**p-Coumaroylnoradrenaline, a novel plant metabolite implicated in tomato defense against pathogens**

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Running title: THT gene family and plant defense in tomato
Abstract

The Avr9 peptide elicitor from the fungus *Cladosporium fulvum*, the bacterial pathogen *Pseudomonas syringae* pathovar *tomato* carrying the avirulence gene *avrPto* (*Pst (avr Pto)*) 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 (*p*-CO) and *p*-coumaroylnoradrenaline (*p*-CN), 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*-CO and *p*-CN were accompanied by elevated mRNA levels of genes encoding phenylalanine ammonia lyase, *p*-coumarate CoA ligase and hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyltransferase (THT), enzymes which are involved in the hydroxycinnamic acid amide biosynthesis. Southern hybridisation indicated that THT is encoded by a multigene family in tomato. Four different THT full-length cDNAs were derived by RT-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 (avr Pto)*. Furthermore *tomTHT1*-*3* showed a more substantial and rapid induction in the incompatible interaction than in the compatible interaction. The cDNAs *tomTHT 1-3, 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.
Introduction

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 (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 (4,5)). 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 near-isogenic tomato lines which 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* which carry the avirulence gene *avrPto*. (*Pst (avrPto)*) (9). These strains cause a programmed cell death reaction, the hypersensitive response (HR), 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 necrotic reaction that resembles the HR, whereas fen plants show only sporadic appearance of necrotic spots (10,11). Both the Fen and Pto signalling pathways to cell death require the Prf gene (12); mutant lines carrying non-functional 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 which 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 as well as 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, catalysed by the enzyme hydroxycinnamoyl-CoA:tyramine N-(hydroxycinnamoyl)transferase (THT; E.C.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. Firstly we have examined the transcriptional activity of several genes (PAL, 4CL and THT) whose products are implicated in p-CO and p-CN biosynthesis. Secondly we have used the sequences of the potato and tobacco THTs 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 (Cf0) and two near-isogenic lines homozygous for the resistance genes *Cf*-9 (*Cf9*) and *Cf*-2 (*Cf2*) were used. Plants were grown either to the sixth leaf stage (six weeks) or to the cotyledon stage (three weeks) in a growth room in Levington’s M3 compost (Levington Horticulture Ltd., Fisons, Ipswich, UK) with a photoperiod of sixteen hours (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 near-isogenic line 76S (*pto* and *fen*) were grown to the sixth leaf stage. In addition the near 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, non-infected 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.

*Pseudomonas syringae* pathovar tomato carrying the *avrPto* gene (*Pst (avrPto)*,(27)) and *Pst (avrPto) Δhrp (K-R)* (28) were grown overnight in King’s B broth (29) containing the respective antibiotics. After centrifugation the bacteria were suspended in 10mM MgCl₂ to the
appropriate concentration and infiltrated in the leaves as described above. For the chemical
elicititation, the fenthion analog, fenitrothion was used. The insecticide is available in the UK
under the product name Dicofen (kindly provided by PBI Agrochemicals Limited, Waltham
Abbey, UK). Mature tomato leaflets were immersed for 30 seconds 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 Scientific Inc., Fair Lawn, NJ., USA). 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 freeze dried plant material was extracted with 70% methanol and
analysed via reverse phase HPLC using a Prodigy 5 ODS-2 column (25 cm x 4.6mm;
Phenomenex Ltd, Macclesfield, U.K.). The following binary gradient, with a flow rate of 1ml
min⁻¹, was applied for elution: t=0min : 10%A, t = 20 min: 16% A; t = 40 min: 50% A; t = 45
min: 75%A; t = 50min: 95%A; t=55min: 10%A). Solvent A was methanol/acetonitrile (1:1
v/v), solvent B was 10 mM ammonium formate pH3. Absorbencies were measured with a
diode array scanning UV detector (Gilson, Middleton, WI, USA). Quantification was
accomplished using the standards derived from the enzyme assays (see below THT enzyme
assay). The molecular weight (Mr) and structures of Peak 6 and 8 were determined by liquid
chromatography/electrospray ionisation 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, 25mM MgSO₄ and rifampicin (50 µg per ml) (31).
The bacterial concentrations were adjusted to 1x10⁴ colony forming units (cfu) per ml by
dilution into new broth. Samples of $p$-CN and $p$-CO derived by HPLC fractionation were
lyophilised and re-dissolved in 35% MeOH to give a stock solution of 1mM. 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, lyophilised and re-dissolved 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 24h. 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 (*Lycopersicon esculentum* cv. Moneymaker), potato (*Solanum tuberosum* cv.
Hunkel), tobacco (*Nicotiana clevelandii*) and barley (*Hordeum vulgare* cv. Franka) was
isolated (described by (32,33)) and digested with the restriction enzyme *EcoRI*. Samples (0.7
µg, 1.4µg, 1.4 µg, 1.4 µg and 2.8µg, respectively) were separated by electrophoresis on a 1%
agarose-gel and transferred to a positively charged nylon membrane (Roche Applied Science).
The non-radioactive hybridisation was performed by using a DIG-labelled potato THT cDNA
(23,34). The blots were hybridised at 36°C and washed in 1x 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, Molecular Biochemicals). For Northern blots, RNA samples (10 µg of total RNA)
were separated on formaldehyde-agarose gels and transferred to Hybond-N nylon membranes
(Nycomed Amersham, Little Chalfont, Buckinghamshire, UK), according to manufacturer´s
protocol. The integrity of the RNA was assessed by visualization of ribosomal RNA by
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’-GAAGTTTCICCAACCC-3’;
t3r: 5’-CCATTITTTGCAGCAATAGAIGCAAC-3’;
t4r 5’-CCATACCTAAAITCATCAAAAATTTCAACTCCC-3’.

RT-PCR (Titan™ One Tube RT-PCR System; Roche, Molecular Biochemicals) 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 hpi. The PCR profile was designed according to the manufacturer’s instructions with slight alterations (incubation temperature: 55°C; denaturation time: 10 sec; 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 Pharmacia Biotech). The starting material consisted of a pool of total RNA samples of various time points after bacterial infection. The 5’ and 3’RACE 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 on request). Finally, full length clones were amplified using primers containing BamHI (5’end) and HindIII (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’RACE nucleotide analysis. The sequences of the four cDNAs were analysed in silico by using the programs BlastP2.2.3...
and ProtParamtool [http://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 cDNA were synthesised 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 Laboratories, München, Germany) featuring a reaction mixture with Sybr Green as fluorescent dye and 1 µl template cDNA in dilutions of 1:5, 1:10, 1:20 and 1:50 (38); details for primer sequences and PCR profile on request. Amplicons were subjected to melting curve analysis (60°C-95°C, 0.5°C increments) and digested with HinfI, 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-Nos. X53043) was used as constitutive standard. The level of THT cDNAs were normalised 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 BamHI and HindIII and the insert DNA was ligated into the expression vector pQUE-30 (Quiagen, 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 10mM 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 synthesised essentially as described by Meng *et al* (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 lyophilised and its purity was checked by HPLC. For the THT assay, 50 µl THT enzyme solution (4 µg/µl) were mixed with 200 µl 10 mM Tris-HCl (pH 7.5), 10 µl p-coumaroyl-CoA (1 mM) and 5 µl tyramine, dopamine, octopamine and noradrenaline to a final concentration ranging from 0.01 mM to 0.5 mM. After 10 min at 25°C, the reaction was stopped by addition of 50 µl of 50% w/v TCA. 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 hours post inoculation (hpi) a greying necrosis could be seen in the incompatible interaction (Fig. 2A, *Cf9*) whereas no macroscopic symptoms could be detected for the *Cf-0* plants (Fig. 2A, *Cf0*). The production of soluble phenolics during these interactions was monitored by HPLC analysis of extracts from the infiltrated leaf areas. Out of thirteen detectable compounds only two (referred to as peak 6 and 8) showed a reliable alteration over three independent time course experiments (Fig. 2A and Fig. 3A). 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 values were approximately 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. 3A). The maximum level was approximately 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 24h 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 to the *Cf-9* interaction (peak ratio P8/P6 = ~3/1), although the maximum levels were approximately ten 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. 2B, *Pto*). Infiltration of the same inoculum in the near-isogenic-susceptible relative 76S, carrying the *pto* allele, resulted in water-soaked necrotic lesions (Fig. 2B, *pto*). Strong induction of Peak 6 and Peak 8 was only seen in the incompatible (HR) interaction with 76R (Fig. 2B). 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. 3B). Maximum levels of peak 8 were ten 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 peak 8 compounds (data not shown).

The cultivar 76R was also inoculated with the *Pst (avrPto) Δhrp* (K-R) mutant, which carries a deletion within the *hrp* gene cluster (28). Genes within this cluster encode components of a TypeIII 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. 3B). *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 to 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 to 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 48h the 76R plants developed dark, large, necrotic lesions covering the whole leaflet (Fig. 2C, Fen). In contrast, challenge of 76S and the mutant line prf3 derived from 76R resulted only in sporadic appearance of necrotic spots (Fig. 2C, fen). At the given time point the level of the peak 6 and peak 8 compounds were ten and three times higher in the Fen plants compared to 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 24h the whole leaf area showed dry, dark green, necrotic lesions, which were not accompanied by the induction of the phenolics in question (Fig. 2D). 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 characterisation of the peak 6 and 8 compounds**

Comparison of the absorbance spectra of peak 6 and 8 (λ<sub>max</sub> 289 and 305 nm) with the UV spectrum of the standard p-coumaroyltlyramine (Fig. 4B, 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-coumaroylnoradrenaline (p-CN) (Fig. 4A, C). This was indicated by a [M+H]<sup>+</sup> 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-H2O]<sup>+</sup> 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-H2O]<sup>+</sup>. 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 MS analysis of peak 8 revealed a 16 Da lower [M+H]<sup>+</sup> peak at m/z 300.2 indicating the presence of a compound with one hydroxy function less than p-coumaroylnoradrenaline. (Fig. 4D, 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-coumaroyloctopamine (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 RT values of Peak 6 and 8 with the retention times of synthesised products derived from the THT enzyme assays using noradrenaline and octopamine as substrates (see results section *Enzymatic properties of recombinant THT enzymes*) gave further evidence for the identity of p-CN and p-CO as the two compounds in question.

**Antimicrobial activity of p-CN against Pst (avr Pto)**

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 24h culture period. Control cultures
received either water or lyophilised HPLC buffer (blank control), which had a small but reproducible growth-promoting activity compared to 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 to the blank control. At 10 µM the effect was smaller and a two-fold reduction was observed. The inhibitory activity of \( p\)-CN appeared to be lower when calculated using the water control; 100µM \( p\)-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 catalysed by the enzyme THT. This protein is conjugating \( p\)-coumaroyl-CoA (synthesised through the action of PAL and 4CL) and a variety of amines for example tyramine, octopamine or noradrenaline. We tested for 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 hybridisation could be observed with the monocot plant barley, the four solanaceous species (potato, pepper, tobacco and tomato) showed multiple strong hybridisation 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 \( 4CL\) cDNA’s 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 RT-PCR followed by 5’-3’RACE. The degenerate primers were designed on the consensus of THT genes from tobacco (Accession No AJ005062) and potato (23). Four different tomato THT clones could be identified. The clones tomTHT1-3 and tomTHT1-4 (Accession-Nos. AY081905 and AY081908, respectively) could be amplified with the primer combination t1f/t3r. The predicted coding regions were 724 or 729 nt 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 nt, respectively (Accession-Nos. AY081906 and AY081907).

The predicted proteins encoded by the four cDNAs showed a high degree of amino acid sequence similarity (Fig. 7); tomTHT1-3, 7-1 and 7-8 all exhibited 93 % sequence similarity to each other, and all were 73% similar to tomTHT1-4. The major difference in tomTHT1-4 compared to the other predicted tomato proteins was in the amino acid stretch 98 to 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 *Solanum 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% / 76% respectively; the similarity between sequences of tobacco and tomTHT1-3 / 7-1 / 7-8 / 1-4 was 86% / 83% / 84% / 70% respectively. 

*In silico* analysis of tomTHTs 1-3, 7-8, 7-1 and 1-4 predicted a molecular weight 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 analysed 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 inoculation and were 63 fold higher at 4h after inoculation than in unchallenged plants. This induction was followed by a decline of transcript levels to approximately 50% of the maximum level at 16h pi (Fig. 8A). 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.8B). Furthermore the constitutive levels of tomTHT1-3 transcripts were 6 times lower in susceptible compared to resistant plants. The accumulation profiles of transcripts for *tomTHT* 7-8, 1-4, 7-1 were clearly different from that of *tomTHT1-3*. Although in unchallenged plants *tomTHT* 7-8, 1-4, 7-1 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 tomTHT 1-3, 7-8 and 7-1 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 (Table1). 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; 7-8) were all enzymatically active. All three exhibited highest affinity for tyramine although the K_m value of tomTHT7-8 was about ten-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 (13,14,37,42,43). 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 Phytophthora infestans (42) and with both the non-host and gene-for-gene determined resistance reactions of pepper to Xanthomonas campestris (37). Biosynthesis of FT is induced in tobacco by tobacco mosaic virus (TMV) infection (14). In tomato, potato and tobacco accumulation of FT occurs after wounding with a haemostat 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 (HCTs) 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
HCTs. Grandmaison et al showed that FT induced hyphal branching but reduced total growth of mycorhizal fungi (45). Newman et al demonstrated antibacterial activity of both FT and CT against *X. campestris* (37). In contrast McLusky et al failed to detect any antimicrobial activity of HCTs from onion induced by attempted *Botrytis* infection against *Botrytis* spp. (43). We have shown that *p*-CN has a relatively weak activity against *Pst (avrPto)*, (a ten-fold growth reduction at 100 µM; a two-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 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 (avr Pto)*. In addition as discussed by Newman et al the localisation 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 (37). 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 4h after bacterial inoculation. In compatible interactions by contrast, the amount of *PAL* and 4CL transcripts increased at a later time point (at 16h after bacterial inoculation), although there was still an increase in *THT* mRNA levels at 4h pi. 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 4h 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 \( X.\) \( c.\) ampestris and is associated with differential synthesis of FT and \( p\)-CT (46).

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 \( \text{tomTHT1-4} \) encoded a truncated inactive THT because of the presence of a premature stop codon.

Examination of the tomato EST database at http://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 \( \text{tomTHT1-3} \) 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 \( \text{tom THT1-3}, \text{tomTHT7-1} \) and \( \text{tomTHT7-8} \) 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 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’UTR 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 \( \text{tomTHT1-3} \) seems to be highly expressed following bacterial challenge in whole leaf tissue. Furthermore \( \text{tomTHT1-3} \) shows a more substantial and rapid induction in resistant compared to 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 (http://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 to “house-keeping” genes such as those encoding alpha-tubulin or glyceraldehyde-3-phosphate dehydrogenase.

By comparison with mammalian spermine/spermidine N-acetyl transferases (SSATs), Schmidt et al have proposed that the amino acid sequence RKLGMGS in potato THT (residues 176-182) is responsible for acyl CoA binding and that the residues shown in bold within this sequence are essential for catalytic activity (23). The three active tomato THTs have the amino acid sequence RKLGMGS/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 ten 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. 2A 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 biosynthesis of the phenolic amine conjugates and the outcome of plant–microbe interactions.

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**Figure legends**

**Fig. 1 Biosynthetic pathway of \( p \)-coumaroyltyramine**

Tyramine hydroxycinnamoyl transferase (THT) catalyses the synthesis of \( p \)coumaroyltyramine from the thioester \( p \)-coumaroyl-CoA and the amine tyramine. The enzymes phenylalanine ammonia lyase (PAL) and \( p \)-coumaroyl-CoA ligase (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).

**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 *Cladosporium fulvum* (IFAvr9) was inoculated into leaves of resistant (\( Cf9 \)) and susceptible (\( Cf0 \)) 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 near isogenic susceptible cultivar 76S (pto). C: Leaves of sensitive (76R, *Fen*) and insensitive (76S, *fen*) tomato cultivars were immersed for 30 seconds 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.

**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 (IF0Δ). Susceptible Cf-0 plants 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) Δ hrp (K-R) (Δ) at the concentration of 10^7
cfu/ml. Each data point represents the mean of two independently extracted samples each
comprising of a pool of 2 leaves.

**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, F)
together with UV-absorbance spectra (B, E) are shown.

**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 24h growth was determined by
plating out serial dilutions. Values given are the mean and standard deviation 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 per 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.

**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 grey.

**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 24hpi (black, dark grey, middle grey, light grey 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.
Table 1. 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 references 20 and 23 respectively.
Fig. 1

Phenylpropanoid pathway:

- Phenylalanine
- Cinnamate
- Tyrosine
- Tyramine
- p-Coumaroyl-CoA

Reactions:

- Phenylalanine + cinnamate → 4Cl + S-CoA
- Tyrosine + NH₂
- Tyramine + TDC

Result:

p-Coumaroyltarminate
Fig. 2

A. 20 hpi. with the Avr9 peptide

B. 16 hpi. with Pst avrPto

C. 48 hpi. with 1% fenitrothion

D. 24 h p. wounding with carborundum
Fig. 4

A) Peak 6

B) 

C) 

D) Peak 8

E) 

F) 

$p$-coumaroylnoradrenaline

$p$-coumaroyloctopamine
|                | Pto + Pst avrPto | pto + Pst avrPto | water |
|----------------|------------------|------------------|-------|
|                | 0 4 10 16 24     | 0 4 10 16 24     | 4 10 16 |
| PAL            |                 |                 |       |
| 4CI            |                 |                 |       |
| THT            |                 |                 |       |
| rRNA           |                 |                 |       |

**Fig. 6**

- **PAL**: Phenylalanine ammonia-lyase
- **4CI**: 4-Ci-3-hydroxyphenylalanine
- **THT**: Trans-3-hydroxytyrosine
- **rRNA**: Ribosomal RNA
Fig. 8

A

B

normalized fluorescence

THT1-3  THT7-8  THT1-4  THT7-1

THT1-3  THT7-8  THT1-4  THT7-1

normalized fluorescence
p-coumaroylnoradrenaline, a novel plant metabolite implicated in tomato defense against pathogens

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