p-Coumaroylnoradrenaline, a Novel Plant Metabolite Implicated in Tomato Defense against Pathogens*

Edda von Roepenack-Lahaye‡§, Mari-Anne Newman‡¶, Sebastian Schornack‖, Kim E. Hammond-Kosack‡, Thomas Lahaye‖, Jonathan D. G. Jones‡, Michael J. Daniels‡, and J. Maxwell Dow‡ ‡‡**

From the ‡Sainsbury Laboratory, Norwich Research Park, Colney Lane, Norwich, NR4 7UH, United Kingdom and ‖Institut für Genetik, Martin-Luther Universität Halle-Wittenberg, Halle (Saale) D-06120, Germany

The Avr9 peptide elicitor from the fungus *Cladosporium fulvum*, the bacterial pathogen *Pseudomonas syringae* pathovar *tomato* carrying the avirulence gene *avrPto* (*Pst* (*avrPto*)), and the organophosphorous insecticide fenitrothion induce resistance-related responses in tomato lines carrying the *Cf-9*, *Pto*, and *Fen* genes, respectively. These responses were associated with synthesis of *p*-coumaroyloctopamine and *p*-coumaroylnoradrenaline, a novel compound for plants. In susceptible near isogenic tomato lines (*Cf-0*, *pto*, *fen*) and wounded tomato leaves, the levels of these compounds were reduced or undetectable. The elevated levels of *p*-coumaroyloctopamine and *p*-coumaroylnoradrenaline were accompanied by elevated mRNA levels of genes encoding phenylalanine ammonia lyase, *p*-coumarate CoA ligase, and hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT), enzymes that are involved in the hydroxycinnamic acid amide biosynthesis. Southern hybridization indicated that THT is encoded by a multigene family in tomato. Four different THT full-length cDNAs were derived by reverse transcriptase-PCR using degenerate primers based on potato and tobacco THT sequences. Transcripts for all four homologs were present in unchallenged tomato leaves, but only *tomTHT1-3* was highly expressed following challenge with *Pst* (*avrPto*). Furthermore, *tomTHT1-3* showed a more substantial and rapid induction in the incompatible interaction than in the compatible interaction. The cDNAs *tomTHT1-3*, *tomTHT7-1*, and *tomTHT7-8* encoded proteins with a high degree of amino acid sequence homology, although the recombinant proteins had different preferences for octopamine and noradrenaline. The fourth cDNA, *tomTHT1-4*, directed synthesis of a truncated enzymatically inactive protein due to the presence of a premature stop codon.

Plant defense against microbial attack is associated with activation of plant responses that serve to restrict the growth of the pathogen and/or to eliminate it. Considerable progress has been made in elucidating many of these responses and how they are triggered following recognition of molecules of pathogen origin by the plant (reviewed in Refs. 1–3). It is widely observed that plant resistance to pathogen challenge is correlated with alterations in phenolic metabolism and a rapid increase in the transcriptional activity of genes involved in the phenylpropanoid pathway. A number of products synthesized by this pathway have established roles in disease resistance in different plants; these include the signal molecule salicylic acid, antimicrobial phytoalexins, and lignin that can reinforce the plant cell wall (reviewed in Refs. 4 and 5). The work described in this paper had the initial aim of identifying further metabolites, originating from the phenylpropanoid pathway, that might play roles in disease resistance in tomato. We have compared the synthesis of soluble phenolics in response to both biotic (fungal elicitors, pathogenic bacteria) and abiotic agents (the organophosphorous insecticide fenitrothion) using nearly isogenic tomato lines that differ in their response to these agents. The resistance of tomato (*Lycopersicon esculentum* L.) to the fungus *Cladosporium fulvum* (*C. fulvum*) is conditioned by the presence of *Cf* resistance gene(s) in the plant together with the cognate avirulence (*avr*) gene(s) in the pathogen (6). The products of a number of *avr* genes are peptides that are found in intercellular fluids derived from *C. fulvum*-infected tomato leaves. These intercellular fluids induce chlorosis and necrosis in tomato in a *Cf* gene-dependent fashion (7).These responses are associated with increases in the levels of salicylic acid, indicating activation of the phenylpropanoid pathway (8). Tomato lines carrying the *Pto* gene are resistant to strains of *Pseudomonas syringae* pathovar *tomato* that carry the avirulence gene *avrPto* (*Pst* (*avrPto*)) (9). These strains cause a programmed cell death reaction, the hypersensitive response (HR),[1] when infiltrated into leaves. In contrast, tomato lines carrying the recessive *pto* allele do not show HR and are susceptible to infection by *Pst* (*avrPto*). The *Fen* gene, which is closely linked to *Pto* and belongs to the *Pto* gene family of serine threonine protein kinases, confers sensitivity to the organophosphorous insecticide fenthion (10). Treatment of *Fen* plants with fenthion or its analog fenitrothion results in a confluent

[1] The abbreviations used are: HR, hypersensitive response; *p*-CO, *p*-coumaroyloctopamine; *p*-CN, *p*-coumaroylnoradrenaline; HPLC, high pressure liquid chromatography; cfu, colony-forming units; hpi, hours post inoculation; ESI, electrospray ionization; MS, mass spectrometry; LC, liquid chromatography; *p*-CT, *p*-coumaroyltyramine; FT, feruloyltyramine; EST, expressed sequence tag; THT, hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase; PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate CoA ligase.
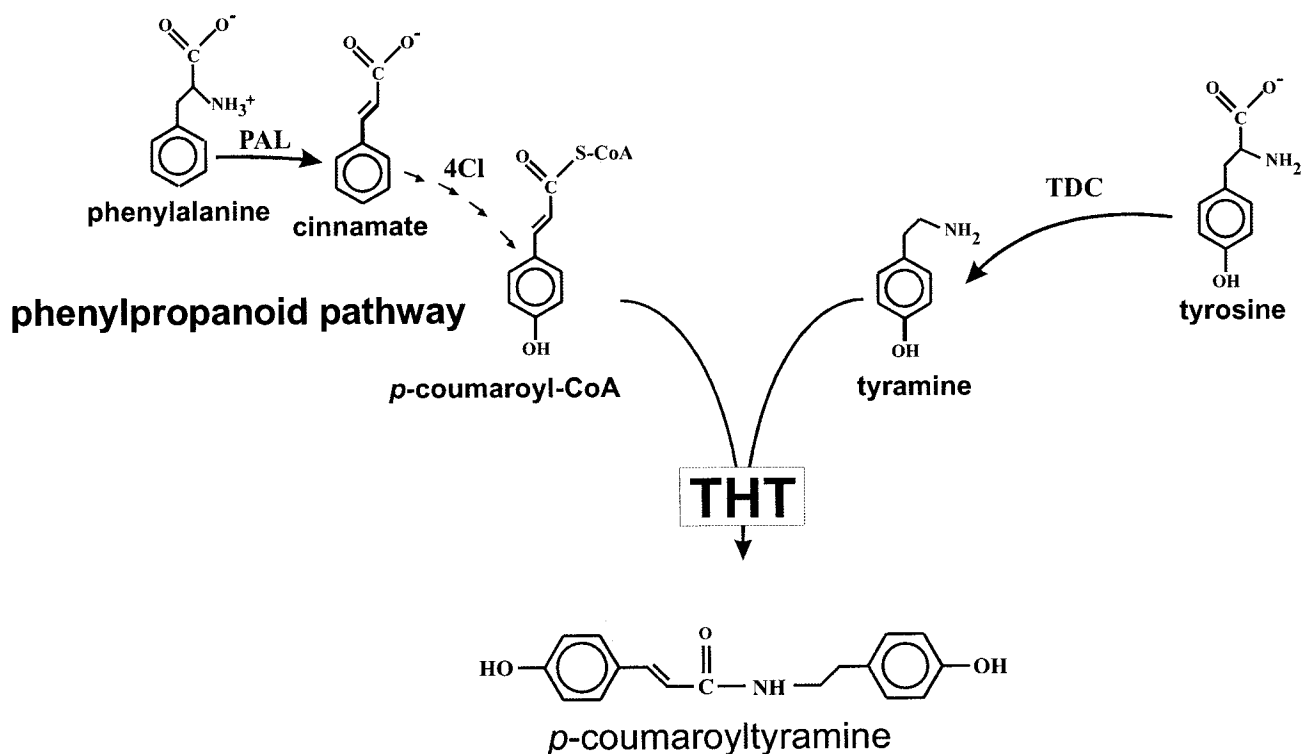
FIG. 1. **Biosynthetic pathway of *p*-coumaroyltyramine.** THT catalyzes the synthesis of *p*-coumaroyltyramine from the thioester *p*-coumaroyl-CoA and the amine tyramine. The enzymes PAL and 4CL of the phenylpropanoid pathway are involved in the synthesis of *p*-coumaroyl-CoA. Tyramine is derived from tyrosine by the action of tyrosine decarboxylase (*TDC*).

necrotic reaction that resembles the HR, whereas *fen* plants show only sporadic appearance of necrotic spots (10, 11). Both the *Fen* and *Pto* signaling pathways to cell death require the *Prf* gene (12); mutant lines carrying nonfunctional alleles (*prf*) show much lower sensitivity to fenthion and no HR in response to *Pst* (*avrPto*). By monitoring the profile of soluble phenolics, we have shown preferential induction of the synthesis of two phenolic compounds in those interactions that are associated with resistance. This is seen with both biotic elicitation and fenitrothion treatment. We identified these two metabolites as *p*-coumaroyloctopamine (*p*-CO) and *p*-coumaroylnoradrenaline (*p*-CN). Conjugates of hydroxycinnamic acids with tyramine and derivatives of tyramine, such as dopamine, methoxytyramine, and octopamine, are found in a wide range of plants. Synthesis of these amides has been shown previously to be activated by treatment with fungal elicitors, by attempted infection by fungi, viruses, and bacteria, and in some cases by wounding (13–19). However, as far as we are aware, this is the first description of the occurrence of *p*-CN in plants and consequently of its association with plant defense. The biosynthesis of *p*-CO and *p*-CN occurs by the condensation of the CoA derivative of *p*-coumaric acid with octopamine and noradrenaline, catalyzed by the enzyme hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT; EC 2.3.1.110). The enzymes phenylalanine ammonia lyase (PAL; EC 4.3.1.5), and 4-coumarate CoA ligase (4CL; EC 6.2.1.12) are involved in the synthesis of *p*-coumaroyl-CoA; octopamine and noradrenaline are synthesized in a number of steps from tyrosine. The biosynthetic pathway for *p*-coumaroyltyramine is shown in Fig. 1. THT has been purified from potato, tobacco, and opium poppy, and the specificity for different acceptors has been established (20–22). All enzymes have wide substrate specificity, although in the case of tobacco and potato, noradrenaline is the least favored acceptor (no experiments were performed with THT from opium poppy using noradrenaline as acceptor). In potato and tobacco, it has been shown that THT is encoded by a multigene family (23, 24). Nevertheless, it is not known whether all genes encode active proteins with similar substrate preferences. So far, three *THT* cDNAs have been cloned from tobacco, one from potato as well as one from pepper (23–25). We have taken several approaches to investigate possible mechanisms underlying the enhanced synthesis of *p*-CO and *p*-CN during defense reactions in tomato. First, we have examined the transcriptional activity of several genes (*PAL*, *4CL*, and *THT*) whose products are implicated in *p*-CO and *p*-CN biosynthesis. Second, we have used the sequences of the potato and tobacco *THT* genes to isolate four different tomato *THT* homologs and have examined the expression of these different *THT* genes after bacterial challenge. Finally, we have compared the acceptor preferences for the different recombinant THT enzymes. We report that the increased *p*-CO and *p*-CN synthesis is associated with the accumulation of transcripts for *PAL*, *4CL*, and *THT*, although gene-specific analysis indicates that the different *THT* genes are differentially regulated upon bacterial challenge of plants. We also report that the different THT isoforms in tomato have different acceptor preferences and that the isoform that is most highly expressed upon bacterial challenge has a significantly higher affinity for noradrenaline than either the potato or tobacco enzymes.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—For experiments with *C. fulvum* elicitors, the tomato cultivar Moneymaker (Cf-0) and two nearly isogenic lines homozygous for the resistance genes *Cf-9* and *Cf-2* were used. Plants were grown either to the sixth leaf stage (6 weeks) or to the cotyledon stage (3 weeks) in a growth room in Levington's M3 compost (Levington Horticulture Ltd., Fisons, Ipswich, UK) with a photoperiod of 16 h (light intensity of 100 microeinsteins). Growth room temperatures were 20 °C in the light and 16 °C in the dark. For the bacterial infection experiments, the tomato cultivar Rio Grande 76R carrying a functional *Pto* and *Fen* gene and a nearly isogenic line 76S (*pto* and *fen*) were grown to the sixth leaf stage. In addition, the nearly isogenic fast neutron-induced mutant lines cv. Rio Grande 76R *prf3*, *prf9*, and *prf2* lacking a functional *Prf* gene were included in the

experiments (12). Plants to be challenged by bacteria or the insecticide fenitrothion were grown under the same light conditions as stated above but at a constant temperature of 22 °C.

Fungal, Bacterial, and Chemical Elicitation and Wounding—The source of *C. fulvum* peptide elicitors Avr2 and Avr9 were intercellular washing fluids originating from tomato plants heavily infected by *C. fulvum* race 5 (referred to as IFAvr9) (26). For controls, intercellular fluids from healthy, noninfected plants (referred to as IF0) were used. For the time course analyses, the IF preparations were infiltrated with a blunt syringe in the intercellular space of tomato cotyledons previously nicked by a razor blade. For all other experiments, adult leaves were infiltrated.

P. syringae pathovar *tomato* carrying the *avrPto* gene (*Pst* (*avrPto*)) (27) and *Pst* (*avrPto*) Δ*hrp* (*K-R*) (28) were grown overnight in King's B broth (29) containing the respective antibiotics. After centrifugation, the bacteria were suspended in 10 mM $MgCl_2$ to the appropriate concentration and infiltrated in the leaves as described above. For the chemical elicitation, the fenthion analog, fenitrothion, was used. The insecticide is available in the United Kingdom under the product name Dicofen (kindly provided by PBI Agrochemicals Ltd., Waltham Abbey, UK). Mature tomato leaflets were immersed for 30 s in a solution of 1% (w/v) fenitrothion containing 0.05% L-77-Silwett (Union Carbide). For wounding, plants were subjected to mechanical injury by gently rubbing the leaves with carborundum (320 grit powder; Fisher). The abrasive was then removed by careful rinsing with tap water.

Analysis of Free Phenolics—Extraction and analysis of the phenolics were performed essentially as described previously (30). In short, 3–4 mg of freeze-dried plant material was extracted with 70% methanol and analyzed via reverse phase HPLC using a Prodigy 5 ODS-2 column (25 cm × 4.6 mm; Phenomenex Ltd., Macclesfield, UK). The following binary gradient, with a flow rate of 1 ml min^{-1}, was applied for elution: $t = 0$ min, 10% A; $t = 20$ min, 16% A; $t = 40$ min, 50% A; $t = 45$ min, 75% A; $t = 50$ min, 95% A; $t = 55$ min, 10% A). Solvent A was methanol/acetonitrile (1:1, v/v), solvent B was 10 mM ammonium formate, pH 3. Absorbences were measured with a diode array-scanning UV detector (Gilson, Middleton, WI). Quantification was accomplished using the standards derived from the enzyme assays (see "THT Enzyme Assay"). The molecular weight (M_r) and structures of peaks 6 and 8 were determined by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI MS). The conditions for LC/ESI MS have been stated before (30).

Test for Antimicrobial Activity—Cultures of *Pst* (*avrPto*) were grown overnight at 28 °C in a rotary shaker in M9 minimal medium supplemented with 0.4% citrate, 25 mM $MgSO_4$, and rifampicin (50 μg/ml) (31). The bacterial concentrations were adjusted to 1×10^4 colony-forming units (cfu)/ml by dilution into new broth. Samples of *p*-CN and *p*-CO derived by HPLC fractionation were lyophilized and redissolved in 35% MeOH to give a stock solution of 1 mM. To exclude inhibitory activity of putative impurities in the volatile HPLC fractions, a blank HPLC run was subjected to the same collection procedure as applied for *p*-CN and *p*-CO. The two blank fractions were combined, lyophilized, and redissolved in methanol in the same way. The phenolics and the blank control were added to the diluted bacterial cultures, which were incubated as above for a further 24 h. Bacterial viable counts were then determined by plating serial dilutions onto Kings B agar plates containing rifampicin. The experiment was performed on triplicate cultures and repeated three times.

Southern Analysis—High molecular weight DNA from pepper (*Capsicum annuum* cv. Early Calwonder ECW 10R), tomato (*L. esculentum* cv. Moneymaker), potato (*Solanum tuberosum* cv. Hunkel), tobacco (*Nicotiana clevlandii*), and barley (*Hordeum vulgare* cv. Franka) was isolated (as described in Refs. 32 and 33) and digested with the restriction enzyme *Eco*RI. Samples (0.7, 1.4, 1.4, 1.4, and 2.8 μg, respectively) were separated by electrophoresis on a 1% agarose gel and transferred to a positively charged nylon membrane (Roche Applied Science). The nonradioactive hybridization was performed by using a digoxygenin-labeled potato THT cDNA (23, 34). The blots were hybridized at 36 °C and washed in 1× SSC at 50 °C.

RNA Isolation and Characterization for Northern Blot Analysis—Total RNA was extracted from frozen leaf material at various time points after inoculation with *Pst* (*avrPto*) (10^7 cfu/ml) or from control tissue using the Tripure Isolation Reagent (Roche Applied Science). For Northern blots, RNA samples (10 μg of total RNA) were separated on formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Amersham Biosciences), according to the manufacturer's protocol. The integrity of the RNA was assessed by visualization of ribosomal RNA by ethidium bromide staining. The cDNA probes for *PAL* (35),

THT (23), and *4CL* (36) were labeled with ^{32}P, hybridized, washed, and exposed as described previously (37).

Cloning of the Tomato THT Genes—Two degenerate primer pairs were designed based on the consensus sequence of potato and tobacco THT (23, 24): t1f, 5′-CATGAATATCATAATTATACTC-3′; t2f, 5′-GAA-GTTTCICCAACCCC-3′; t3r, 5′-CCATTITTTGCAGCAATAGAIGCAA-C-3′; t4r, 5′-CCATACCTAAAITCATCAAAIATTTCAACTCCC-3′. Reverse transcriptase-PCR (Titan™ One Tube reverse transcriptase-PCR system; Roche Applied Science) was carried out by using the primer combinations t1f/t3r and t2f/t4r on total RNA extracted from 76R plants 0, 4, 10, and 16 h postinoculation (hpi). The PCR profile was designed according to the manufacturer's instructions with slight alterations (incubation temperature, 55 °C; denaturation time, 10 s; annealing temperature, 50 °C; 10 + 39 cycles). The amplification products were loaded on 1% agarose gels and separated by electrophoresis. Amplified DNA was extracted from gels and subcloned into the vector pGEM-T (Promega). Ten clones from each primer combination per given time point were sequenced. For the isolation of the tomato THT full-length cDNAs, mRNA was purified by using oligo(dT)-cellulose spin columns (Amersham Biosciences). The starting material consisted of a pool of total RNA samples of various time points after bacterial infection. The 5′- and 3′-rapid amplification of cDNA ends was carried out with gene-specific primers designed for each of the four candidate clones (Marathon™ cDNA amplification kit; Clontech; details for primer sequences and PCR profile available on request). Finally, full-length clones were amplified using primers containing *Bam*HI (5′-end) and *Hin*dIII (3′-end) restriction sites to facilitate subsequent transfer into an expression vector. Candidate clones were sequenced to check for congruent results of full-length amplification and 5′-3′-rapid amplification of cDNA ends nucleotide analysis. The sequences of the four cDNAs were analyzed *in silico* by using the programs BlastP2.2.3 and ProtParamtool (available on the World Wide Web at www.expasy.ch). The alignment of the various sequences was performed applying the clustal method (gap penalty, 30; gap length penalty, 20; weight table, Pam 250).

Real Time PCR Analysis of the Tomato THT Homologs—The tomato cultivars Rio Grande 76R and 76S were infiltrated with *Pst* (*avrPto*) at a concentration of 10^7 cfu/ml. Single leaf discs (1 cm diameter) were cut out at 0, 4, 10, 16, and 24 hpi and frozen in liquid nitrogen. RNA was extracted using the RNAeasy plant miniprep kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Subsequently, 2.5 μg of cDNA were synthesized by reverse transcription using the Revert Aid First Strand Synthesis Kit as described in the manual (Fermentas, St. Leon-Rot, Germany). Real time PCR was carried out on an iCycler (Bio-Rad) featuring a reaction mixture with Sybr Green as fluorescent dye and 1 μl of template cDNA in dilutions of 1:5, 1:10, 1:20, and 1:50 (38); details for primer sequences and PCR profile are available on request. Amplicons were subjected to melting curve analysis (60–95 °C, 0.5 °C increments) and digested with *Hin*fI, resulting in sequence-specific restriction patterns to assure amplification specificity. Message levels were determined as octuplicates at different dilutions (duplicates of each dilution; see above). Tomato Ef1α (accession number X53043) was used as constitutive standard. The level of THT cDNAs were normalized using the level of Ef1α cDNA as described in the ABI user bulletin 2.

Expression of Tomato THT cDNAs in Escherichia coli—The plasmids containing the coding region of the THT clones were digested with *Bam*HI and *Hin*dIII, and the insert DNA was ligated into the expression vector pQUE-30 (Qiagen, Hilden, Germany).The construct was transformed into the *E. coli* strain M15(pREP4). Growth and induction of positive clones were performed as described by the manufacturer. The bacterial cultures were diluted to OD 0.3. After centrifugation of a 250-μl aliquot, the pellet formed was resuspended in 25 μl of SDS-polyacrylamide gel electrophoresis loading buffer, sonicated, and boiled for 5 min. These samples were spun down, and the supernatants were separated on 10 or 15% SDS-polyacrylamide gels (39), followed by staining with Colloidal Brilliant Blue (Sigma). For determination of THT activity, bacterial pellets were resuspended in 10 mM Tris-HCl, pH 7.5, and were broken by three passages through the French press. After centrifugation to remove cell debris, the supernatant was stored at −80 °C.

THT Enzyme Assay—The substrate *p*-coumaroyl-CoA was enzymatically synthesized essentially as described by Meng and Campbell (40) using a recombinant tobacco 4CL (36). The reaction mixture was applied to a C18 Varian Bond Elut Extraction Cartridge (Phenomenex), and the CoA-conjugate was selectively eluted with acetonitrile/water (1:3, v/v). The material was lyophilized, and its purity was checked by HPLC. For the THT assay, 50 μl of THT enzyme solution (4 μg/μl) were

mixed with 200 μl of 10 mM Tris-HCl (pH 7.5), 10 μl of *p*-coumaroyl-CoA (1 mM), and 5 μl of tyramine, dopamine, octopamine, and noradrenaline to a final concentration ranging from 0.01 to 0.5 mM. After 10 min at 25 °C, the reaction was stopped by the addition of 50 μl of 50% (w/v) trichloroacetic acid. The assay mixture was spun down, and 200 μl of the supernatant were subjected to reverse phase HPLC analysis on a C18 column, exactly as described earlier for the phenolic analysis. The amine conjugates were identified by retention time and UV spectra. The K_m values for the different acceptor amines were calculated from Lineweaver-Burk plots from double measurements at each concentration.

RESULTS

Differential Accumulation of Phenolics in Response to Fungal Elicitors—Intercellular fluid containing the *C. fulvum* Avr9 and *Avr2* peptides (IFAvr9) was used for infiltration of resistant (*Cf-9*) and susceptible (*Cf-0*) tomato plants. At 12–16 hpi, a graying necrosis could be seen in the incompatible interaction (Fig. 2*A*, *Cf-9*), whereas no macroscopic symptoms could be detected for the *Cf-0* plants (Fig. 2*A*, *Cf-0*). The production of soluble phenolics during these interactions was monitored by HPLC analysis of extracts from the infiltrated leaf areas. Of 13 detectable compounds, only two (referred to as peaks 6 and 8) showed a reliable alteration over three independent time course experiments (Figs. 2*A* and 3*A*). Tomato *Cf-9* cotyledons infiltrated with IFAvr9 showed an accumulation of the peak 6 compound starting at 15 hpi and the peak 8 compound starting at 6 hpi. The maximum level of the two substances induced in *Cf-9* plants were achieved at 21 hpi; thereafter, the levels declined rapidly. The levels at the maximum were ~20- and 80-fold higher, respectively, than in *Cf-0* plants. In *Cf-9* cotyledons infiltrated with IF0 (containing no Avr peptides), an accumulation of the peak 8 compound was observed beginning at 6 hpi and reaching a maximum value at 9 hpi (Fig. 3*A*). The maximum level was ~10-fold lower than that seen with IFAvr9; thereafter, the level declined slowly. There was no accumulation of the peak 6 compound in this interaction. To show that the induction of the two phenolics was not just specific for interactions involving *Cf-9*, a comparable experiment using *Cf-2* plants was carried out. At 24 h after IFAvr9 inoculation, chlorosis could be seen in the incompatible *Cf-2* interaction, correlating with an induction of peaks 6 and 8. The accumulation profiles of these two compounds were comparable with the *Cf-9* interaction (peak ratio P8/P6 = ~3:1), although the maximum levels were approximately 10 times lower than in the *Cf-9* interaction (data not shown). The triggering of the synthesis of the two phenolic compounds in these experiments could be a specific response of tomato to fungal elicitors rather than a broad defense mechanism capable of being activated by a range of pathogens. To test this, we monitored the levels of phenolics in tomato leaves challenged with an avirulent bacterial pathogen and with a derivative of fenthion that triggered programmed cell death in tomato.

Synthesis of Phenolics after Bacterial Challenge—In the tomato cultivar 76R, the *Pto* locus confers resistance to *Pst* strains expressing the avirulence gene *avrPto* (9). Infiltration of *Pst* (*avrPto*) into 76R at concentrations of 10^7 cfu/ml lead to an HR, indicated by dry necrosis, at about 16 hpi (Fig. 2*B*, *Pto*). Infiltration of the same inoculum in the nearly isogenic-susceptible relative 76S, carrying the *pto* allele, resulted in water-soaked necrotic lesions (Fig. 2*B*, *pto*). Strong induction of peaks 6 and 8 was only seen in the incompatible (HR) interaction with 76R (Fig. 2*B*). Time course analysis revealed that the peak 8 compound started to accumulate at 4 hpi, and maximum accumulation was reached at 16 hpi. The peak 6 compound started to accumulate at 10 hpi but again showed a maximum accumulation at 16 hpi (Fig. 3*B*). Maximum levels of peak 8 were 10 times higher than that of peak 6. By contrast, in the compatible interaction with 76S, levels of both compounds were about 30 times lower than the corresponding values with 76R. Control plants infiltrated with water showed no induction of the peak 6 and 8 compounds (data not shown).

The cultivar 76R was also inoculated with the *Pst* (*avrPto*) Δ *hrp* (K-R) mutant, which carries a deletion within the *hrp* gene cluster (28). Genes within this cluster encode components of a Type III secretion system believed to deliver the AvrPto protein to its site of recognition within the plant cell. The deletion mutant strain does not trigger HR and showed no induction of the phenolics in the infiltrated leaf area (Fig. 3*B*). *Pst* (*avrPto*) was also inoculated into the *prf2*, *prf3*, and *prf9* mutant lines derived from 76R (12). These lines have mutations in the *Prf* gene, which is involved in the signal transduction pathway leading to HR, and hence are susceptible to *Pst* (*avrPto*). All of these lines gave very low levels of peak 6 and peak 8 compounds, comparable with that seen in the susceptible cultivar 76S (data not shown). Overall, these results showed that the greatly enhanced synthesis of the two phenolics in tomato/*Pst* interactions is dependent on the presence of *avrPto*, *Pto*, and the signal transduction component *Prf* and hence is correlated with the triggering of HR and the expression of resistance.

Synthesis of Phenolics after Chemical Elicitation—The tomato cultivar 76R carries the *Fen* gene, which is tightly linked to *Pto* and conditions sensitivity to the organophosphorous insecticide fenthion, leading to a necrotic reaction comparable with the HR (10, 11). The near isogenic tomato cultivar 76S (*fen*) shows a high degree of insensitivity to fenthion. We challenged these plants with the fenthion analog fenitrothion. After 48 h, the 76R plants developed dark, large, necrotic lesions covering the whole leaflet (Fig. 2*C*, *Fen*). In contrast, challenge of 76S and the mutant line *prf3* derived from 76R resulted only in sporadic appearance of necrotic spots (Fig. 2*C*, *fen*). At the given time point, the level of the peak 6 and peak 8 compounds were 10 and 3 times higher in the *Fen* plants compared with the *fen* or *prf3* plants. Interestingly, after fenitrothion treatment, peak 6 is prevalent within the spectrum of phenolics, whereas with fungal elicitors or bacteria peak 8 is predominant.

Synthesis of Phenolics after Wounding—Tomato leaves were gently rubbed with carborundum and carefully washed to remove the abrasive. After 24 h, the whole leaf area showed dry, dark green, necrotic lesions, which were not accompanied by the induction of the phenolics in question (Fig. 2*D*). This result established that the synthesis of the peak 6 and peak 8 phenolics was not a general response to tissue necrosis caused by any agent but was specific to the programmed cell death responses associated with disease resistance.

Structural Characterization of the Peak 6 and 8 Compounds—Comparison of the absorbance spectra of peak 6 and 8 (λ_{max} 289 and 305 nm) with the UV spectrum of the standard *p*-coumaroyltyramine (Fig. 4, *B* and *E*) showed that they belong to the same family of phenolic amines. The identity of peak 6 was revealed by LC/ESI MS to be *p*-CN (Fig. 4, *A* and *C*). This was indicated by a $(M + H)^-$ peak at *m/z* 316.2 and fragment ions at *m/z* 147.0 (*p*-coumaroyl moiety; fragmentation occurred between C-10 and the nitrogen atom) and $(M + H-H_2O)^+$ at *m/z* 298.2 (water loss at the carbon skeleton of the noradrenaline residue). Generally, in the amine conjugates with a hydroxy function at C-7′, the ESI mass spectra display a prominent $(M + H-H_2O)^{+\cdot}$ These ions can be used to differentiate between 7′-hydroxy compounds and those with hydroxyl substitution on the benzene ring of the amine moiety (23). To our knowledge, this is the first description of this conjugate in plants. The mass spectrometry analysis of peak 8 revealed a 16-Da lower $(M + H)^-$ peak at *m/z* 300.2, indicating the presence of a compound
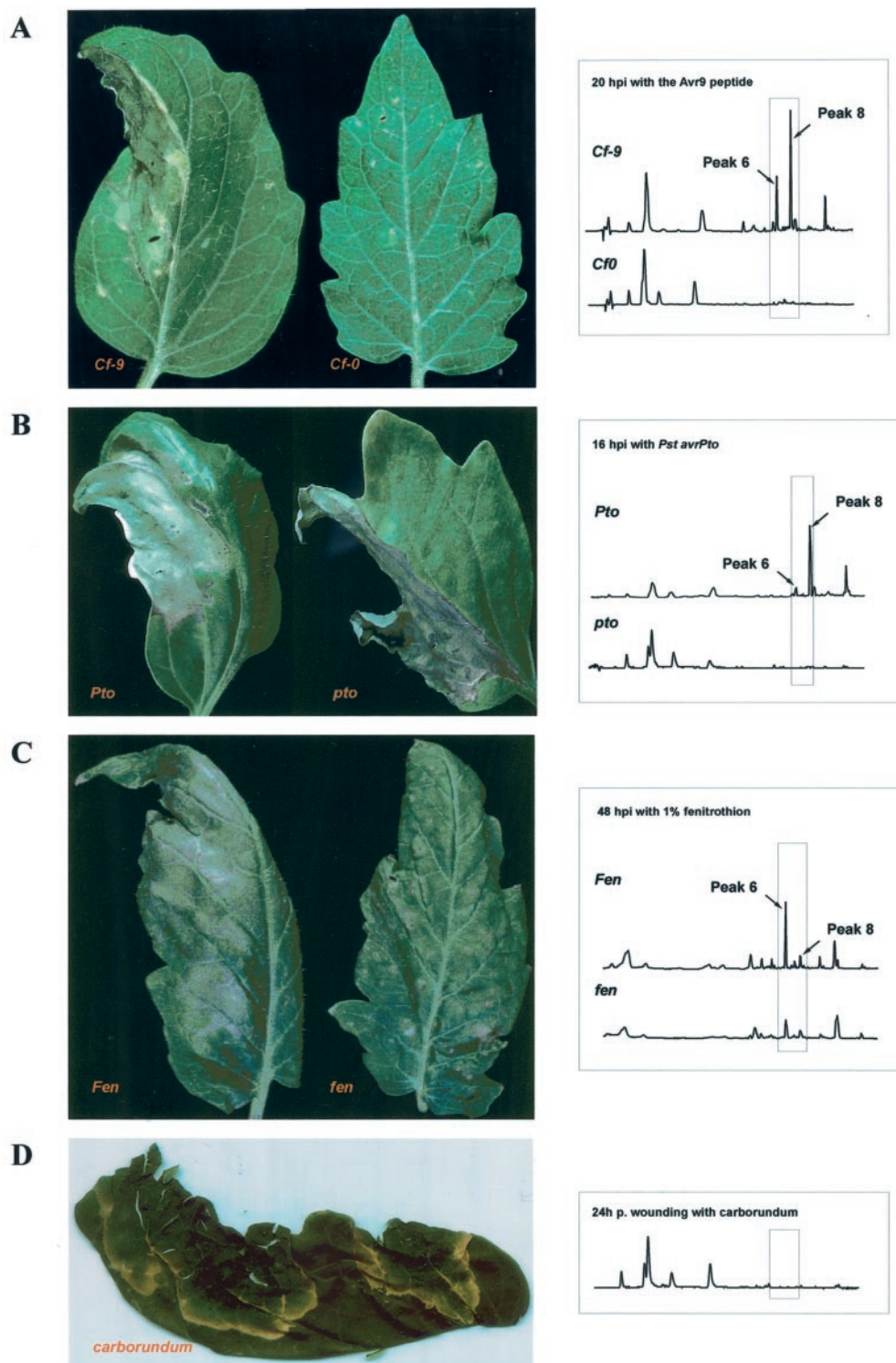
FIG. 2. **The accumulation of phenolic conjugates in tomato in response to biotic and abiotic stresses.** *A*, intercellular washing fluid containing the Avr9 peptide of the fungus *C. fulvum* (IFAvr9) was inoculated into leaves of resistant (*Cf-9*) and susceptible (*Cf-0*) tomato plants cv. Moneymaker. *B*, suspensions (10^7 cfu/ml) of the bacterium *Pseudomonas syringae* pathovar *tomato* carrying the avirulence gene *avrPto* were infiltrated in the tomato line (76R) carrying the resistance gene *Pto* and a nearly isogenic susceptible cultivar 76S (*pto*). *C*, leaves of sensitive (76R, *Fen*) and insensitive (76S, *fen*) tomato cultivars were immersed for 30 s in a 1% solution of the organophosphorous insecticide fenitrothion. *D*, leaves of the tomato cv. Moneymaker were wounded by gently rubbing the leaf surface with the abrasive carborundum. For all treatments, both the appearance of the leaves at the sampling time and subsequent HPLC analyses of the soluble phenolics are shown. In the chromatograms, the two differentially accumulating phenolic conjugates are highlighted.

with one hydroxy function less then *p*-coumaroylnoradrenaline (Fig. 4, *D* and *F*). The fragment ions at *m/z* 147.1 and 282.1 (water loss at the carbon skeleton of the octopamine residue) verified it to be *p*-CO. This metabolite has already been identified in cell cultures of *Solanum khasianum* and *S. tuberosum*

(23, 41). In addition to the MS analysis comparison of the retention time values of peaks 6 and 8 with the retention times of synthesized products derived from the THT enzyme, assays using noradrenaline and octopamine as substrates (see "Enzymatic Properties of Recombinant THT Enzymes") gave further

FIG. 3. **Time course analysis of peak 6 and 8 compounds in tomato after treatment with fungal elicitors or bacteria.** *A*, accumulation of peak 6 and 8 compounds in resistant *Cf-9* plants infiltrated with intercellular washing fluid containing the Avr9 peptide (IFAvr9) (●) or with IF derived from noninfected tomato plants (IF0) (▲). Susceptible *Cf-0* plant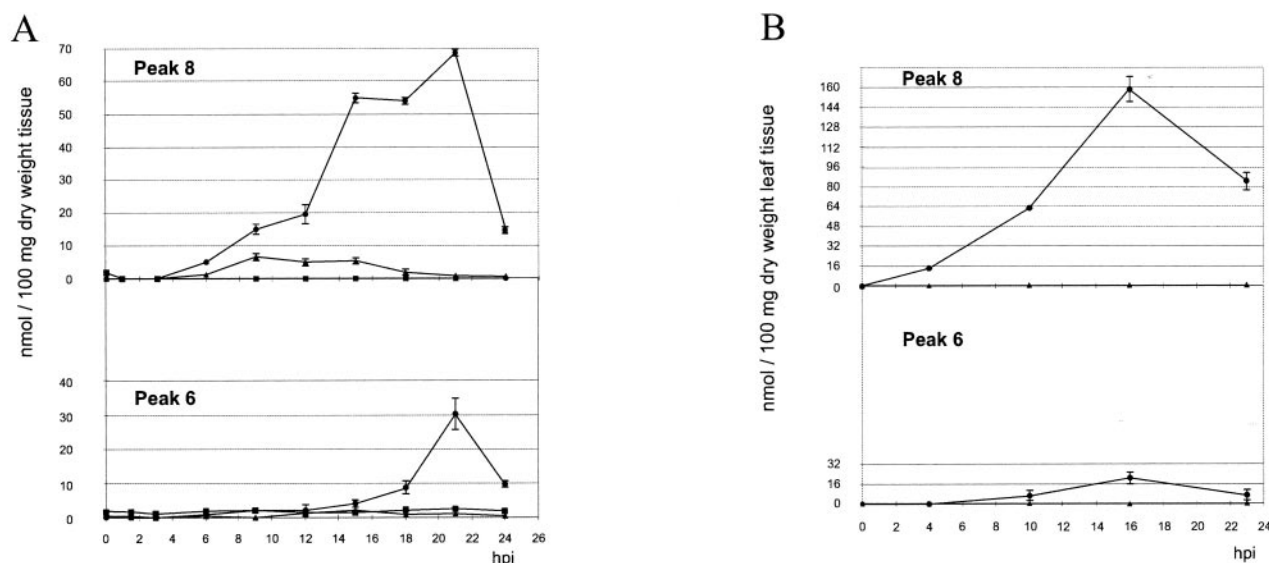s were also inoculated with IFAvr9 ■. Each data point represents the mean of two independently extracted samples each comprising of a pool of four cotyledons. *B*, accumulation of peak 6 and 8 compounds in tomato plants carrying the *Pto* resistance gene after infiltration with the bacteria strains *Pst(avrPto)* (●) and *Pst(avrPto)Δhrp* (K-R) (▲) at a concentration of 10^7 cfu/ml. Each data point represents the mean of two independently extracted samples each comprising of a pool of two leaves.

evidence for the identity of *p*-CN and *p*-CO as the two compounds in question.

Antimicrobial Activity of p-CN against Pst (avrPto)—The phenolic conjugates *p*-CN and *p*-CO were purified by HPLC from extracts of challenged leaves and were added to liquid cultures of *Pst* (*avrPto*) to various final concentrations. Effects on bacterial growth were monitored after a 24-h culture period. Control cultures received either water or lyophilized HPLC buffer (blank control), which had a small but reproducible growth-promoting activity compared with water (Fig. 5). Preliminary experiments showed that at a range of concentrations up to 100 μM, *p*-CO had no significant effect on bacterial growth or viability. At 100 μM, *p*-CN reduced the growth of *Pst* (*avrPto*) 10-fold in comparison with the blank control. At 10 μM, the effect was smaller, and a 2-fold reduction was observed. The inhibitory activity of *p*-CN appeared to be lower when calculated using the water control; 100 μM *p*-CN reduced bacterial growth by 7-fold, and 10 μM *p*-CN had no significant inhibitory effect.

Association of p-CN and p-CO Accumulation with Transcript Alterations of THT, PAL, and 4CL—As outlined in the Introduction, the last step in the biosynthesis of *p*-CN and *p*-CO in tomato is catalyzed by the enzyme THT. This protein conjugates *p*-coumaroyl-CoA (synthesized through the action of PAL and 4CL) and a variety of amines (*e.g.* tyramine, octopamine, or noradrenaline). We tested for the presence of *THT* genes in tomato and other plants by performing a botanical garden blot using a potato *THT* cDNA as probe. Although only very weak signs of hybridization could be observed with the monocot plant barley, the four solanaceous species (potato, pepper, tobacco, and tomato) showed multiple strong hybridization signals (data not shown). This indicated the presence of a multigene family in each of these four species. The transcript levels for *THT*, *PAL*, and *4CL* were measured in both incompatible and compatible interactions of *Pst* (*avrPto*) with tomato cultivars 76R and 76S using the potato *THT* cDNA and tomato *PAL* and *4CL* cDNAs as probes (Fig. 6). Untreated tomato leaves showed a moderate, constitutive expression of *THT*, but no transcripts for *PAL* or *4CL* could be detected. In the incompatible interaction, a strong accumulation of *THT* transcripts in response to

Pst (*avrPto*) could be observed as early as 4 hpi, with high transcript levels being maintained up to 24 hpi. In contrast, transcripts for *PAL* and *4CL* revealed increased levels at 4 hpi but declined slowly within the next 20h (Fig. 6, *Pto* + *Pst (avrPto)*). In the compatible interaction, *THT* transcript levels showed an increase at 4 hpi, although to a lower level than in the incompatible interaction. Subsequently, the THT transcript levels dropped at 10 hpi before a second phase of accumulation at 16 and 24 hpi, although again to a lower level than that seen in the incompatible interaction. Transcripts for *PAL* and *4CL* only accumulated at 16 hpi and were reduced by 24 hpi (Fig. 6, *pto* + *Pst (avrPto)*). Infiltration of water into the plants also triggered the accumulation of transcripts for *THT* at 4 hpi and for *PAL* at 16 hpi, although no accumulation of transcripts for *4CL* was detected at any time point (Fig. 6, *water*). The patterns of *THT* and *PAL* transcript accumulation in compatible interactions with *Pst* (*avrPto*) could thus be mimicked by water inoculation alone, whereas the accumulation of *4CL* transcripts required the presence of bacteria.

Isolation and Characterization of Tomato THT cDNA Clones—RNA was isolated from the leaves of tomato cultivar 76R responding to *Pst* (*avrPto*) and used to isolate *THT* clones by degenerative reverse transcriptase-PCR followed by 5′–3′-rapid amplification of cDNA ends. The degenerate primers were designed on the consensus of *THT* genes from tobacco (accession number AJ005062) and potato (23). Four different tomato *THT* clones could be identified. The clones *tomTHT1-3* and *tomTHT1-4* (accession numbers AY081905 and AY081908, respectively) could be amplified with the primer combination t1f/t3r. The predicted coding regions were 724 or 729 nucleotides, respectively. With the primer pair t2f/t4r, two additional *THT* genes, *tomTHT7-1* and *tomTHT7-8*, could be isolated with coding regions of 741 and 723 nucleotides, respectively (accession numbers AY081906 and AY081907).

The predicted proteins encoded by the four cDNAs showed a high degree of amino acid sequence similarity (Fig. 7); tomTHT1-3, tomTHT7-1, and tomTHT7-8 all exhibited 93% sequence similarity to each other, and all were 73% similar to tomTHT1–4. The major difference in tomTHT1–4 compared with the other predicted tomato proteins was in the amino acid
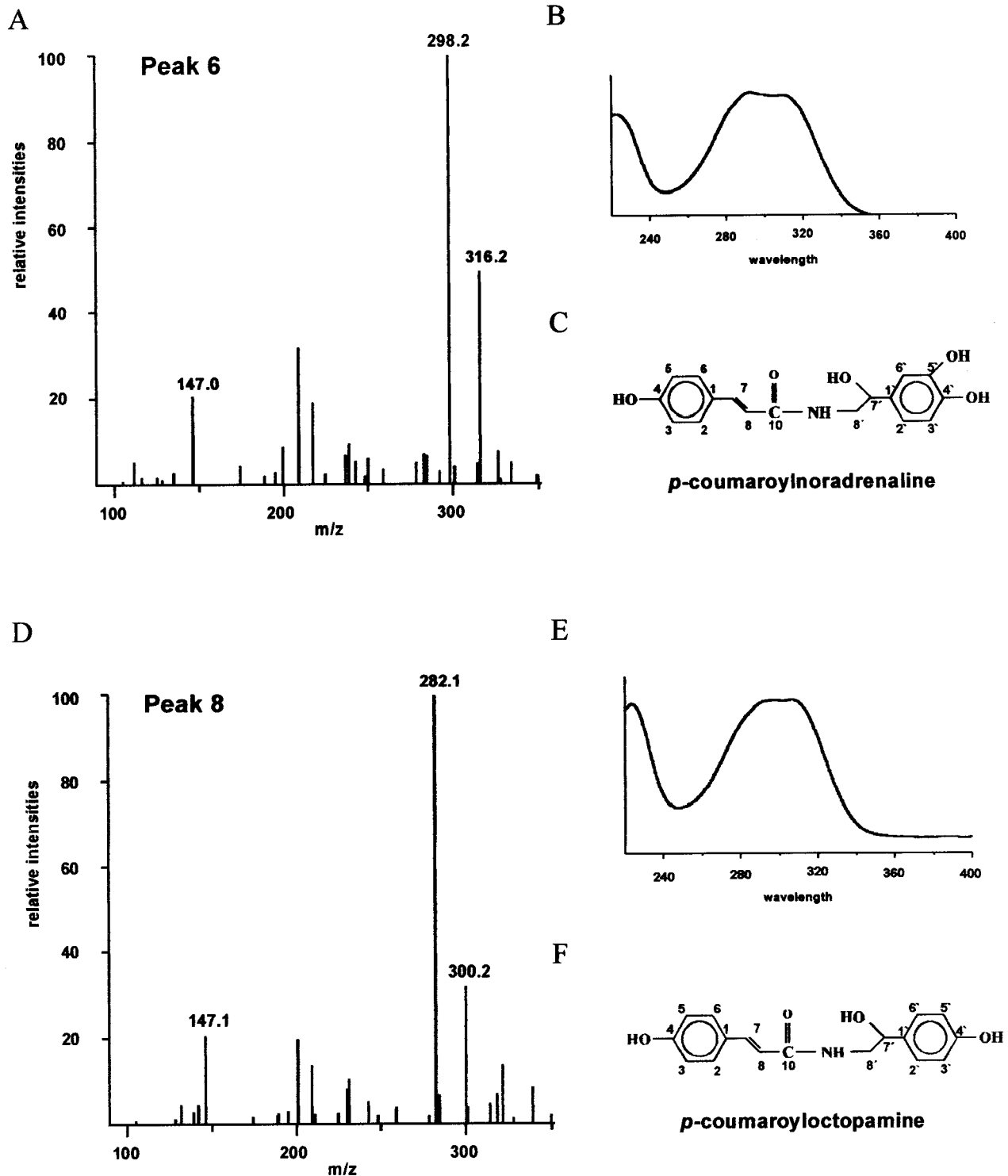
FIG. 4. **ESI MS spectra, absorbance spectra and chemical structure of peak 6 and 8 compounds.** The compounds in peak 6 and peak 8 were identified by mass spectrometry as *p*-coumaroylnoradrenaline (*A*) and *p*-coumaroyloctopamine (*D*), and the structures (*C* and *F*) together with UV-absorbance spectra (*B* and *E*) are shown.

stretch 98–122, which ended in a premature stop signal. In terms of amino acid sequence, the THTs from tomato were more closely related to the THT of *S. tuberosum* than to THT from tobacco. The similarity between sequences of potato and tomTHT1-3/7-1/7-8/1-4 was 95, 89, 93, and 76%, respectively; the similarity between sequences of tobacco and tomTHT1-3/7-1/7-8/1-4 was 86, 83, 84, and 70%, respectively.

In silico analysis of tomTHT1-3, tomTHT7-8, tomTHT7-1, and tomTHT1-4 predicted a molecular mass of 26.5, 27.1, 28.0,

and 13 kDa for the encoded proteins with pI values of 5.4, 5.4, 5.8, and 9.2, respectively.

Gene-specific Transcript Analysis of the Four Tomato THT cDNA Clones—The accumulation of transcripts for the four tomato THT cDNAs was analyzed by real time PCR in both the incompatible and compatible interaction of *Pst (avrPto)* with the tomato cultivar 76R and 76S, respectively. In the incompatible interaction, levels of transcripts corresponding to *tomTHT1-3* showed a substantial and rapid increase upon in-
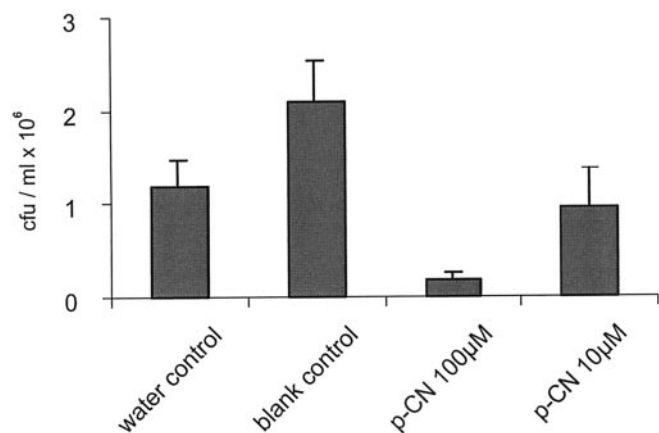
FIG. 5. **Antimicrobial activity of *p*-coumaroylnoradrenaline.** Cultures of *Pst (avrPto)*, grown in minimal medium, were supplemented with *p*-coumaroylnoradrenaline (final concentration of 100 or 10 μM), water, or lyophilized HPLC buffer (blank control). The number of bacterial colonies after a 24-h growth was determined by plating out serial dilutions. Values given are the mean and S.D. of three separate measurements.

FIG. 6. **Expression of THT in tomato during compatible and incompatible interactions with bacteria.** Accumulation of transcripts for *THT*, *PAL*, and *4CL* was measured in resistant (*Pto*) and susceptible (*pto*) tomato plants in response to *Pst (avrPto)* (10^7 cfu/ml start inoculum). Total RNA was extracted from leaves at different times (h) after inoculation with bacteria or with water (mock inoculation). The integrity and equal loading of the RNA was assessed by visualization of ribosomal RNA by ethidium bromide staining.

oculation and were 63-fold higher at 4 h after inoculation than in unchallenged plants. This induction was followed by a decline of transcript levels to ~50% of the maximum level at 16 hpi (Fig. 8*A*). Elevated levels of *tomTHT1-3* transcripts could also be seen in the compatible interaction. In this case, however, maximum induction occurred at a much later time point (16 hpi) and to a lesser extent (about 16-fold less) than in the incompatible interaction (Fig. 8*B*). Furthermore, the constitutive levels of *tomTHT1-3* transcripts were 6 times lower in susceptible compared with resistant plants. The accumulation profiles of transcripts for *tomTHT7-8, tomTHT1-4*, and *tomTHT7-1* were clearly different from that of *tomTHT1-3*. Although in unchallenged plants *tomTHT7-8*, *tomTHT1-4*, and *tomTHT7-1* had approximately the same transcript level as *tomTHT1-3*, after challenge the levels of transcripts of all three genes declined in both compatible and incompatible interactions.

Enzymatic Properties of Recombinant THT Enzymes—The tomato THTs were expressed in *E. coli* using the vector pQE30. Analysis of the extracts from the induced cultures by SDS-polyacrylamide gel electrophoresis confirmed the slightly different sizes of tomTHT1-3, tomTHT7-8, and tomTHT7-1 predicted from the translated DNA sequence of the cDNA clones. The expression of tomTHT1-4 could also be induced, but this (truncated) protein was only detectable on a 15% SDS gel (data not shown).

Crude bacterial extracts containing the recombinant tomato THTs were tested for their enzymatic activity and substrate specificity with *p*-coumaroyl-CoA as acyl donor and various amines as possible acceptors (Table I). Extracts from bacteria carrying the empty expression vector pQE30 or expressing the truncated protein tomTHT1–4 showed no activity with any of the acceptor substrates. The other three recombinant tomTHTs (1-3, 7-1, and 7-8) were all enzymatically active. All three exhibited the highest affinity for tyramine, although the K_m value of tomTHT7-8 was about 10-fold higher than that of tomTHT1-3 and tomTHT7-1. For tomTHT1-3 and tomTHT7-1, the affinities for the different acceptor decreased in the order tyramine > octopamine > dopamine > noradrenaline and tyramine > dopamine > octopamine > noradrenaline, respectively. In contrast, the affinity of tomTHT7-8 for noradrenaline was higher than for both dopamine and octopamine.
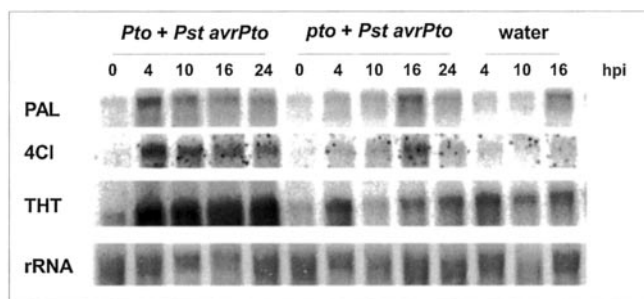
DISCUSSION

Conjugates of hydroxycinnamic acids with tyramine or with derivatives of tyramine, such as dopamine, methoxytyramine, and octopamine, are found in a wide range of plants including onion, potato, pepper, and tobacco (13–16, 37). It has been shown that their synthesis is activated in response to fungal elicitors as well as in response to attempted infection by fungi, viruses, and bacteria and, in some cases, wounding. Increased synthesis of feruloyl- and *p*-coumaroyl- derivatives of tyramine (FT and *p*-CT) and octopamine (FO and *p*-CO) is associated with *Phytophthora infestans* elicitor stimulation of potato cell suspension cultures (23, 44). Increased synthesis of FT and CT is also associated with the resistance of potato plants to *P. infestans* (15) and with both the nonhost- and gene-for-gene-determined resistance reactions of pepper to *Xanthomonas campestris* (37). Biosynthesis of FT is induced in tobacco by tobacco mosaic virus infection (14). In tomato, potato, and tobacco, accumulation of FT occurs after wounding with a hemostat or a pressure-applying tool, although in wounded pepper and nightshade no FT can be detected (19). Here we have shown that markedly enhanced synthesis of *p*-CO and *p*-CN (a novel compound for plants) is specifically associated with resistance reactions triggered in tomato by a range of agents (fungal elicitors, avirulent bacteria, and the chemical fenitrothion). However, wounding with the abrasive carborundum causing necrotic cell death did not lead to the induction of these two compounds.

Two major roles for hydroxycinnamoyl tyramines in plant defense have been proposed. They can be incorporated into the plant cell wall to strengthen it against microbial degradation, or potentially they can act as direct antimicrobial agents. We have not addressed the issue of incorporation of *p*-CN and *p*-CO into the plant cell wall, but it could account for the reduction of the levels of soluble *p*-CO and *p*-CN at later time points after treatment with *C. fulvum* elicitors and *Pst (avrPto)*. There are only a few reports of antimicrobial activity of hydroxycinnamoyl tyramines. Grandmaison *et al.* (42) showed that FT induced hyphal branching but reduced total growth of mycorhizal fungi. Newman *et al.* (37) demonstrated antibacterial activity of both FT and CT against *X. campestris*. In contrast, McLusky *et al.* (16) failed to detect any antimicrobial activity of hydroxycinnamoyl tyramines from onion induced by attempted *Botrytis* infection against *Botrytis* spp. We have shown that *p*-CN has a relatively weak activity against *Pst (avrPto)* (a 10-fold growth reduction at 100 μM; a 2-fold growth reduction at 10 μM). The maximum value of *p*-CN accumulating in resistant tomato leaves after challenge with *Pst (avrPto)* was 21 nmol/100 mg dry weight leaf tissue (Fig. 3*B*). This is equivalent
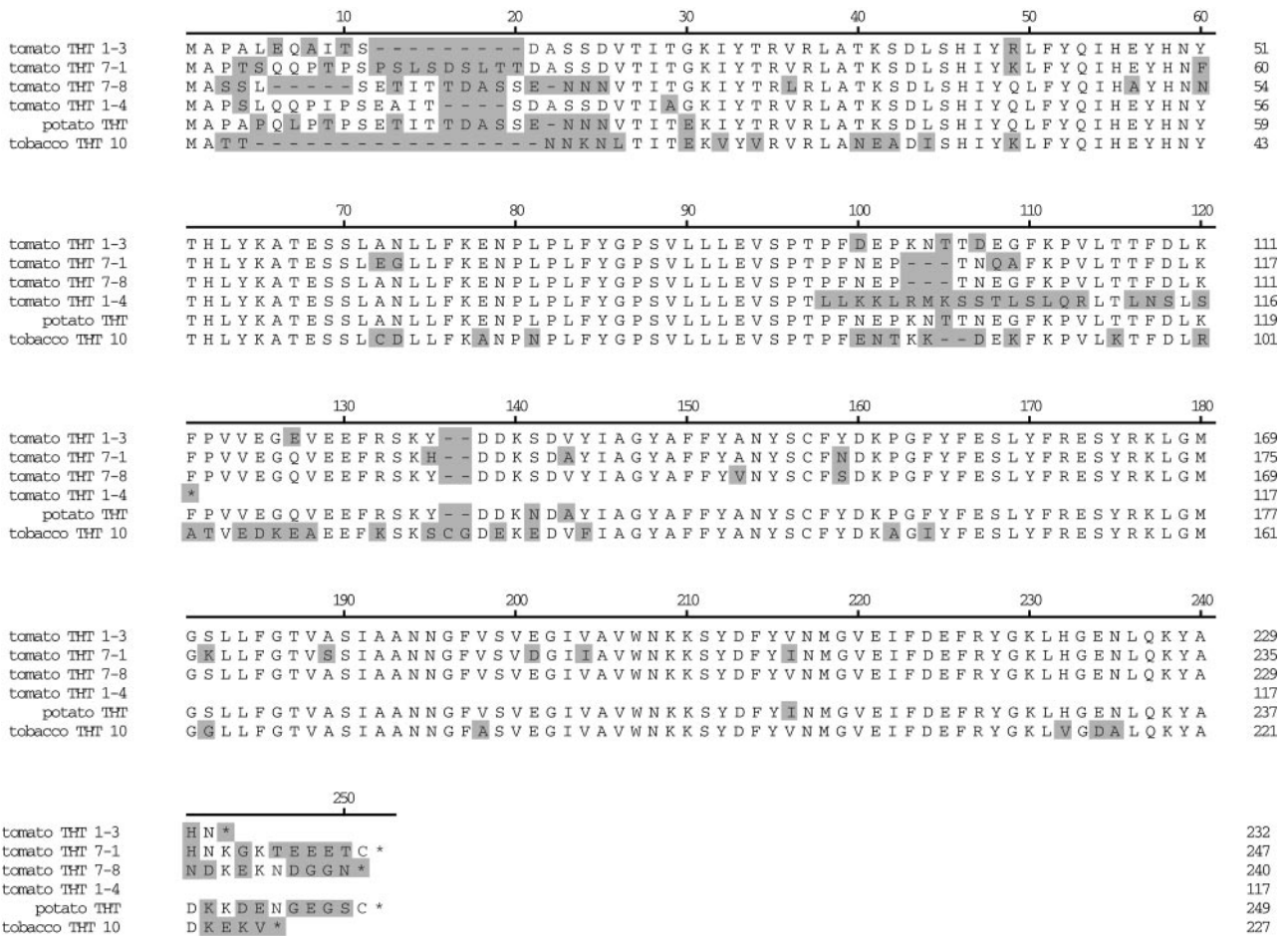
		10	20	30	40	50	60
tomato THT 1-3	M A P A L E Q A I T S - - - - - - - - - D A S S D V T I T G K I Y T R V R L A T K S D L S H I Y R L F Y Q I H E Y H N Y						51
tomato THT 7-1	M A P T S Q Q P T P S P S L S D S L T T D A S S D V T L G K I Y T R V R L A T K S D L S H I Y K L F Y Q I H E Y H N F						60
tomato THT 7-8	M A S S L - - - - - S E T I T T D A S S E - N N N V T I T G K I Y T R L R L A T K S D L S H I Y Q L F Y Q I H A Y H N N						54
tomato THT 1-4	M A P S L Q Q P I P S E A I T - - - - S D A S S D V T I A G K I Y T R V R L A T K S D L S H I Y Q L F Y Q I H E Y H N Y						56
potato THT	M A P A P Q L P T P S E T I T T D A S S E - N N N V T I T E K I Y T R V R L A T K S D L S H I Y Q L F Y Q I H E Y H N Y						59
tobacco THT 10	M A T T - - - - - - - - - - - - - - - N N K N L T I T E K V Y V R V R L A N E A D I S H I Y K L F Y Q I H E Y H N Y						43

		70	80	90	100	110	120
tomato THT 1-3	T H L Y K A T E S S L A N L L F K E N P L P L F Y G P S V L L L E V S P T P F D E P K N T T D E G F K P V L T T F D L K						111
tomato THT 7-1	T H L Y K A T E S S L E G L L F K E N P L P L F Y G P S V L L L E V S P T P F N E P - - - T N Q A F K P V L T T F D L K						117
tomato THT 7-8	T H L Y K A T E S S L A N L L F K E N P L P L F Y G P S V L L L E V S P T P F N E P - - - T N E G F K P V L T T F D L K						111
tomato THT 1-4	T H L Y K A T E S S L A N L L F K E N P L P L F Y G P S V L L L E V S P T L L K K L R M K S S T L S L Q R L T L N S L S						116
potato THT	T H L Y K A T E S S L A N L L F K E N P L P L F Y G P S V L L L E V S P T P F N E P K N T T N E G F K P V L T T F D L K						119
tobacco THT 10	T H L Y K A T E S S L C D L L F K A N P N P L F Y G P S V L L L E V S P T P F E N T K K - - D E K F K P V L K T F D L R						101

		130	140	150	160	170	180
tomato THT 1-3	F P V V E G E V E E F R S K Y - - D D K S D V Y I A G Y A F F Y A N Y S C F Y D K P G F Y F E S L Y F R E S Y R K L G M						169
tomato THT 7-1	F P V V E G Q V E E F R S K H - - D D K S D A Y I A G Y A F F Y A N Y S C F N D K P G F Y F E S L Y F R E S Y R K L G M						175
tomato THT 7-8	F P V V E G Q V E E F R S K Y - - D D K S D V Y I A G Y A F F Y V N Y S C F S D K P G F Y F E S L Y F R E S Y R K L G M						169
tomato THT 1-4	*						117
potato THT	F P V V E G Q V E E F R S K Y - - D D K N D A Y I A G Y A F F Y A N Y S C F Y D K P G F Y F E S L Y F R E S Y R K L G M						177
tobacco THT 10	A T V E D K E A E E F K S K S C G D E K E D V F I A G Y A F F Y A N Y S C F Y D K A G I Y F E S L Y F R E S Y R K L G M						161

		190	200	210	220	230	240
tomato THT 1-3	G S L L F G T V A S I A A N N G F V S V E G I V A V W N K K S Y D F Y V N M G V E I F D E F R Y G K L H G E N L Q K Y A						229
tomato THT 7-1	G K L L F G T V S S I A A N N G F V S V D G I I A V W N K K S Y D F Y I N M G V E I F D E F R Y G K L H G E N L Q K Y A						235
tomato THT 7-8	G S L L F G T V A S I A A N N G F V S V E G I V A V W N K K S Y D F Y V N M G V E I F D E F R Y G K L H G E N L Q K Y A						229
tomato THT 1-4							117
potato THT	G S L L F G T V A S I A A N N G F V S V E G I V A V W N K K S Y D F Y I N M G V E I F D E F R Y G K L H G E N L Q K Y A						237
tobacco THT 10	G G L L F G T V A S I A A N N G F A S V E G I V A V W N K K S Y D F Y V N M G V E I F D E F R Y G K L V G D A L Q K Y A						221

	250	
tomato THT 1-3	H N *	232
tomato THT 7-1	H N K G K T E E E T C *	247
tomato THT 7-8	N D K E K N D G G N *	240
tomato THT 1-4		117
potato THT	D K K D E N G E G S C *	249
tobacco THT 10	D K E K V *	227

FIG. 7. **Comparison of the amino acid sequences encoded by the four tomato THTs.** Multiple sequence alignments of the amino acid sequences of the four tomato THT homologs with those of potato and tobacco THT. The alignment was performed applying the clustal method (gap penalty, 30; gap length penalty, 20; weight table, Pam 250). Differences are highlighted in *gray*.
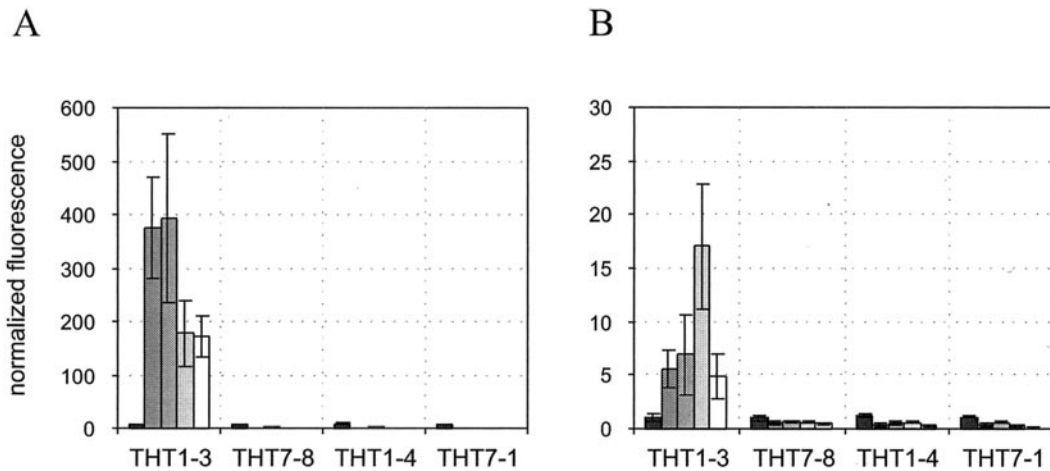

A

B

FIG. 8. **Quantitative gene-specific analysis of the tomato THT homologs.** Real time PCR analysis of the four tomato THT homologs in an incompatible (*A*) and compatible interaction (*B*) at 0, 4, 10, 16, and 24 hpi (*black*, *dark gray*, *medium gray*, *light gray*, and *white bar*). Message levels were determined as octuplicates at different dilutions (1:5, 1:10, 1:20, and 1:50) and normalized with the constitutive standard tomato Ef1α.

to a concentration of 24 μM p-CN in the tomato leaf, assuming the compound was distributed throughout the cellular water. This concentration lies within the range in which p-CN has antimicrobial activity against *Pst (avrPto)*. In addition, as discussed by Newman *et al.* (37), the localization of p-CN to the apoplast could increase its effective concentration in the immediate bacterial environment, so that we do not exclude the possibility that p-CN contributes to resistance through its antimicrobial action. It is also possible that hydroxycinnamoyl

conjugates including p-CN and p-CO have further roles in plant-pathogen interactions that are as yet undefined but which could include activity as signal molecules.

The increased synthesis and accumulation of p-CN and p-CO in tomato during incompatible interactions with *Pst (avrPto)* is associated with elevated mRNA levels of the genes encoding PAL, 4CL, and THT at 4 h after bacterial inoculation. In compatible interactions, by contrast, the amount of *PAL* and *4CL* transcripts increased at a later time point (at 16 h after

TABLE I
Substrate specificity of the recombinant tomato THT enzymes in comparison with literature data for the tobacco and recombinant potato THT proteins

THT homolog	Substrate[a]	K_m
		μM
tomTHT1-3	Tyramine	4
	Dopamine	22
	Octopamine	12
	Noradrenaline	137
tomTHT7-1	Tyramine	4
	Dopamine	33
	Octopamine	290
	Noradrenaline	322
tomTHT7-8	Tyramine	60
	Dopamine	183
	Octopamine	345
	Noradrenaline	130
Potato THT[b]	Tyramine	40
	Dopamine	430
	Octopamine	30
	Noradrenaline	2110
Tobacco THT[b]	Tyramine	4
	Dopamine	202
	Octopamine	11
	Noradrenaline	1570

[a] With *p*-coumaroyl-CoA as acyl donor for tomato homologs and feruloyl-CoA for potato and tobacco enzymes.

[b] Data for tobacco and potato THT are taken from Ref. 20 and 23, respectively.

bacterial inoculation), although there was still an increase in *THT* mRNA levels at 4 hpi. The induction of *PAL* and *THT* seen in compatible interactions may be a response to the inoculation procedure, since inoculation with water produced very similar effects. Induction of *4CL*, however, was specific to bacterial interactions and was not induced by water. The induction of *THT* alone at 4 h in compatible interactions and after water inoculation was not sufficient to allow synthesis of *p*-CO and *p*-CN. Elevated *THT* mRNA level accompanies the elicitor-induced accumulation of *p*-CO, *p*-CT, FT, and FO in potato suspension culture (23). Similar differential induction of transcript levels of PAL and THT has been described in pepper in response to avirulent and virulent strains of *X. campestris* and is associated with differential synthesis of FT and *p*-CT (43).

Southern analysis indicated that, as in potato and tobacco, *THT* in tomato is encoded by a multigene family. Four different cDNAs were cloned. One of these cDNAs, *tomTHT1-4*, encoded a truncated inactive THT because of the presence of a premature stop codon. Examination of the tomato expressed sequence tag (EST) data base (available on the World Wide Web at tigrblast.tigr.org/tgi/) indicates the presence of two further highly homologous *THT* isoforms, defined by ESTs TC98928 and TC98925 (92 and 74% homology to *tomTHT1-3* on the DNA level). We did not detect these two homologs, most likely due to the design of the degenerate primers and subsequent preference of the other clones. The three cDNAs *tomTHT1-3*, *tomTHT7-1*, and *tomTHT7-8* encode proteins that are overall highly similar to each other but show divergence particularly in the N terminus and extreme C terminus. The tobacco THT isoforms defined through cDNA cloning are much less divergent (24); two predicted proteins are identical, and a third is altered in only two amino acid positions. It is not known whether these alterations give rise to alterations in substrate preference. The two tobacco cDNAs encoding proteins with identical amino acid sequence differ in their 3′- and 5′-untranslated region sequences, which may reflect differences in their regulation.

Using real time PCR we have shown differential expression of the genes encoding the tomato THT homologs during incompatible and compatible interactions with *Pst* (*avrPto*). The transcript analysis suggests that transcripts for all four homologs are present in unchallenged tomato leaves but that only *tomTHT1-3* seems to be highly expressed following bacterial challenge in whole leaf tissue. Furthermore, *tomTHT1-3* shows a more substantial and rapid induction in resistant compared with susceptible plants. These results reflect those of the Northern blot analysis, where all *THT* transcripts would be measured together. Differential expression of the tomato THT family members is also indicated by their EST distribution profile (available on the World Wide Web at tigrblast.tigr.org/tgi/). All of the *THT* gene family members show alterations in their expression patterns in different plant tissues (roots, shoots, or mature green fruit) under a variety of developmental stages or in leaves under elicitation conditions. Transcripts for *tomTHT1-3* (EST TC98926) are the most abundant *THT* gene transcripts among ESTs derived from bacterially challenged leaf tissue as well as callus tissue. The expression level is comparable with "housekeeping" genes such as those encoding α-tubulin or glyceraldehyde-3-phosphate dehydrogenase.

By comparison with mammalian spermine/spermidine *N*-acetyl transferases (SSATs), Schmidt *et al.* (23) have proposed that the amino acid sequence RKLGMGS in potato THT (residues 176–182) is responsible for acyl-CoA binding and that the residues underlined within this sequence are essential for catalytic activity. The three active tomato THTs have the amino acid sequence RKLGMG(S/K) at the homologous position, with conservation of all three essential residues. Tyramine is the preferred substrate for all five THT proteins discussed. However, all three tomato isoforms have a lower K_m for noradrenaline than either the potato and tobacco enzymes (measured with feruloyl-CoA as acyl donor) (20, 23). Other differences between the tomato isoforms and potato and tobacco enzymes are seen in their relative affinities for dopamine and octopamine. For THT1-3, the affinity for octopamine is higher than for dopamine. For THT7-1 and THT7-8, the affinity for octopamine is lower than for dopamine, whereas for the tobacco and potato enzymes the reverse is true and the affinity for dopamine is at least 10 times lower than for octopamine. In addition, THT7-8 seems to have apart from tyramine the highest affinity for noradrenaline.

It is evident from Fig. 2*A* that although *p*-CO and *p*-CN synthesis is induced in tomato by fungal elicitor, bacterial inoculation, and fenitrothion treatment, the relative levels of the two products differ in each case. Other hydroxycinnamic amides such as *p*-CT were also detected at lower concentrations; however, no reproducibly significant difference between resistant and susceptible responses were observed. The pattern of the hydroxycinnamoyl conjugates seen will depend on the relative level and substrate specificity of the THT isoforms present in the tissue but presumably more importantly on the availability of the different acceptors and of the acyl donors. Differential induction of THT isoforms with different specificity in tomato represents one possible mechanism to alter the pattern of products in response to different stimuli and perhaps also in plant development. The mechanisms that determine the relative availability of tyramine and its hydroxylation derivatives octopamine and noradrenaline under different elicitation conditions are not known. The work described here provides correlative evidence for a role for *p*-CN and *p*-CO in tomato resistance against microbial attack. Future studies involving overexpression and gene silencing of the THT family members will increase our insight into the interrelation of the biosyn-

thesis of the phenolic amine conjugates and the outcome of plant-microbe interactions.

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