene expression. HCAAs also accumulated in transgenic NahG tomato plants over-expressing a bacterial salicylic

hydroxylase. However, treatment of plants with the ethylene biosynthesis inhibitor, aminoethoxyvinilglycine, led to a reduction in the accumulation of *THT* transcripts and HCAAs. Together, the results suggest that pathogen-induced induction of ethylene is essential for HCAAs synthesis, while salicylic acid is not required for this response. In addition, notable antibacterial and antioxidant activities were found for the new HCAAs, thus indicating that they could play a role in the defense of tomato plants against bacterial infection.

INTRODUCTION

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, and consequently most plants are resistant to many pathogens of plants (Dixon et al. 1994; Dangl and Jones 2001). These mechanisms of defense are induced upon infection by pathogens leading to the so called acquired resistance, but a pre-existing 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 is then 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 can also 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 (2-hydroxybenzoic acid, SA) (Dempsey et al. 1999), the synthesis of pathogenesis-related proteins (Van Kan et al. 1992; Van Loon et al. 2006b), and natural antimicrobial products (phytoalexins) (Hammerschmidt 1999; Dixon 2001). 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 phenylproponaoid metabolites (Thomma et al. 1999; Verberne et al. 2000; Dixon et al. 2002; Niggeweg et al. 2004). Cinnamic acid (phenylpropenoid acid) is the product of deamination of phenylanine catalysed 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 (HCAAs) (Strack 1997; Facchini et al. 2002). 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 (Negrel and Martin 1984; Keller et al. 1996; Pearce et al. 1998; Schmidt et al. 1998; Newman et al. 2001). The pivotal regulatory enzyme responsible for the synthesis of HCAAs is the enzyme hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyl transferase (THT; EC 2.3.1.110) (Negrel and Martin 1984; Negrel and Javelle 1997; Facchini et al. 2002). Recently, Von Roepenack-Lahaye et al. (2003) have found that the tomato cultivar Rio Grande, challenged with the bacterial pathogen

Pseudomonas syringae pv. t*omato*, 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 to SA, to elicit the up-regulation of defense proteins in tomato and cucumber plants (Bellés et al. 1999; Bellés et al. 2006). Interestingly, this secondary metabolite did not accumulate when tomato plants were infiltrated with the necrotizing pathogen *Ps. 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 *Ps. 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 *THT* gene in *Ps. syringae*-infected tomato leaves. The antimicrobial and antioxidant activities of these compounds were also studied. All of them presented a positive antioxidant activity and the new HCAAs of dopamine had a notable bactericidal action. To gain information on the possible signals implicated in the activation of HCAAs synthesis after bacterial challenging, we have also explored the implication of ethylene and SA on HCAAs accumulation. Interestingly, inhibition experiments of *Ps. syringae*-induced

ethylene with aminoethoxyvinylglycine (AVG) strongly suggest that ethylene is essential to elicit HCAAs synthesis. However, exogenous application SA had no effect on HCAAs levels in tomato leaves, and NahG tomato plants accumulated HCAAs upon bacterial challenge, thus further supporting the notion that SA does not affect the pathogen-induced induction of HCAAs synthesis.

RESULTS AND DISCUSSION

Characterization of HCAAs in Rutgers tomato challenged with *Ps. syringae*

Rutgers tomato leaves inoculated with the bacterial pathogen *Ps. syringae* pv. *tomato* developed a hypersensitive-like response, manifested as a bacterial concentration-dependent necrotic reaction. In this paper we applied a bacterial concentration of 10^7 colony-forming units (CFU)/ml. The method employed to inoculate the bacteria first produced water soaking in the leaves, which disappeared within approximately one hour. The observation of the hypersensitive reaction began with the glossy appearance of the inoculated leaf surface area (approx. 6 mm in diameter) and was evident as early as 12 hours after bacterial infiltration. The pathogen response culminated at approximately 72 h post-inoculation with the appearance of non-spreading 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 *Ps. syringae* accumulated SA (Bellés et al. 1999) a phenolic associated with the hypersensitive response of plants to pathogens (Métraux and Raskin 1993; Dempsey et al. 1999). Therefore, to extend the search for other metabolites induced in tomato by *Ps. syringae*, we decided to carry out a more general analysis of UV-

absorbing methanol-soluble phenolic compounds in extracts from *Ps. syringae*-infected tomato leaves. Figure 1 depicts representative HPLC chromatograms of methanol-soluble extracts from *Ps. syringae*-infected Rutgers tomato leaves 24 h after the challenge. 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 *Ps. 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 consistently observed and we were not able to identify its chemical nature. It is likely to correspond to an artefact due to irregularities in the separation process.

To precisely identify these unknown compounds corresponding to peaks 1-4, an HPLC-MS analysis combined with electrospray ionization (ESI) of the soluble-methanol phenolic fraction from infected tomato leaves was performed. Table 1 shows the mass spectra fragments of peaks 1-4 from methanolic tomato extracts obtained by ESI in the positive ion mode. 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 1-4 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 co-chromatographed with synthesised 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, as compared to 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 $[M+H-H_2O]^+$ 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 co-chromatographed with, and have identical UV spectra to that of authentic HCAAs that had been chemically synthesised. To our knowledge, this is the first evidence of the presence of HCAAs of dopamine in plants.

Accumulation of HCAAs in tomato infected with *Ps. syringae*

Results of the analysis of the time course of induction of p-CD, FD, p-CT, and FT in Rutgers tomato leaves upon *Ps. syringae* challenge is presented in Figure 2A. The relative timing of the maximum accumulation of HCAAs, about 24 h post-inoculation, preceded the formation of necrotic brown spots, which appear after 72 h post-inoculation. The accumulation profiles for FD and FT were quite similar and different from that of CD and CT that were also similar. The levels of all HCAAs 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, while the levels of CD and CT remained at this comparatively level during the subsequent two 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 HCAAs, we compared the levels present in the inoculated area and the immediately surrounding tissue (approx. 1 mm) with the accumulation in the remaining non-inoculated leaflet. The increase in HCAAs was primarily confined to and around the inoculated region (Fig. 2B). This location was similar to that previously reported for HCAAs of tyramine in tomato (Pearce et al. 1998) and tobacco (Guillet and De Luca 2005) leaves after wounding.

The four HCAAs described above as being associated with the tomato Rutgers-*Pseudomonas syrinagae* interaction, were accumulated in a similar way in two other additional tomato cultivars (Ailsa Craig and Moneymaker) upon *Pseudomonas syringae* pv. *tomato* inoculation (data not shown). The fact that these compounds had not been detected in Rio Grande tomato infected with *Ps. syringae* (Von Roepenack-Lahaye et al. 2003) and that we have not found the HCAAs described by these authors, may be due to differential cultivar behaviour. The time course of induction and relative timing of the accumulation of these amine conjugates in Rutgers tomato leaves challenged with *Ps. syringae* was comparable to 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 *Ps. syringae* C Increased synthesis of *p*-coumaroyloctopamine and *p*-coumaroylnoradrenaline associated with *Ps. 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 *Xanthomons 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 HCAAs as an alternative to their biosynthetic pathway. Therefore, we have also investigated the possible presence of glycosyl derivatives of the accumulated amides in tomato upon bacterial infection. After treatment of methanolic extracts from *Ps. 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 et al. (2001) for CT and FT in pepper leaves elicited by *Xanthomonas campestris*.

Recently, Niehl et al. (2006), studying the compatible interaction between *Solanum tuberosum* and *potato virus X*, have hypothesised that tyramine and dopamine (both highly induced upon infection), can contribute to active plant defense responses by forming hydroxcinnamic acid amides, which increases cell wall stability and fortification. However, the authors were not able to directly identify the HCAAs of tyramine and dopamine in infected leaf material. In this work, we have found that these HCAAs accumulated in tomato plants after infection with a necrotizing pathogen. The results presented in this report extend to other plant system and to other class of HCAAs 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 HCAAs accumulation in tomato infected with *Ps. syringae*

Total RNA was isolated at different time points after the onset of bacterial infiltration and subjected to RT-PCR analysis to determine the expression levels of mRNA encoding the THT1-3 isoenzyme (Roepenack-Lahaye et al. 2003). Figure 3A shows the kinetic pattern of *THT* transcript accumulation at the specified times. *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 HCAAs accumulation (Fig. 2A) demonstrated an association between HCAAs production and alterations in transcription of *THT*. As expected, the increment of the *THT* gene expression occurred earlier than that of HCAAs accumulation. These results suggest that activation of the *THT* gene expression results in an increase of HCAAs synthesis in a similar manner 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 increment in the level of *THT* transcripts. The time course of the induction of *THT* transcripts observed in Rutgers cultivar is in agreement with that observed in Rio Grande cultivar upon *Ps. syringae* infection (Von Roepenack-Lahaye et al. 2003), and is comparable with that observed in potato cell cultures after *Pseudomonas infestans* elicitor treatment (Schmidt et al. 1999) or pepper infected with *Xanthomonas campestris* (Newman et al. 2001).

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

Initial studies on the interaction between Rutgers tomato and the bacterial pathogen, *Ps. 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 HCAAs accumulation in Rutgers tomato. Accordingly, tomato plants were treated with exogenous ethylene (50 µL/L), as indicated in Material and Methods. Figure 4B shows that ethylene treatment led to an increase in the expression levels of *THT* transcripts, as compared with control plants by 24 h. This result is consistent with the elevated levels of HCAAs in ethylene-treated plants (Fig. 4A). The increment of *THT* expression in ethylene-treated plants were comparable to that in *Ps. 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 *Ps. syringae* induced-ethylene in eliciting *THT* expression and HCAAs 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 treatements 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 HCAAs accumulation was monitored at the specific times after challenging the leaves. Fig. 5A shows the time course of ethylene production in *Ps. syringae*-challenged tomato leaves which is similar, although with an anticipated induction, to that found in tomato leaves infected with *Xanthomonas campestris* (Ciardi et al. 2000).

Ethylene synthesis began to increase at 5 h after bacterial inoculation and peaked around 17 h after infection, markedly declined thereafter to levels comparable to 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 post-inoculation 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 HCAAs 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 HCAAs 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 HCAAs synthesis. These results suggest that ethylene, generated after *Ps. syringae* infection, is essential for the induction of *THT* gene expression and HCAAs accumulation. These observations are in accordance with the conclusive evidence obtained on the crucial role of ethylene signalling in tomato to respond to pathogen infections (Lund et al. 1998; Díaz et al. 2002), and to elicit pathogenesis-related proteins (Conejero et al. 1990). Moreover, a correlation between infection or wounding and *THT* production had been previously reported (Negrel et al. 1993, 1995; Pearce et al. 1998; Ishihara et al. 2000). Similarly it is well known that wounding stimulates the 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 HCAAs synthesis

SA is a crucial signal to elicit plant defense reactions and its levels strongly increase after pathogen invasion (Métraux and Raskin 1993; Dempsey et al. 1999). Previously, we had found that Rutgers tomato infected with *Ps. syringae* produced a rapid and sharp increase of SA after bacterial inoculation (Bellés et al. 1999). To study the possible role of SA on the induction of HCAAs in this plant-pathogen interaction, tomato plants were treated with SA. Surprisingly, no induction of HCAAs was detected (data not shown) when Rutgers plants were treated with SA at concentrations known to induce defense responses in tomato (Van Kan et al. 1995; Bellés et al. 1999). NahG tomato plants, over-expressing a bacterial salicylic hydroxylase which converts SA into catechol (Brading et al. 2000) were used to confirm that SA is not implicated in HCAAs induction. To this purpose, these plants were infected with *Ps. syringae*. Non infected plants were employed as controls. Figure 6A represents the HCAAs accumulation at 24 and 48 h post-inoculation. 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 *Ps. 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 HCAAs 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 HCAAs

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 *Ps. syringae*, as compared to the tetracycline chlorhydrate as a control, thus suggesting these two new HCAAs of dopamine can act as direct antimicrobial agents. Surprisingly, no significant antibacterial effect was observed for HCAAs of tyramine (Table 2A). These results differ to those reported by Newman et al. (2001) working with other bacterium. These authors found a marked antibacterial activity of FT and CT against *Xanthomonas campestris* pv. *campestris*. In this respect, other reports have also shown no conclusive evidence for antimicrobial action of FT and CT against fungi, i.e.: Grandmaison et al. (1993) reported that FT reduces the development of vesicular-arbuscular mycorrhizal fungi, however McLusky et al. (1999) found that FT and CT had no antifungal effect on *Botrytis allii* and *Botrytis cinerea*. Other class of HCAAs have been shown to posses 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 HCAAs of tyramine and dopamine has not been investigated. In this work, we present a potent CD and FD DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity comparable to the positive control BHT. These results demonstrate the notable antioxidant activity of these compounds (Table 2B) similar to that observed for a series of phenolic acid derivatives (Cos et al. 2002), and to that of 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 2B). 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 et al. (2002), our results confirm that the presence of dopamine in an HCAAs 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 et al. (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 HCAAs 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 HCAAs of dopamine in plants has not been reported so far. This is the first report describing the possible implication of these HCAAs in a plant-pathogen interaction and further support the role of HCAAs 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 HCCA of dopamine as both direct barriers against pathogen attack and as antioxidants and bactericidal agents in tomato-pathogen

interaction *in vivo*. Similar strategies have been applied in tobacco and rice and in both cases accumulation of similar metabolites was observed (Jang et al. 2004; Hagel and Facchini 2005). 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 (*Solanum lycopersicum* cv. Rutgers) (Western Hybrid Seeds Inc. CA) or from transgenic tomato plants over-expressing a bacterial hydroxylase (*naHG* gene; Brading *et al*. 2000) kindly provided by Dr. Jonathan D.G. Jones (John Innes Centre, Norwich, UK) were used in the experiments. The plants (one per pot) were grown in a controlled growth room at 27 °C/23 °C (16 h day/8 h night), relative humidity from 50% to 70% in 15-cm-diameter pots containing a mixture of peat (Biolan) and vermiculite 1:1. The pots were subirrigated with a nutrient solution as described (Naranjo et al. 2003). Infection of five-week-old tomato plants at the 5- to 6-leaf stage with *Ps. 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 C 3 agar medium (Oxoid, Basington, UK) supplemented with 0.45 g of KH_2PO_4 per liter, 2.39 g of $Na_2HPO_4.12H_2O$ (pH 6.8) per liter. Bacterial colonies were then resuspended in 10 mM $MgSO_4$ to a final concentration of OD_{600}: 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 in Collinge et al. (1987). Briefly, aliquots of 100 µl of this bacterial suspension were injected into the abaxial side of each leaflet (3-4 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

385 control leaflets were mock-inoculated with 10 mM $MgSO_4$.

Extraction and HPLC analysis of HCAAs and SA

Extraction of methanol-soluble HCAAs and SA from tomato leaflets was done according to protocols previously published (Naranjo et al. 2003; Bellés et al. 2006).
390 Briefly, leaflets (0.3-0.5 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 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 ºC using glass tubes of 5 ml. One half was
395 dissolved in 1 ml of 5% perchloric acid and kept for 1 h at 4 ºC and centrifuged at 14,000 g for 15 min to remove polymers. The supernatant was extracted with 2.5 ml of cyclopentane/ethyl acetate 1:1 using glass tubes of 5 ml. The organic upper phase was collected and dried at 40 ºC 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 &
400 Schuell), nylon filters (Waters). 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), 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 g for 15 min, the remaining supernatant was
405 extracted as above and the dried residue resuspended in 200 µl of methanol and filtered through 0.45 µm nylon filters. Forty microliters aliquots were injected with a Waters 717 autosampler into a reverse-phase Symmetry 5 µm C18 (4.6 x 150 mm; Waters) column equilibrated in 1% acetic acid. Eluents were 1% acetic acid (eluent A) and methanol (eluent

B). A lineal gradient starting with 100% eluent A and 0% eluent B and ending with 0% of 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 were again applied and the column was allowed to equilibrate with 1% acetic acid for 10 min, with a total run time of 40 minutes. The temperature of the oven was 30 ºC. HCAAs were photometrically detected (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). HCAAs and SA were quantified with the Waters Millennium[32] software using synthesised HCAAs and authentic SA, respectively, as standards. Data were corrected for losses in the extraction procedure, and recovery of metabolites ranged between 50 and 80%.

Identification of HCAAs

To identify the structure of the major peaks from methanol soluble extracts of *Ps. syringae*-infected tomato leaves, samples, extracted as indicted 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 2500 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 l and 60 l per min, respectively. Other mass spectrometer conditions were: low mass resolution 13.5, high mass resolution 13.5,

ion energy 0.5, 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 per scan. Samples (20 µl) from methanolic extracts were injected at room temperature into a reverse-phase Symmetry 5 µm C18 (4.6 x 150 mm; Waters) column. A 20-min linear gradient of 1% (v/v) acetic acid (J. T. Baker) in Milli Q water to 100% methanol (J. T. Baker) 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 HCAAs

The HCAAs 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 tetrahydrofurane (THF) (20ml), 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 H_2O and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and evaporated to dryness to give viscous oil, which was purified by a column of flash 60 silica gel (Merck, 0.040-0063 mm) with a mixture of dichlorometane/ethyl acetate 1:1, as the mobile phase to obtain the resultant *trans*-amide. The identity of the product was checked by ^{1}HNMR 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.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA from control and *Ps. syringae*-infected tomato plants or leaves treated with 50 µl/L ethylene or 1 mM AVG were prepared using the TRIZOL reagent (Gibco BRL). *Ps. infected*-infected material (0.5 g) was collected at the specified times (h) after inoculation. Ethylene-treated leaves were collected 24 hours after the treatment. Ten micrograms of total RNA were reverse-transcribed with 100 U of M-MLV reverse transcriptase (Promega) at 37 ºC for 60 min in a final volume of 50 µl, using a 18-mer oligo (dT) as a primer. Five microliters of reversetranscribed DNA were amplified by PCR using standard procedures with the following conditions: 1 min denaturation at 94 ºC followed by 25 cycles of 1 min denaturation at 94 ºC, 1 min annealing at 55 ºC and 1 min extension at 72 ºC, finished with a final extension step of 5 min at 72 ºC. 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 µl 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 disc diffusion (Cole 1994). *Ps. syringae*, maintained in 80% glycerol at -80 ºC, were reactivated in 20-ml Petry dishes with a KB solid medium containing 40 g proteose peptone, 20 g glycerol, 980 ml H_2O, 10 ml of 10% K_2HPO_4, and 10 ml of 10% $MgSO_4$, were incubated for 48 h at 28 ºC. Then, bacterial colonies were cultured in 15 ml of LB medium (Pronadisa) overnight at 28 ºC.

One milliliter culture containing approximately 10^6 CFU was mixed with 15 ml of culture medium Plate Count Agar (Difco) in a Petri dish. When the medium was completely solidified, three Whatman discs (nº 113, 0.5 cm diameter) impregnated with different concentrations of each HCAAs dissolved in 10 µl of methanol, were added in the Whatman discs. The plates were incubated for 24 h at 28ºC 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 tetracyicline chlorhydrate (10 µg/cm^2), was performed. Bactericidal activity was determined measuring the inhibition zone developed around the paper disc indicating a zone of no growth and each assay was performed three times. Minimal Inhibitory Concentation (MIC) 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 HCAAs was evaluated using the assay based on the scavenging of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Hirota et al.1997) as described in Alfaro et al. (2003). Two milliliters of ethanolic solution containing the corresponding compounds at different concentrations were 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 (ED_{50}). Butylated hydroxytoluene (BHT) (Sigma) was tested as a positive control. Data of the experiments represent the mean ± SE of three

individual samples.

AVG and ethylene treatments

For AVG and ethylene treatments five-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 (0.3-0.5 g fresh weight) from non-treated, AVG-treated, and infected plants were detached at different times to determine ethylene production, RNA preparation, and for HCAAs analysis. For ethylene treatments, plants were placed during 24 h into 125 L sealed containers in a conditioned chamber at 25 °C and a photoperiod of 16 h. A mixture of ethylene in air (50 µl/L) (Lynde) is passed through the containers at a constant flow rate of 15 L/h.

Analysis of ethylene

At the specific times, ethylene was measured by sealing a single equivalent leaflet (0.3- 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 (60m length, 0.56 mm I.D.) connected to a flame-ionization detector in a 4890 Hewlett-Packard (Palo Alto, CA) gas chromatograph equipped with a 3395 Hewlett-Packard integrator. The temperature of the oven was 60°C. 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.

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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 post-inoculation. 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 minutes, respectively.

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 post-inoculation (p. i.) with *Pseudomonas syringae* pv**.** *tomato* at 10^7 (CFU)/ml. Results are the mean ± SE from three replicates. **B,** Accumulation of CD, FD, CT, and FT at 24 h post-inoculation in the inoculated area and the immediately surrounding tissues (approx. 1 mm) as compared to the accumulation in the adjacent tissue. FW, fresh weight.

Fig. 3. Expression of tyramine hydroxycinnamoyl transferase (*THT*) transcripts in Rutgers tomato infected with *Pseudomonas syringe* pv. *tomato* (*Pst*) at 10^7 (CFU)/ml. **A,** *THT* cDNA. **B,** ribosomal RNA loading control. Ten micrograms of total RNA were prepared from *Pst*-infected and control tomato leaves and reverse-transcribed using *THT*-specific primers as stated in Materials and Methods. The resulting RT-PCR products were separated on 1% agarose gels. Two micrograms of total RNA 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.

Fig. 4. Effect of exogenous ethylene treatment of Rutgers tomato plants on **A,** Hydroxycinnamic acid amides (HCAAs) accumulation, and **B,** Tyramine hydroxycinnamoyl transferase (*THT*) gene expression. Plants were treated for 24 h with either 50 µl/L of ethylene or air and then samples were processed to measure HCAAs content and for RNA extraction and RT-PCR analysis as described in Figure 3. Measures of HCAAs are the mean ± SE of three replicates. *p*-coumaroyldopamine (CD), feruloyldopamine (FD), *p*-coumaroyltyramine (CT), and feruloyltyramine (FT). 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 aminoethoxyvinilglycine (AVG) and then inoculated with the bacteria. Time course of ethylene production in the corresponding controls are also included. Samples were collected at the indicated times for ethylene measurements as described in Materials and Methods. Results are the mean ± SE from three replicates. **B,** Effect of AVG on tyramine hydroxycinnamoyl transferase (*THT*) gene expression. Relative *THT* mRNA amounts obtained by RT-PCR before infection (T_0) and at the indicated times post-inoculation were determined as described in Materials and Methods. Similar results were observed in two separate experiments. **C,** Effect of AVG on hydroxycinnamic acid amides (HCAAs)

accumulation in Rutgers tomato plants infected by *Ps. syringae* at 24 and 48 h post-inoculation. Measures of HCAAs are the mean ± SE of three replicates. *p*-coumaroyldopamine (CD), feruloyldopamine (FD), *p*-coumaroyltyramine (CT), and feruloyltyramine (FT). FW, fresh weight.

Fig. 6. **A,** Accumulation of hydroxycinnamic acid amides (HCAAs) in NahG transgenic tomato plants infected with *Pseudomonas syringae* pv. *tomato* at 10^7 (CFU)/ml. Samples were collected at 24 and 48 h post-inoculation (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 *Pseudomonas syringae*-pv. *tomato* and in the corresponding control. Samples were collected at 24 and 40 h post-inoculation. Results are the mean of are the mean ± SE of three replicates. C, Levels of total salicylic acid (SA) (the sum of free SA and its conjugated glucoside, SAG) in Moneymaker tomato infected with *Pseudomonas syringae* pv. tomato and in the corresponding control. Samples were collected at 24 and 40 h post-inoculation. Results are the mean of are the mean ± SE of three replicates. *p*-coumaroyldopamine (CD), feruloyldopamine (FD), *p*-coumaroyltyramine (CT), and feruloyltyramine (FT). FW, fresh weight.

Table 1. ESI mass spectra in positive ion detection mode [*m/z* (%)], absorbance maxima (nm), and retention times of peaks 1, 2, 3, and 4 (a) and of synthetic HCAAs (b).

Table 2. **A,** Antibacterial activity of hydroxycinnamic acid amides (HCAAs) measured as
785 Minimal Inhibition Concentration (MIC). Values are the mean ± SE of three individual experiments. **B,** 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of HCAAs, hydroxycinnamic acids, and β-phenylethyl-alkaloids. Values represent the concentration of product necessary to reduce to 50% the absorbance of DPPH and are the mean ± SE of three individual experiments.