TITLE

Transgenic tomato plants overexpressing tyramine *N*-hydroxycinnamoyltransferase exhibit elevated hydroxycinnamic acid amide levels and enhanced resistance to *Pseudomonas syringae*

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ABSTRACT

Hydroxycinnamic acid amides (HCAA) are secondary metabolites involved in plant development and defence which have been widely reported throughout the plant kingdom. These phenolics show antioxidant, antiviral, antibacterial and antifungal activities. Hydroxycinnamoyl-CoA: tyramine *N*-hydroxycinnamoyl transferase (THT) is the key enzyme in HCAA synthesis, and is induced in response to pathogen infection, wounding or elicitor treatments, preceding HCAA accumulation. We have engineered transgenic tomato plants overexpressing tomato *THT*. These plants displayed an enhanced *THT* gene expression in leaves as compared to wild type plants. Consequently, leaves of *THT*-overexpressing plants showed a higher constitutive accumulation of the amide coumaroyltyramine (CT). Similar results were found in flowers and fruits. Moreover, feruloyltyramine (FT) also accumulated in these tissues, being present at higher levels in transgenic plants. Accumulation of CT, FT and octopamine and noradrenaline HCAA in response to *Pseudomonas syringae* pv. *tomato* infection was higher in transgenic plants than in the wild type plants. Transgenic plants

showed an enhanced resistance to the bacterial infection. In addition, this HCAA accumulation was accompanied by an increase in salicylic acid levels and pathogenesis-related gene induction. Taken together, these results suggest that HCAA may play an important role in the defence of tomato plants against *P. syringae* infection.

KEYWORDS

Tyramine *N*-hydroxycinnamoyltransferase (THT), Hydroxycinnamic acid amides (HCAA), tomato, *Pseudomonas syringae*, defence, salicylic acid (SA)

INTRODUCTION

Hydroxycinnamic acid amides (HCAA) are low molecular weight phenolic compounds which have been found in a wide range of plant families (Martin-Tanguy et al., 1978). They are formed by the condensation of hydroxycinnamic acids, such as *p*-coumaric (4-hydroxycinnamic acid) and ferulic (4-hydroxy-3-methoxycinnamic acid) acids, with different amines. Among these, polyamines such as putrescine and spermidine, or the β-phenylethylamine-alkaloids tyramine (2-[4-hydroxyphenyl] ethylamine) and octopamine (2-hydroxy-2-[4-hydroxyphenyl] ethylamine), have been described to form part of HCAA (Strack, 1997; Facchini et al., 2002). The presence of HCAA has been reported throughout the plant kingdom, usually as main phenolic constituents of flowers, seeds, and pollen grains (Bottcher et al., 2008; Luo et al., 2009; Handrick et al., 2010). These phenolic compounds, showing strong antioxidant and chemotherapeutic effects, are often considered as nutraceuticals (Zhang et al., 1997; Kawashima et al.,

1998; Nagatsu et al., 2000; Park and Schoene, 2002; Roh et al., 2004). Besides, they represent an important class of bioactive compounds with antiviral, antibacterial, antifungal and insecticidal activities (Grandmaison et al., 1993; Lee et al., 2004; Tebayashi et al., 2007).

HCAA play an important role in plant defence, and their synthesis is induced in response to various stresses, including pathogen infection, wounding or elicitor treatments in different plant tissues (Hahlbrock and Scheel, 1989; Keller et al., 1996; Pearce et al., 1998; Schmidt et al., 1998; Newman et al., 2001). They have been particularly described in solanaceous plants (Clarke, 1982; Keller et al., 1996; Negrel and Javelle, 1997). To this respect, tomato plants challenged with the bacterial pathogen *Pseudomonas syringae* pv. t*omato* have been reported to synthesize *p*-coumaroyloctopamine and *p*-coumaroylnoradrenaline in response to infection (von Roepenack-Lahaye et al., 2003).

Two major roles have been proposed for HCAA in plant defence. On the one hand, they can be incorporated into the plant cell wall in order to strengthen it against microbial degradation. Some studies have reported that HCAA of tyramine are synthesized in the cytosol and transported into the cell wall through a peroxidase-mediated process, in response to wounding or pathogen challenge (Clarke, 1982; Negrel and Lherminier, 1987; Hagel and Facchini, 2005). The accumulation of HCAA together with other cell wall-bound phenolics creates a barrier against pathogens, by reducing the digestibility of the cell wall and/or by directly inhibiting the growth of fungal hyphae (Grandmaison et al., 1993).

On the other hand, HCAA can act directly as antimicrobial agents. For instance, coumaroyltyramine (CT) and feruloyltyramine (FT), which accumulate in pepper plants

infected with the bacterial pathogen *Xanthomonas campestris*, display antibacterial activity *in vitro* (Newman et al., 2001). In addition, FT isolated from Allium roots has been described to present antifungal activity (Fattorusso et al., 1999). Moreover, it has been found that inoculation of tomato cv. Rutgers with *Pseudomonas syringae* pv. *tomato* led to the accumulation of CT and FT as well as dopamine HCAA (coumaroyldopamine and feruloyldopamine) upon bacterial infection. Interestingly, the HCAA of dopamine showed a notable bactericidal action. This HCAA accumulation was accompanied by a rapid and sharp induction of salicylic acid (SA) (Zacarés et al., 2007). This compound has been described as an important intermediary signal in the activation of certain plant defence responses to biotic and abiotic stress agents (Delaney et al., 1994). The accumulation of SA in the plant induces the synthesis of pathogenesis related (PR) proteins, such as PR-1 (Tornero et al., 1997; van Loon et al., 2006).

Hydroxycinnamoyl-CoA:tyramine *N*-hydroxycinnamoyl transferase (THT; EC 2.3.1.110) is the key regulatory enzyme responsible for the synthesis of HCAA (Negrel and Martin, 1984; Negrel and Javelle, 1997; Schmidt et al., 1999; Facchini et al., 2002) and is induced in response to elicitor treatment, wounding and pathogens (Villegas and Brodelius, 1990; Negrel et al., 1993; Schmidt et al., 1998). This suggests a general role for THT in plant defence responses. The enzyme was first isolated from tobacco (Negrel and Martin, 1984), and has been purified from other species such as potato, tobacco, and opium poppy. In order to establish the specificity for different acceptors, purified enzymes have been characterized, and wide substrate specificity has been reported for all of them (Hohlfeld et al., 1996; Negrel and Javelle, 1997; Yu and Facchini, 1999). It is generally assumed that THT displays the highest affinity to tyramine, followed by octopamine and dopamine. In tomato, THT is encoded by a multigene family. Four

different tomato *THT* isoforms have been identified, being *tomTHT1-3* highly expressed upon *Pseudomonas syringae* pv. *tomato* infection (von Roepenack-Lahaye et al., 2003).

In order to study the role of THT in HCAA biosynthesis, transgenic plants overexpressing the *THT* gene had been engineered. In this regard, transgenic tobacco lines, with an increase in THT activity, showed high levels of CT and FT in leaves in response to wounding (Hagel and Facchini, 2005). Besides, overexpression of the pepper *THT* gene in rice increased HCAA content upon exogenous tyramine application (Lee et al., 2007). These authors proposed that the absence of HCAA accumulation in healthy or untreated transgenic plants may be due to possible amine substrate limitation. Therefore, it could be interesting to transform species presenting higher basal levels of amines. That is the case of tomato, which contains constitutively elevated levels of tyramine and serotonin (Feldman and Lee, 1985; Kang et al., 2009).

Thus, in the present study we have generated transgenic tomato plants (*Solanum lycopersicum*) overexpressing the tomato *THT1-3* isoform. We have extensively characterized these plants by analyzing the accumulation of HCAA in the different tissues and studying their response to infection by *Pseudomonas syringae* pv. *tomato.*

RESULTS

Generation and selection of transgenic tomato plants overexpressing *THT*

To study the biological function of the HCAA accumulation, transgenic tomato plants expressing the *tomTHT1-3* gene under the control of the constitutive double cauliflower mosaic virus (CaMV) 35S promoter were generated. To this purpose, wild-type tomato

(*S. lycopersicum* cv. Moneymaker) plants were transformed with the binary vector pBI-THT via *Agrobacterium* (see Materials and Methods). Different independent transgenic lines were regenerated *in vitro* in the presence of kanamycin. Plants from all these lines were first assayed for GUS activity, and then tested by RT-PCR for neomycin phosphotransferase II (*Npt II*) and *THT* gene expression. Lines 3, 4 and 7 displayed the highest levels of *THT* mRNA, presented positive GUS activity and all of them accumulated *Npt II* mRNA. These lines were selected for further studies.

THT **gene expression and HCAA accumulation in transgenic tomato leaves**

Transgenic lines 3, 4 and 7 were analyzed to accurately obtain the level of *THT* mRNA accumulation. For this purpose, tomato leaves from both THT transgenic lines and wild type plants were used to perform quantitative RT-PCR, using specific primers for the tomato *THT* gene. As Figure 1A shows, all the transgenic lines analyzed presented higher constitutive *THT* gene expression when compared to the parental plants. The *THT* gene expression levels largely varied among the different transgenic lines, showing line 4 the lowest levels of *THT* mRNA accumulation, and line 7 the highest ones. In fact, *THT* gene expression in line 7 was approximately 12-fold higher by comparison with that observed in wild type plants.

In order to study the correlation between *THT* gene expression and HCAA accumulation in the different lines, leaves from control and THT transgenic tomato plants were subjected to UPLC-PDA-Micromass Q-TOF analysis to measure the HCAA content (see Materials and Methods). Previous studies reported that HCAA of tyramine accumulated to high levels in tomato in response to various stresses, such as wounding or pathogen infection (Pearce et al., 1998; Zacarés et al., 2007). Among them, we have detected the constitutive accumulation of amides formed by tyramine with ferulic or

158 coumaric acids in non-infected transgenic tomato plants. Although FT was barely
159 detectable (data not shown), all the transgenic lines presented CT contents higher than
160 those found in wild type plants (Figure 1B). These CT accumulation levels correlated
161 well with the observed *THT* mRNA levels (Figure 1A). Line 7 presented the highest
162 levels of CT accumulation, containing up to 10 times the content found in wild type
163 plants. Statistical analysis showed that the differences observed in the three lines with
164 respect to wild type plants were statistically significant, exhibiting lines 3 and 7 the
165 highest levels. Therefore these two independent lines were selected for further assays.

166

167 ***THT* gene expression and HCAA accumulation in flowers of transgenic tomato**
168 **plants**

169 To better characterize the overexpressing transgenic plants, we used quantitative RT-
170 PCR to analyze the *THT* mRNA accumulation in flowers at the anthesis stage for
171 transgenic tomato and wild type plants. As Figure 2A shows, both transgenic lines
172 constitutively expressed elevated *THT* mRNA levels. In line 7, *THT* mRNA levels
173 resulted to be around 3-fold higher than those observed in wild type flowers. On the
174 other hand, to determine whether the *THT* expression levels in flowers were correlated
175 with HCAA accumulation, flowers at the anthesis stage from wild type and transgenic
176 tomato plants were subjected to UPLC-PDA-Micromass Q-TOF analysis (see Materials
177 and Methods). Unlike what was found in leaves, accumulation of both CT and FT was
178 detected in tomato flowers. As shown in Figure 2B, the level of CT was quite similar in
179 transgenic and wild type flowers, showing no statistical differences. However, the
180 accumulation of FT was significantly higher in both transgenic lines as compared to WT

plants. Line 7 presented FT levels up to 4-fold higher than the levels detected in WT flowers.

THT gene expression and HCAA accumulation in fruits of transgenic tomato

In a similar manner as previously described for leaves and flowers, we tested both constitutive *THT* gene expression and HCAA accumulation in WT and transgenic tomato fruits. First, mature (red) fruits from transgenic tomato lines and wild type plants were analyzed by quantitative RT-PCR using specific primers for the tomato *THT* gene expression. As observed in leaves and flowers, the level of *THT* gene expression in transgenic fruits was higher (approximately 6-fold in line 7) than that observed in the wild type fruits (Figure 3A).

HCAA levels were also examined in wild type and transgenic tomato fruits. For this analysis, mature fruits from transgenic tomato and wild type plants were collected and processed as described in Materials and Methods. Then, samples were subjected to UPLC-PDA-Micromass Q-TOF analysis to measure the content of HCAA. Similarly to what we have observed in flowers, levels of CT and FT were higher in transgenic plants than in the wild type (Figure 3B). While CT levels were significantly higher for both analyzed transgenic lines, differences in FT levels were only significant for line 7. In this line, both CT and FT levels were 4-fold higher than those observed in the wild type plants. The increase in HCAA content in fruits of THT transgenic tomato plants correlates with the high levels observed in flowers.

HCAA accumulation in *Pseudomonas syringae*-infected transgenic tomato plants

Previous studies have reported the induction of *THT 1-3* in tomato plants upon *P. syringae* infection, thus indicating a possible role of this gene in plant defence (von Roepenack-Lahaye et al., 2003). Moreover, it has been described that 'Rutgers' tomato leaves infected with *Pseudomonas syringae* pv. *tomato* showed a rapid *THT* activation, followed by HCAA accumulation (Zacarés et al., 2007; López-Gresa et al., 2011). In order to analyze the HCAA accumulation upon a virulent *P. syringae* infection, time-course studies were performed on WT and THT tomato plants. As described in Materials and Methods, infected and mock-inoculated leaves from WT and THT tomato plants were harvested at 0, 24 and 48 hours after *P. syringae* infiltration. Results of the time-course analysis for CT and FT accumulation in leaves from THT and WT tomato plants upon *P. syringae* challenge are presented in Figure 4. As a general overview, all plants displayed enhanced levels of CT and FT along the compatible infection, both amides increasing in a time-dependent manner. The absolute levels of these metabolites differed substantially depending on HCAA, the highest values corresponding to CT. Lines 3 and 7 displayed higher HCAA levels as compared to WT plants. Differences between line 3 and WT plants were statistically significant for CT levels at both 24 and 48 hpi, and for FT levels at 48 hpi. Line 7 presented significantly higher values of both CT and FT at 24 and 48 h after the bacterial inoculation. In this line, the accumulation of tyramine HCAA upon bacterial infection was approximately 2-fold higher when compared to that observed in wild type plants. CT and FT levels showed a slight increase in the mock-inoculated ones, probably as a consequence of the wounding produced by the inoculation method.

We have also detected the accumulation of HCAA of octopamine and noradrenaline in the transgenic tomato plants infected with *P. syringae*. This is in agreement with our previous results (López-Gresa et al., 2011), where the same amides were found to accumulate in 'Rutgers' tomato leaves at 24 and 48 hours after bacterial infiltration. Figure 5 shows our results of coumaroyloctopamine (CO), feruloyloctopamine (FO), coumaroylnoradrenaline (CNA) and feruloylnoradrenaline (FNA) accumulation in leaves from THT and WT tomato plants 48 hours after *P. syringae* challenge. We observed that plants from line 3 exhibited statistical differences in the content of FO when compared to wild type plants. Differences were statistically significant for all the amides (CO, FO, CNA and FNA) in line 7. In these plants, the accumulation of these HCAA was approximately 3-fold higher upon *Pseudomonas syringae* infection when compared to that observed in wild type plants. In contrast with HCAA of tyramine, HCAA of octopamine and noradrenaline were not detected in mock-inoculated leaves during 48 hours experiments.

Resistance in *P. syringae*-infected transgenic tomato plants

To investigate the effect of *THT* overexpression and the consequent high accumulation of HCAA in plant defence, we studied the response of transgenic tomato plants to infection with the bacterial pathogen *P. syringae* pv. *tomato.* To this purpose, wild type and transgenic tomato plants were infiltrated with *P. syringae* and harvested at 24 and 48 hours after inoculation, in order to test bacterial growth (see Materials and Methods). Results of the time-course analysis corresponding to bacterial growth in leaves from transgenic (THT-3 and THT-7) and wild type (WT) tomato plants upon *P. syringae* challenge are presented in Figure 6. Bacterial growth was significantly reduced in both

transgenic lines with respect to the control plants at 48 hpi. For line 7, a 32% decrease was observed in the number of colony-forming units (CFU) with respect to wild type plants, at 48 hours after inoculation. Comparison of bacterial growth between lines 3 and 7 reveals a dosage effect, thus correlating resistance with HCAA accumulation (Figures 4 and 5). Concerning the symptoms, we observed the appearance of necrosis and chlorosis in the infected leaves, without clear macroscopic differences between WT and transgenic infected plants.

SA accumulation and *PR-1* gene induction in *P. syringae*-infected transgenic tomato plants

It is generally admitted that salicylic acid (SA) plays a central role in plant defence. (Métraux and Raskin, 1993; Dempsey et al., 1999). Previous studies reported that 'Rutgers' tomato plants infected with *P. syringae* produced a rapid and sharp increase of SA after bacterial inoculation (Bellés et al., 1999). In order to test whether the resistance induced by *THT* overexpression might be related to signalling, SA levels were followed in transgenic and wild type plants along the infection with *P. syringae*. Results of this time-course analysis are shown in Figure 7A. We observed no significant differences in SA levels between WT and transgenic lines at 24 hpi. Nevertheless, 48 h after inoculation both transgenic lines presented significantly higher levels of SA when compared with the levels detected in the WT plants. This is in accordance with the results shown here, where significant differences in bacterial growth occurred at 48 hpi between transgenic lines and WT. Among them, SA levels in line 7 were 3-fold higher in *P. syringae*-inoculated plants when compared to the corresponding wild type plants.

Since SA is a major regulator of pathogenesis-related (*PR*) protein gene induction, total RNA was isolated at different time points after the onset of bacterial infiltration and

274 subjected to quantitative RT-PCR analysis in order to determine the expression levels of
275 *PR-1* mRNA (Figure 7B). Correlating with the observed SA accumulation, the
276 expression of *PR-1* increased as a consequence of the infection, being higher in both
277 THT lines. Expression of *PR-1* gene was approximately 1.5-fold higher in line 7 when
278 compared to that observed in wild type plants at 48 hours after the inoculation, being
279 this difference statistically significant.

280

281 **DISCUSSION**

282 Tyramine *N*-hydroxycinnamoyltransferase (THT) is the key enzyme in HCAA
283 synthesis, catalyzing the condensation of hydroxycinnamic acids with different amines.
284 The resulting amides have been described as an important class of bioactive compounds
285 with antiviral, antibacterial, antifungal and insecticidal activities (Grandmaison et al.,
286 1993; Lee et al., 2004; Tebayashi et al., 2007). Besides, *THT* induction and the
287 consequent HCAA accumulation occurs upon pathogen infection or wounding, all this
288 pointing to their role in plant defence (Hahlbrock and Scheel, 1989; Grandmaison et al.,
289 1993; Keller et al., 1996; Pearce et al., 1998; Schmidt et al., 1998; Newman et al., 2001;
290 Lee et al., 2004; Tebayashi et al., 2007). To better understand the biological function of
291 this accumulation, in the present study we have generated and characterized transgenic
292 tomato plants overexpressing *THT*. In addition, we have studied their response to the
293 bacterial pathogen *P. syringae* pv. *tomato* as compared to the response displayed by the
294 WT plants.

295 Several overexpressing THT transgenic tomato lines have been generated, showing an
296 abundant *THT* gene expression levels in the leaves. Specifically, the best line presented

297 a *THT* mRNA accumulation approximately 12-fold higher than the wild type levels.
298 This overexpression was directly coupled with a high HCAA accumulation. This line
299 showed the highest coumaroyltyramine (CT) accumulation as well, reaching levels 10-
300 fold higher than the observed in the wild type plants.

301 The accumulation of tyramine derivatives, such as CT and ferouloyltyramine (FT), has
302 been previously reported in transgenic *THT*-overexpressing plants obtained in different
303 species. For instance, transgenic tobacco plants overexpressing *THT* have been reported
304 to rapidly accumulate FT and CT in response to wounding (Hagel and Facchini, 2005).
305 In these plants, HCAA levels were higher in THT wounded leaves when compared to
306 those observed in the wild type plants. Unlike our results in tomato, no constitutive
307 HCAA accumulation was detected in transgenic THT tobacco leaves. Related to this,
308 transgenic rice lines overexpressing a *THT* gene from pepper have been obtained (Lee et
309 al., 2007). Similarly to what was observed in tobacco, CT and FT contents were barely
310 detectable when analyzing transgenic 10-day-old rice shoots. However, leaves of these
311 seedlings supplemented with tyramine produced five times more CT and FT relative to
312 those grown in its absence, showing the transgenic leaves the highest CT and FT
313 contents. The correlation between CT and FT levels and the presence of tyramine also
314 indicate a limited tyramine pool in rice plants.

315 Interestingly, we have observed that CT constitutively accumulated in leaves of
316 transgenic tomato plants overexpressing *THT*, indicating that tyramine content is high
317 enough in this species, as described by Ly and associates (2008), and does not play a
318 substrate rate-limiting role in HCAA synthesis. To this respect, basal levels of tyramine
319 of WT and THT transgenic tomato plants were measured and no clear differences were
320 observed (data not shown). Constitutive HCAA accumulation was also described in

transgenic tomato plants overexpressing SHT, an enzyme implicated in HCAA synthesis that accepts serotonin with the highest substrate affinity (Kang et al., 2009). These authors have described HCAA accumulation in transgenic SHT tomato plants upon wounding. We have also observed a significantly enhanced accumulation of CT in wounded THT transgenic tomato leaves when compared to the wild type, while FT levels remained practically unchanged (e-Xtra, Figure X1).

We further characterized the transgenic tomato plants by studying *THT* gene expression in flowers. Similarly to what was observed in the leaves, flowers showed an increase in *THT* mRNA accumulation with respect to the wild type plants. In contrast to what was observed in leaves, both amides CT and FT were detected in flowers. The level of CT was quite similar, whereas the accumulation of FT was 4-fold higher in flowers from transgenic line 7 compared to WT. In accordance with the results observed in leaves, the presence of high levels of tyramine HCAA in flowers was coupled with enhanced levels of *THT* expression. Interestingly, the levels of HCAA of tyramine (CT and FT) in flowers of this line were approximately 12-fold higher than the levels detected in the leaves.

Similarly to what we have observed, pepper flowers have been reported to accumulate high levels of HCAA. Besides, flowers resulted to be the organs with the highest levels of tyramine in pepper, thus indicating that HCAA levels correlated with those of the amine substrates (Kang and Back, 2006). In accordance with this, the higher levels of HCAA that we have observed in transgenic tomato flowers may be due to the basal content of tyramine present in these organs. This HCAA accumulation suggest an important function for these compounds, acting as antimicrobial defence against pathogens, reinforcing the cell wall during anthesis or controlling sexual organogenesis

345 (Martin-Tanguy et al., 1987; Negrel and Lherminier, 1987; Hahlbrock and Scheel,
346 1989; Liyama et al., 1994).

347 *THT* gene expression was also tested in mature (red) tomato fruits from transgenic
348 plants, resulting in approximately 6-fold higher levels in transgenic fruits when
349 comparing line 7 to wild type. This elevated *THT* expression was accompanied by a
350 higher FT and CT content. A high HCAA accumulation in fruits has been proposed to
351 contribute to the defensive response. In this respect, Kang and associates (2010) have
352 recently reported the accumulation of the HCAA caffeoylserotonin (CaS) in pepper
353 fruits challenged with the anthracnose fungus *Colletotrichum gloeosporoides*. These
354 authors suggested that CaS, which is induced by pathogen infection and exhibits
355 antifungal activity, plays a key role in pepper plants defence. In agreement with these
356 results, the overaccumulation of CT and FT found in THT tomato fruits may act as a
357 constitutive defence against possible infections. In fact, CT and FT have been long
358 implicated in plant defence against pathogen attack, and several studies showed that
359 their synthesis in plants is activated in response to infection by fungi, virus or bacteria.
360 For instance, potato plants infected with *Phytophthora infestans* have been described to
361 increase the synthesis of CT and FT (Keller et al., 1996), and FT biosynthesis was
362 induced in tobacco upon Tobacco mosaic virus infection (Negrel and Jeandet, 1987).

363 In order to study the response of *THT*-overexpressing plants to pathogen attack,
364 transgenic tomato plants were infected with the bacterial pathogen *Pseudomonas*
365 *syringae* pv. *tomato.* We observed that CT and FT accumulated in a time-dependent
366 manner along the infection, the highest values corresponding to CT. The accumulation
367 of CT and FT was up to 2-fold higher in *P. syringae*-inoculated transgenic plants from
368 line 7 compared to the corresponding infected wild type plants. In addition,

accumulation of other HCAA of octopamine (CO and FO) and noradrenaline (CNA and FNA) was also detected, with levels approximately 3-fold higher in infected transgenic tomato plants from line 7 as compared to wild type. Hence, THT transgenic tomato plants overaccumulated HCAA upon *P. syringae* infection.

We have previously reported the accumulation of HCAA of tyramine, octopamine and noradrenaline in tomato leaves after bacterial infiltration. Interestingly, feruloylnoradrenaline displays a very outstanding antioxidant activity, indicating that it may be a component of the tomato chemical defence response against pathogens (Zacarés et al., 2007; López-Gresa et al., 2011). Thus, supporting the idea that HCAA overaccumulation may lead to an enhanced resistance to pathogens.

To investigate this possibility, we studied the response of THT tomato plants to the bacterial pathogen *P. syringae* pv. *tomato.* It is worth noting that bacterial growth was significantly reduced in transgenic plants 48 hours after inoculation. To our knowledge, this is the first report indicating that transgenic plants overexpressing *THT* exhibit resistance to bacteria. Our results suggest that this enhanced resistance is most probably due to HCAA overaccumulation, Comparison of two independent transgenic lines reveals a dosage effect, thus reinforcing the idea that HCAA levels correlate with inhibition of bacterial growth. This is in accordance with the previously described relationship between HCAA accumulation and expression of resistance, since HCAA levels in pepper and tomato subjected to incompatible interactions resulted to be higher when compared with the corresponding compatible ones (Newman et al., 2001; von Roepenack-Lahaye et al., 2003). HCAA levels detected in our infected *THT* overexpressing lines are comparable, and even higher, that the concentration found in

392 the incompatible interactions described in tomato by von Roepenack-Lahaye and
393 associates (2003).

394 In *Arabidopsis thaliana*, AtACT catalyzes the last reaction in HCAA biosynthesis. In
395 response to *Alternaria brassicicola* challenge, *AtACT* transcripts rapidly increased and
396 HCAA accumulation of agmatine and putrescine was detected in rosette leaves (Muroi
397 et al., 2009). Interestingly, Arabidopsis *AtACT* mutant showed enhanced susceptibility
398 to the pathogenic fungus, indicating that HCAA accumulation is a part of the effective
399 defence mechanisms of *A. thaliana*. On the other hand, overexpression of *AtACT* in
400 transgenic torenia (*Torenia hybrida*) plants resulted in HCAA accumulation,
401 accompanied by an enhanced resistance against the necrotrophic fungus *Botrytis cinerea*
402 (Muroi et al., 2012). Our present results further support the relationship between these
403 amides and resistance.

404 SA plays a central role in plant defence signalling and it has been described to
405 accumulate in tomato plants upon challenging with *P. syringae* (Bellés et al., 1999;
406 Dempsey et al., 1999). Thus, levels of SA were measured in THT transgenic and wild
407 type plants infected with *P. syringae*. SA levels were approximately 3-fold higher in
408 THT plants from line 7. Considering that SA is a major regulator of pathogenesis-
409 related (*PR*) gene induction (van Loon et al., 2006), the expression level of *PR-1* was
410 also tested at different time-points after the onset of bacterial infiltration. We have
411 observed that levels of *PR-1* increased along the infection, being higher in THT plants
412 as compared to wild type plants. These results correlated well with the SA accumulation
413 levels observed in the *THT* overexpressing plants.

414 HCAA have been described to promote resistance by creating a barrier against
415 pathogens or directly acting as antimicrobial agents (Clarke, 1982; Negrel and Martin,

1984; Fattorusso et al., 1999; Newman et al., 2001). Our results show that the accumulation of these compounds is accompanied with the SA synthesis, leading to the activation of plant defence. It is increasingly admitted that phenolamides have to be regarded also as metabolic intermediates, rather than just final products (Bassard et al., 2010). Further experiments to test whether exogenous HCAA application could activate the plant defence system in order to prevent possible infections would result of particular interest.

To summarize, this study shows that transgenic tomato plants overexpressing *THT* display an enhanced *THT* gene expression levels in leaf tissue, coupled with a constitutive CT overaccumulation. The same was observed when analyzing transgenic flowers and fruits, and FT was also found to accumulate constitutively in these tissues. Besides, the accumulation of these two amides together with CO, FO, CNA and FNA, was higher in *Pseudomonas*-infected THT tomato plants when compared to wild type plants. This increase in HCAA content in the transgenic tomato plants was accompanied by increased SA levels and *PR* gene induction. Finally, THT transgenic tomato plants showed enhanced resistance against *P. syringae* infection, suggesting a defensive role for HCAA in this interaction. The present study highlights the value of these engineered transgenic plants as a tool to provide new evidences of the HCAA role in this and further plant-pathogen interactions.

MATERIALS AND METHODS

Vector construction and tomato transformation

438 The full-length cDNA of tyramine *N*-hydroxycinnamoyltransferase gene (*THT 1-3*)
439 (von Roepenack-Lahaye et al., 2003) was amplified by RT-PCR using total RNA
440 obtained from tomato leaves infected by the bacterial pathogen *Pseudomonas syringae*
441 pv. *tomato*. Primers used were 5'-
442 CCGGATCCTCTAGAATGGCTCCTGCTCTTGAACA-3' as the forward primer, and
443 5'-CCGGATCCCTAACAGCTCCCTTTCGCCGT-3' as the reverse primer, both
444 oligonucleotides containing the *Bam HI* restriction site. The resulting PCR product
445 (THT) was cloned into the pGEM-T Easy vector (Stratagene) and sequenced. The
446 generation of the binary vector for plant transformation was carried out in two steps.
447 First, the DNA band obtained by digestion of pGEM-THT with the restriction enzyme
448 *Bam HI* was cloned into a pBluescript modified vector containing a double region of the
449 35S Cauliflower Mosaic Virus and a *nos* termination sequence. Then, the
450 [2XCaMV35S::THT::nos] cassette was released by digestion with the restriction
451 enzyme *Hind III* and inserted into pBI121 vector. This vector carries the neomycin
452 phosphotransferase gene (*NPT II*) as a transgenic selectable marker. The final
453 construction (pBI-THT) was used to transform *Agrobacterium tumefaciens* LBA4404.
454 This *A. tumefaciens* was used then for co-culture with tomato (*Lycopersicon esculentum*
455 cv. Moneymaker) cotyledons. Explant preparation, selection and regeneration followed
456 the methods described by Ellul and associates (2003). Transformation frequencies were
457 around 15%. Transformants were selected in kanamycin-containing medium and
458 propagated in soil for obtaining T_1 seeds. Wild type tomato seeds used in all the studies
459 came from *in vitro* regenerated plants, which were obtained in parallel with the
460 transgenic plants.

461 **β-glucuronidase activity**

462 For the histochemical detection of β-glucuronidase activity, fresh tissue samples were
463 infiltrated under vacuum for 25 minutes in a solution containing 0.5 mg X-Gluc (5-
464 bromo-4-chloro-3-indolyl glucuronide) and 0.1% Triton X-100 in 50 mM phosphate
465 buffer at pH 7.2, and incubated at 37 °C for 10 to 16 hours. Tissue samples were then
466 destained with successive washes of 70% ethanol.

467 **Plant material and treatments**

468 Seeds from tomato (*Lycopersicum esculentum* cv. Moneymaker, *Tm2*^{2}, *pto*), and
469 transgenic tomato plants overexpressing the tomato tyramine *N*-
470 hydroxycinnamoyltransferase (*THT*) gene were used in this study. Tomato Moneymaker
471 seeds were kindly provided by Professor J. D. G. Jones (John Innes Centre, Norwich,
472 UK). The *Tm2*^{2} gene confers resistance to the Tomato Mosaic Virus (ToMV). These
473 plants lack the *Pto* resistance gene that recognizes the AvrPto effector from
474 *Pseudomonas syringae* pv *tomato* DC3000, therefore they are susceptible to this
475 pathogen.

476 Plants (one per pot) were grown under standard greenhouse conditions at a temperature
477 range from 20 ºC to 25 ºC, 16-h light/8-h dark photoperiod and a relative humidity of
478 50% to 70%, in 12-cm diameter pots containing a 1:1 mixture of peat (Biolan, Kauttua,
479 Findland) and vermiculite. The pots were subirrigated once a day with a nutrient
480 Hoagland solution (Naranjo et al., 2003, Bellés et al., 2006).

481 Three different types of tissue were analyzed for Moneymaker control and T_1 THT
482 transgenic tomato plants: leaves, flowers and fruits. For leaf tissues, the third and fourth
483 leaves from 4-week-old tomato plants at the five- to six-leaf stage were harvested.
484 Flowers were harvested from the flower stalk in the anthesis stage. Tomato fruits were

485 collected when ripe (red). Each fruit was washed and cut, and seeds and placentas were
486 removed, so that the tissue sample consisted of the fleshy part of the fruit (mesocarp and
487 endocarp).

488 Wounding treatments were performed with 4-week-old plants by crushing the third and
489 fourth compound leaves per plant with forceps, according to Lisón and associates
490 (2006). Samples were collected 24 hours after wounding.

491 Plant material was harvested in liquid nitrogen at the indicated time points, and
492 immediately reduced to a fine powder with mortar and pestle or stored frozen at -80 ºC.

493

494 **Bacterial inoculation and CFU determination**

495 The third and fourth leaves from 4-week-old tomato plants (Moneymaker *pto* control
496 and T_1 THT transgenic plants) were infected with a bacterial suspension of
497 *Pseudomonas syringae* pv. *tomato* DC3000 ΔavrPto (kindly provided by Dr. Selena
498 Giménez, Centro Nacional de Biotecnología, Madrid, Spain). There was not *AvrPto-Pto*
499 mediated gene-for-gene interaction; consequently, a compatible interaction was
500 established.

501 Bacteria were grown overnight at 28 ºC in 20-mL Petri dishes with King's B agar
502 medium supplemented with 100 µL rifampicin (50 mg/mL). Then bacterial colonies
503 were transferred to 15 mL King's B medium and grown overnight at 28 ºC. Bacteria
504 were pelleted by centrifugation and resuspended in 10 mM $MgCl_2$ to an optical density
505 of 0.1 at 600 nm. Dilution plating was used to determine the final inoculum
506 concentration, which averaged $1x10^5$ colony-forming units (CFU)/mL. One hundred µL

of this bacterial suspension were injected into the abaxial side of each leaflet with a 1-mL sterilized plastic syringe without needle, as described by Collinge and associates (1987). Equivalent control leaflets were mock-inoculated with a sterile solution of 10 mM $MgCl_2$.

For determinating *in planta* bacterial growth, three leaf disks (1 cm^2 each) from the bacteria-infiltrated leaves were excised from each plant, from a total of five plants per line and per time, at the appropriate time points (24 and 48 hours after inoculation, adapted from Coego and associates (2005). Then, the three leaf disks were macerated in 10 mM $MgCl_2$ in order to obtain the density of the bacterial populations, determined by plating serial dilutions and counting the CFU on King's B medium supplemented with rifampicin.

RNA extraction and quantitative RT-PCR analysis

Total RNA of tomato tissues was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. After extraction, RNA was further precipitated by adding one volume of LiCl 6 M, and then the pellet was washed with LiCl 3 M and dissolved in RNase-free water. Finally, 2 units of TURBO DNase (Ambion) were added per µL RNA to remove contaminating genomic DNA. Quantitative RT-PCR analysis was performed as previously described by Campos and associates (2014). One µg total RNA was used to obtain the corresponding cDNA target sequences using an $oligo(dT)_{18}$ primer and the PrimeScript RT reagent kit (Perfect Real Time, Takara) according to the manufacturer's protocol. Quantitative PCR was carried out in the presence of the double-stranded DNA-specific dye Power SYBR Green PCR Master Mix (Applied

530 Biosystems), and amplification was monitored in real time with the 7500 FAST Real-
531 Time PCR System (Life Technologies). A house-keeping gene transcript, *Elongation*
532 *Factor 1 alpha* (eEF1α), was used as endogenous reference. The PCR primers were
533 designed using the pcrEfficiency software (Mallona et al., 2011), and they are shown in
534 e-Xtra, Table X1.

535

536 **Extraction procedure and UPLC-PDA-Micromass Q-TOF analysis of HCAA**

537 Extraction of methanol-soluble HCAA from tomato leaves, flowers and fruits was done
538 according to López-Gresa and associates (2011). An aliquot of 0.5 g of frozen powdered
539 tissue from each sample was transferred to a mortar and homogenized with 1.5 mL of
540 methanol. The extraction mixture was vortexed for 1 min, then sonicated for 10 min and
541 centrifuged at 14,000 x g for 15 min. The pellet was resuspended in 0.5 mL of
542 methanol, and the same steps were repeated as above. Both supernatants (total volume 2
543 mL) were transferred to 5-mL glass tubes and dried under a flow of nitrogen at 40 ºC.
544 The residue was dissolved in 300 µL of methanol and filtered through 13 mm Nylon
545 0.45 µm Minispike filters (Waters). The solvent was evaporated and the residue
546 dissolved again in 200 µL of methanol. All steps of the extraction were performed under
547 dark conditions to avoid *cis/trans* light-induced isomerization of phenylpropanoid
548 double bonds (Muhlenbeck et al., 1996).

549 A 5 µL aliquot from the final 200 µL sample was analyzed by UPLC-MS using an
550 ACQUITY UPLC-PDA system coupled to a Q-ToF Micromass spectrometer (Waters).
551 Separation was performed on an Acquity BEH C18 column (2.1 mm × 150 mm i.d., 1.7
552 µm). The mobile phase consisted of formic acid:ultrapure water (1:1000 v/v phase A)

553 and formic acid:acetonitrile (1:1000 v/v; phase B). Gradient conditions were as follows:
554 95% to 90% A in 14 min, 90% to 80% A in 15 min, 80% to 65% A in 10 min, 65% to
555 57% A in 1 min, 57% to 0% A in 1 min, held at 100% B for 3 min, returned to 95% A
556 in 1 min, and equilibrated for 4 min before the next injection. The flow rate was 0.4
557 mL/min. The column and sample temperatures were kept at 40 ºC and 4 ºC,
558 respectively. UV spectra were acquired between 210 and 600 nm with a 1.2-nm
559 resolution and 20 points/s sampling rate. MS analysis was performed by electrospray
560 ionization in negative mode. The mass spectrometer was calibrated with sodium
561 formate (10 ng/μL in 90:10 propan-2-ol:water). Analysis conditions were as follows:
562 capillary voltage, 3.0 kV; cone voltage, 45 eV; desolvation temperature, 300 ºC; source
563 temperature, 120 ºC; cone gas flow, 50 L/h; desolvation gas flow, 500 L/h; collision
564 energy,5 eV. MS data were acquired in centroid mode in the mass-to-charge ratio scan
565 range 100 to 1500 with a scantime of 0.52 s and an interscantime of 0.1 s. Leu-
566 enkephalin was used as the lockmass using a LockSpray exact mass ionization source.
567 All data were acquired using Masslynx NT4.1 software (Waters Corp. Mildford, MA,
568 USA). HCAA were quantified using synthesized HCAA as standard. HCAA synthesis
569 was performed as described by Zacarés and associates (2007), and López-Gresa and
570 associates (2011).

571

572 **Extraction and HPLC analysis of SA**

573 Extraction of salicylic acid (SA) from tomato leaflets was done according to protocols
574 previously published (Bellés et al., 2008). Briefly, leaves (0.3 to 0.5 g fresh weight of
575 tissue) were ground with a pestle in a mortar using liquid nitrogen and then
576 homogenized in 1.5 mL of methanol. The extracts were vortexed vigorously, sonicated

for 10 min, and then centrifuged at 14,000 x *g* for 15 min. The pellet was resuspended in 0.5 mL of methanol, and the same steps were repeated as above. The supernatant (2 mL) was divided in two halves and dried under nitrogen at 40 °C using glass tubes of 5 mL. One half was dissolved in 1 mL of 0.1 M sodium acetate (pH 4.5), while the other half was dissolved in 1 mL of 0.1 M sodium acetate (pH 4.5) containing 10 units of almond β-glucosidase (EC 3.2.1.21,14.3 U/mg, Fluka, Buchs, Switzerland), for hydrolysis experiments. Samples were incubated at 37 °C for 3 h, and then perchloric acid (70%) was added to all of them until a final concentration of 5%, to stop the reaction. The supernatant was extracted with 2.5 mL of 1:1 cyclopentane/ethyl acetate using glass tubes of 5 mL, and the organic upper phase was collected and dried at 40 °C under a flow of nitrogen. The pellet was resuspended in 300 µl of methanol and filtered through 13 mm Nylon 0.45 µm Minispike filters (Waters).

Aliquots (30 µL) were injected with a Waters 717 autosampler into a reverse-phase Sun Fire 5-µm C18 (4.6 by 150 mm, Waters) column equilibrated in 1% acetic acid. Eluents were 1% acetic acid (eluent A) and methanol (eluent B). A linear gradient starting with 100% eluent A and 0% eluent B and ending with 0% eluent A and 100% eluent B was applied over 20 min at a flow rate of 1 mL/min. After washing the column with 100% methanol for 10 min, the initial conditions were applied again, and the column was allowed to equilibrate with 1% acetic acid for 10 min, with a total run time of 40 min. The oven temperature was 30 °C. Free and total SA (the sum of free SA and its conjugated glucoside, SAG) was detected with a 470 Waters fluorescence detector (λ excitation 313 nm; λ emission 405 nm). SA was quantified with the Waters Millennium[32] software using authentic SA as standard. Data were corrected for losses in the extraction procedure, and recovery of metabolites ranged between 50 and 80%.

601

602 **Statistical analysis**

603 Results shown in Figures 1 to 5, 7, and S1 correspond to means ± SE of three
604 independent plants from a representative experiment. For the statistical analysis, data
605 coming from three independent growth experiments were used, which corresponds to a
606 total of nine individuals (three from each experiment). For the bacterial growth analysis
607 shown in Figure 6, five individual plants per time were inoculated, and the
608 corresponding statistical analysis was performed for a total of fifteen individuals.

609 Comparison between WT and each transgenic line were performed by a *t*-test analysis,
610 using MS-Excel from Microsoft's Office Suite. A p-value < 0.05 was considered as
611 significant. The *p*-values coming from the *t*-test analysis are presented in e-Xtra Table
612 X2.

613

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621

AUTHOR CONTRIBUTIONS

The work presented here was carried out in collaboration between all authors. José Mª Bellés defined the research theme. Laura Campos, Laura Zacarés and Mª Pilar López-Gresa carried out the laboratory experiments. Purificación Lisón and Ismael Rodrigo contributed to the experimental design and the interpretation of the data. Laura Campos drafted the article. Mª Pilar López-Gresa, Purificación Lisón and Ismael Rodrigo participated in revising it critically for important intellectual content. José Mª Bellés and Vicente Conejero gave the final approval of the version to be published. Each author has participated sufficiently in the work to take public responsibility for the content.

LITERATURE CITED

Bassard, J.E., Ullmann, P., Bernier, F., and Werck-Reichhart, D. 2010. Phenolamides: Bridging polyamines to the phenolic metabolism. Phytochemistry 71:1808-1824.

Bellés, J.M., Garro, R., Fayos, J., Navarro, P., Primo, J., and Conejero, V. 1999. Gentisic acid as a pathogen-inducible signal, additional to salicylic acid for activation of plant defenses in tomato. Mol. Plant-Microbe Interact. 12:227-235.

Bellés, J.M., Garro, R., Pallás, V., Fayos, J., Rodrigo, I., and Conejero, V. 2006. Accumulation of gentisic acid as associated with systemic infections but not with the hypersensitive response in plant-pathogen interactions. Planta 223:500-511.

642 Bellés, J.M., López-Gresa, M.P., Fayos, J., Pallás, V., Rodrigo, I., and Conejero, V.
643 2008. Induction of cinnamate 4-hydroxylase and phenylpropanoids in virus-
644 infected cucumber and melon plants. Plant Sci. 174:524-533.

645 Bottcher, C., von Roepenack-Lahaye, E., Schmidt, J., Schmotz, C., Neumann, S.,
646 Scheel, D., and Clemens, S. 2008. Metabolome analysis of biosynthetic mutants
647 reveals a diversity of metabolic changes and allows identification of a large
648 number of new compounds in Arabidopsis. Plant Physiol 147:2107-2120.

649 Campos, L., Granell, P., Tárraga, S., López-Gresa, M.P., Conejero, V., Bellés, J.M.,
650 Rodrigo, I., and Lisón, P. 2014. Salicylic acid and gentisic acid induce RNA
651 silencing-related genes and plant resistance to RNA pathogens. Plant Physiol.
652 Biochem. 77:35-43.

653 Clarke, D.D. 1982. Active Defence Mechanisms in Plants. Pages 321-322 in: The
654 accumulation of cinnamic acid amides in the cell walls of potato tissue as an
655 early response to fungal attack. R.K.S, Wood ed. Plenum Press, New York.

656 Coego, A., Ramirez, V., Ellul, P., Mayda, E., and Vera, P. 2005. The H_2O_2-regulated
657 Ep5C gene encodes a peroxidase required for bacterial speck susceptibility in
658 tomato. Plant J 42:283-293.

659 Collinge, D.B., Milligan, D.E., Dow, J.M., Scofield, G., and Daniels, M.J. 1987. Gene
660 expression in *Brassica campestris* showing a hypersensitive response to the
661 incompatible pathogen *Xanthomonas campestris* pv *vitians*. Plant Mol. Biol.
662 8:405-414.

663 Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D.,
664 Gaffney, T., Gutrella, M., Kessmann, H., Ward, E., and Ryals, J. 1994. A central
665 role of salicylic acid in plant disease resistance. Science 266:1247-1250.

666 Dempsey, D.A., Shah, J., and Klessig, D.F. 1999. Salicylic acid and disease resistance
667 in plants. Crit. Rev. Plant Sci. 18:547-575.

668 Ellul, P., Garcia-Sogo, B., Pineda, B., Rios, G., Roig, L., and Moreno, V. 2003. The
669 ploidy level of transgenic plants in Agrobacterium-mediated transformation of
670 tomato cotyledons (*Lycopersicon esculentum Mill*.) is genotype and procedure
671 dependent Theor. Appl. Genet. 106:231-238.

672 Facchini, P.J., Hagel, J., and Zulak, K.G. 2002. Hydroxycinnamic acid amide
673 metabolism: physiology and biochemistry. Can. J. Bot. 80:577-589.

674 Fattorusso, E., Lanzotti, V., and Taglialatela-Scafati, O. 1999. Antifungal *N*-feruloyl
675 amides from roots of two *Allium species*. Plant Biosyst. 133:199–203.

676 Feldman, J.M., and Lee, E.M. 1985. Serotonin content of foods: effect on urinary
677 excretion of 5-hydroxyindoleacetic acid. Am. J. Clin. Nutr. 42:639-643.

678 Grandmaison, J., Olah, G.M., Vancalsteren, M.R., and Furlan, V. 1993.
679 Characterization and localization of plant phenolics likely involved in the
680 pathogen resistance expressed by endomycorrhizal roots. Mycorrhiza 3:155-164.

681 Hagel, J.M., and Facchini, P.J. 2005. Elevated tyrosine decarboxylase and tyramine
682 hydroxycinnamoyltransferase levels increase wound-induced tyramine-derived
683 hydroxycinnamic acid amide accumulation in transgenic tobacco leaves. Planta
684 221:904-914.

685 Hahlbrock, K., and Scheel, D. 1989. Physiology and molecular biology of
686 phenylpropanoid metabolism. Ann. Rev. Plant Physiol. Plant Mol. Biol. 40:347-
687 369.

688 Handrick, V., Vogt, T., and Frolov, A. 2010. Profiling of hydroxycinnamic acid amides
689 in Arabidopsis thaliana pollen by tandem mass spectrometry. Anal. Bioanal.
690 Chem. 398:2789-2801.

691 Hohlfeld, H., Scheel, D., and Strack, D. 1996. Purification of hydroxycinnamoyl-
692 CoA:tyramine hydroxycinnamoyltransferase from cell-suspension cultures of
693 *Solanum tuberosum* L. cv. Datura. Planta 199:166-168.

694 Kang, K., Lee, K., Ishihara, A., Park, S., Kim, Y.S., and Back, K. 2010. Induced
695 synthesis of caffeoylserotonin in pepper fruits upon infection by the anthracnose
696 fungus, *Colletotrichum gloeosporioides*. Sci. Hortic. 124:290-293.

697 Kang, K., Lee, K., Sohn, S.O., Park, S., Lee, S., Kim, S.Y., Kim, Y.S., and Back, K.
698 2009. Ectopic expression of serotonin *N*-hydroxycinnamoyltransferase and
699 differential production of phenylpropanoid amides in transgenic tomato tissues.
700 Sci. Hortic. 120:504-510.

701 Kang, S., and Back, K. 2006. Enriched production of *N*-hydroxycinnamic acid amides
702 and biogenic amines in pepper (*Capsicum annuum*) flowers. Sci. Hortic.
703 108:337-341.

704 Kawashima, S., Hayashi, M., Takii, T., Kimura, H., Zhang, H.L., Nagatsu, A.,
705 Sakakibara, J., Murata, K., Oomoto, Y., and Onozaki , K. 1998. Serotonin
706 derivative, *N*-(*p*-coumaroyl) serotonin, inhibits the production of TNF-α, IL-1α,

707 IL-1β, and IL-6 by endotoxin-stimulated human blood monocytes. J. Interferon
708 Cytokine Res. 18:423–428.

709 Keller, H., Hohlfeld, H., Wray, V., Hahlbrock, K., Scheel, D., and Strack, D. 1996.
710 Changes in the accumulation of soluble and cell wall-bound phenolics in
711 elicitor-treated cell suspension cultures and fungus-infected leaves of *Solanum*
712 *tuberosum*. Phytochemistry 42:389-396.

713 Lee, D.E., Kang, K., Lee, S.G., and Back, K. 2007. Enhanced synthesis of
714 feruloyltyramine and 4-coumaroyltyramine is associated with tyramine
715 availability in transgenic rice expressing pepper tyramine *N*-
716 hydroxycinnamoyltransferase. Plant Sci. 172:57-63.

717 Lee, D.G., Park, Y., Kim, M.R., Jung, H.J., Seu, Y.B., Hahm, K.S., and Woo, E.R.
718 2004. Antifungal effects of phenolic amides isolated from the root bark of
719 *Lycium chinense*. Biotechnol. Lett. 26:1125-1130.

720 Lisón, P., Rodrigo, I., and Conejero, V. 2006. A novel function for the cathepsin D
721 inhibitor in tomato. Plant Physiol. 142:1329-1339.

722 Liyama, K., Lam, T.B.T., and Stone, B.A. 1994. Covalent cross links in the cell wall.
723 Plant Physiol. 104:315-320.

724 López-Gresa, M.P., Torres, C., Campos, L., Lison, P., Rodrigo, I., Bellés, J.M., and
725 Conejero, V. 2011. Identification of defence metabolites in tomato plants
726 infected by the bacterial pathogen *Pseudomonas syringae*. Environ. Exp. Bot.
727 74:216-228.

728 Luo, J., Fuell, C., Parr, A., Hill, L., Bailey, P., Elliott, K., Fairhurst, S.A., Martin, C.,
729 and Michael, A.J. 2009. A novel polyamine acyltransferase responsible for the
730 accumulation of spermidine conjugates in Arabidopsis seed. Plant Cell 21:318-
731 333.

732 Ly, D., Kang, K., Choi, J.Y., Ishihara, A., Back, K., and Lee, S.G. 2008. HPLC analysis
733 of serotonin, tryptamine, tyramine, and the hydroxycinnamic acid amides of
734 serotonin and tyramine in food vegetables. J. Med. Food 11:385-389.

735 Mallona, I., Weiss, J., and Marcos, E.C. 2011. pcrEfficiency: a Web tool for PCR
736 amplification efficiency prediction. BMC Bioinformatics 12: 404.

737 Martin-Tanguy, J., Cabanne, F., Perdrizet, E., and Martin, C. 1978. Distribution of
738 hydroxycinnamic acid-amides in flowering plants. Phytochemistry 17:1927-
739 1928.

740 Martin-Tanguy, J., Negrel, J., Paynot, M., and Martin, C. 1987. Hydroxycinnamic acid
741 amides, hypersensitivity, flowering and sexual organogenesis in plants., New
742 York.

743 Métraux, J.P., and Raskin, I. 1993. Role of phenolics in plant disease resistance. Pages
744 191-209 in: Biotechnology in Plant Disease Control. Wiley-Liss, New York.

745 Muhlenbeck, U., Kortenbusch, A., and Barz, W. 1996. Formation of
746 hydroxycinnamoylamides and α-hydroxyacetovanillone in cell cultures of
747 *Solanum khasianum*. Phytochemistry 42:1573-1579.

748 Muroi, A., Ishihara, A., Tanaka, C., Ishizuka, A., Takabayashi, J., Miyoshi, H., and
749 Nishioka, T. 2009. Accumulation of hydroxycinnamic acid amides induced by

pathogen infection and identification of agmatine coumaroyltransferase in *Arabidopsis thaliana*. Planta 230:517-527.

Muroi, A., Matsui, K., Shimoda, T., Kihara, H., Ozawa, R., Ishihara, A., Nishihara, M., and Arimura, G. 2012. Acquired immunity of transgenic torenia plants overexpressing agmatine coumaroyltransferase to pathogens and herbivore pests. Sci. Rep. 2: 689.

Nagatsu, A., Zhang, H.L., Mizukami, H., Okuyama, H., Sakakibara, J., Tokuda, H., and Nishino, H. 2000. Tyrosinase inhibitory and anti-tumor promoting activities of compounds isolated from safflower (*Carthamus tinctorius L.*) and cotton (*Gossypium hirsutum L.*) oil cakes. Nat. Prod. Lett. 14:153-158.

Naranjo, M.A., Romero, C., Bellés, J.M., Montesinos, C., Vicente, O., Serrano, R. 2003. Lithium treatment induces a hypersensitive-like response in tobacco. Panta. 217: 417-424.

Negrel, J., and Martin, C. 1984. The biosynthesis of feruloyltyramine in *Nicotiana tabacum*. Phytochemistry 23:2797-2801.

Negrel, J., and Jeandet, P. 1987. Metabolism of tyramine and feruloyltyramine in TMV inoculated leaves of *Nicotiana tabacum*. Phytochemistry 26:2185-2190.

Negrel, J., and Lherminier, J. 1987. Peroxidase mediated integration of tyramine into xylem cell-walls of tobacco-leaves. Planta 172:494-501.

Negrel, J., and Javelle, F. 1997. Purification, characterization and partial amino acid sequencing of hydroxycinnamoyl-CoA: tyramine *N*- (hydroxycinnamoyl)

771 transferase from tobacco cell-suspension cultures. Eur. J. Biochem. 247:1127-
772 1135.

773 Negrel, J., Javelle, F., and Paynot, M. 1993. Wound induced tyramine
774 hydroxycinnamoyl transferase in potato *(Solanum tuberosum)* tuber disks. J
775 Plant Physiol. 142:518-524.

776 Newman, M.A., von Roepenack-Lahaye, E., Parr, A., Daniels, M.J., and Dow, J.M.
777 2001. Induction of hydroxycinnamoyl-tyramine conjugates in pepper by
778 *Xanthomonas campestris*, a plant defense response activated by *hrp* gene-
779 dependent and *hrp* gene-independent mechanisms. Mol Plant-Microbe Interact.
780 14:785-792.

781 Park, J.B., and Schoene, N. 2002. Synthesis and characterization of *N*-
782 coumaroyltyramine as a potent phytochemical which arrests human transformed
783 cells via inhibiting protein tyrosine kinases. Biochem. Biophys. Res. Commun.
784 292:1104-1110.

785 Pearce, G., Marchand, P.A., Griswold, J., Lewis, N.G., and Ryan, C.A. 1998.
786 Accumulation of feruloyltyramine and *p*-coumaroyltyramine in tomato leaves in
787 response to wounding. Phytochemistry 47:659-664.

788 Roh, J.S., Han, J.Y., Kim, J.H., and Hwang, J.K. 2004. Inhibitory effects of active
789 compounds isolated from safflower (*Carthamus tinctorius* L.) seeds for
790 melanogenesis. Biol. Pharm. Bull. 27:1976-1978.

791 Schmidt, A., Scheel, D., and Strack, D. 1998. Elicitor-stimulated biosynthesis of
792 hydroxycinnamoyltyramines in cell suspension cultures of *Solanum tuberosum*.
793 Planta 205:51-55.

794 Schmidt, A., Grimm, R., Schmidt, J., Scheel, D., Strack, D., Rosahl, S. 1999. Cloning
795 and expression of a potato cDNA encoding hydroxycinnamoyl-CoA:tyramine *N*-
796 (hydroxycinnamoyl) transferase. J. Biol. Chem. 274:4273-80.

797 Strack, D. 1997. Phenolic metabolism. Pages 387-416 in: Plant Biochemistry, P.M.Dey
798 and J.B. Harborne, eds. Academic Press, London.

799 Tebayashi, S.I., Horibata, Y., Mikagi, E., Kashiwagi, T., Mekuria, D.B., Dekebo, A.,
800 Ishihara, A., and Kim, C.S. 2007. Induction of resistance against the leaffminer,
801 *Liriomyza trifolii*, by jasmonic acid in sweet pepper. Biosci. Biotechnol.
802 Biochem. 71:1521-1526.

803 Tornero, P., Gadea, J., Conejero, V., and Vera, P. 1997. Two PR-1 genes from tomato
804 are differentially regulated and reveal a novel mode of expression for a
805 pathogenesis-related gene during the hypersensitive response and development.
806 Mol Plant Microbe Interact. 10:624-634.

807 van Loon, L.C., Rep, M., and Pieterse, C.M.J. 2006. Significance of inducible defense-
808 related proteins in infected plants. Annu. Rev. Phytopathol. 44: 135-162.

809 Villegas, M., and Brodelius, P.E. 1990. Elicitor induced hydroxycinnamoyl-CoA:
810 tyramine hydroxycinnamoyltransferase in plant-cell suspension cultures. Physiol
811 Plant. 78:414-420.

812 von Roepenack-Lahaye, E., Newman, M.A., Schornack, S., Hammond-Kosack, K.E.,
813 Lahaye, T., Jones, J.D.G., Daniels, M.J., and Dow, J.M. 2003. *p*-
814 coumaroylnoradrenaline, a novel plant metabolite implicated in tomato defense
815 against pathogens. J. Biol. Chem. 278:43373-43383.

816 Yu, M., and Facchini, P.J. 1999. Purification, characterization, and immunolocalization
817 of hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase from
818 opium poppy. Planta 209:33-44.

819 Zacarés, L., López-Gresa, M.P., Fayos, J., Primo, J., Bellés, J.M., and Conejero, V.
820 2007. Induction of *p*-coumaroyldopamine and feruloyldopamine, two novel
821 metabolites, in tomato by the bacterial pathogen *Pseudomonas syringae*. Mol.
822 Plant-Microbe Interact. 20:1439-1448.

823 Zhang, H.L., Nagatsu, A., Watanabe, T., Sakakibara, J., and Okuyama, H. 1997.
824 Antioxidative compounds isolated from safflower (*Carthamus tinctorius L.*) oil
825 cake. Chem. Pharm. Bull. 45:1910-1914.

826

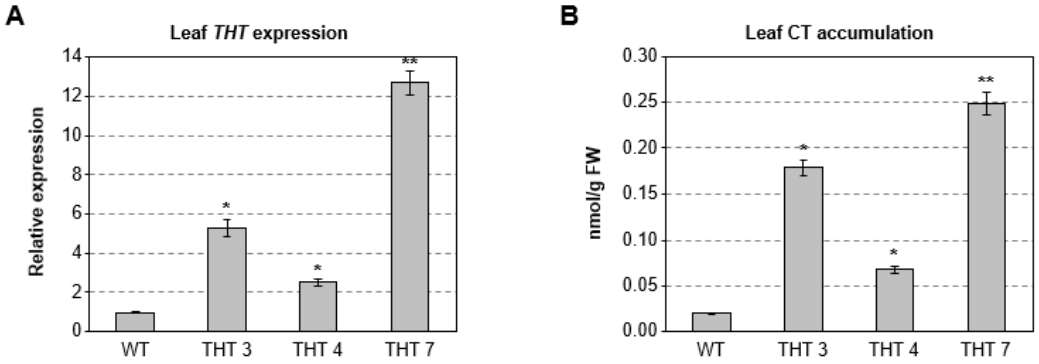
827

828 **FIGURE CAPTIONS**

829 **Figure 1. *THT* gene expression and HCAA content in leaves of THT transgenic**
830 **tomato plants.** (A) Quantitative RT-PCR analysis of *THT* mRNA accumulation in
831 leaves from different transgenic tomato lines (3, 4 and 7), and wild type (WT) plants.
832 *Elongation Factor 1 alpha* (*eEF1α*) gene was used as endogenous reference. (B)
833 Accumulation of coumaroyltyramine (CT) in leaves from the plants shown in (A),

834 analyzed by UPLC-PDA-Micromass Q-TOF. Results correspond to means ± SE of three
835 independent plants from a representative experiment. A *t*-test analysis was performed
836 with the data coming from three independent experiments. Asterisks (*) indicate
837 statistical significance with *p*-value < 0.05 with respect to WT plants. Double asterisks
838 (**) indicate statistical significance with *p*-value < 0.01 with respect to WT plants.

Figure 1



839

840 **Figure 2. *THT* gene expression and HCAA content in flowers of THT transgenic**
841 **tomato plants.** (A) Quantitative RT-PCR analysis of *THT* mRNA accumulation in
842 flowers harvested from the flower stalk at the anthesis stage from wild type (WT) plants
843 and transgenic lines THT-3 and THT-7. *Elongation Factor 1 alpha* (*eEF1α*) gene was
844 used as endogenous reference. (B) Accumulation of coumaroyltyramine (CT) and
845 feruloyltyramine (FT) in flowers from the plants shown in (A), analyzed by UPLC-
846 PDA-Micromass Q-TOF. Results correspond to means ± SE of three independent plants
847 from a representative experiment. A *t*-test analysis was performed with the data coming
848 from three independent experiments. Asterisks (*) indicate statistical significance with
849 *p*-value < 0.05 with respect to WT plants. Double asterisks (**) indicate statistical
850 significance with *p*-value < 0.01 with respect to WT plants.

Figure 2

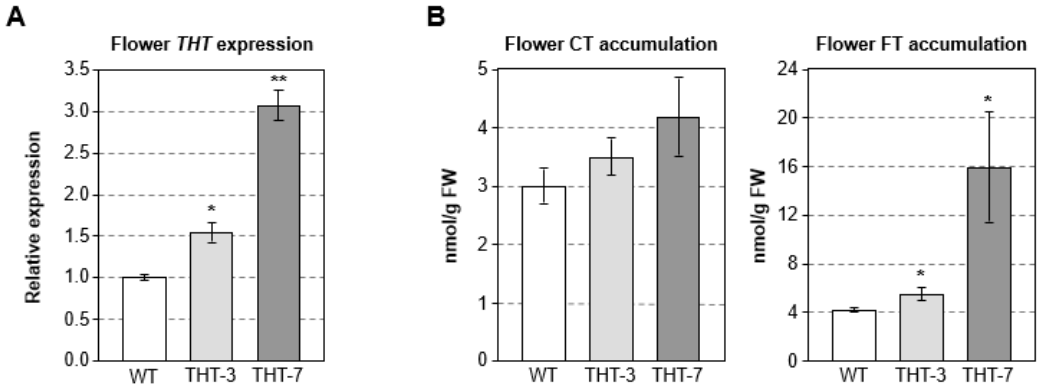


Figure 3. *THT* gene expression and HCAA content in fruits of THT transgenic tomato plants. (A) Quantitative RT-PCR analysis of *THT* mRNA accumulation in mature fruits from wild type (WT) plants and transgenic lines THT-3 and THT-7. *Elongation Factor 1 alpha* (*eEF1α*) gene was used as endogenous reference. (B) Accumulation of coumaroyltyramine (CT) and feruloyltyramine (FT) in mature fruits from the plants shown in (A), analyzed by UPLC-PDA-Micromass Q-TOF. Results correspond to means ± SE of three independent plants from a representative experiment. A *t*-test analysis was performed with the data coming from three independent experiments. Asterisks (*) indicate statistical significance with p-value < 0.05 with respect to WT plants. Double asterisks (**) indicate statistical significance with p-value < 0.01 with respect to WT plants.

Figure 3

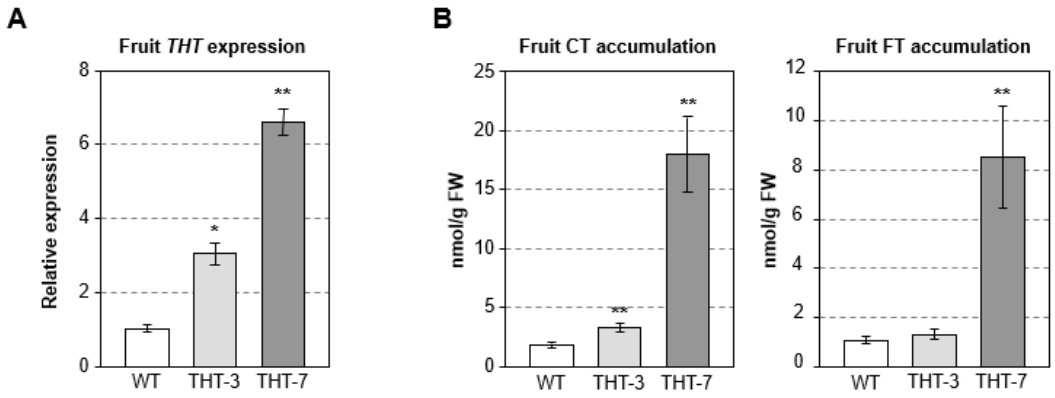
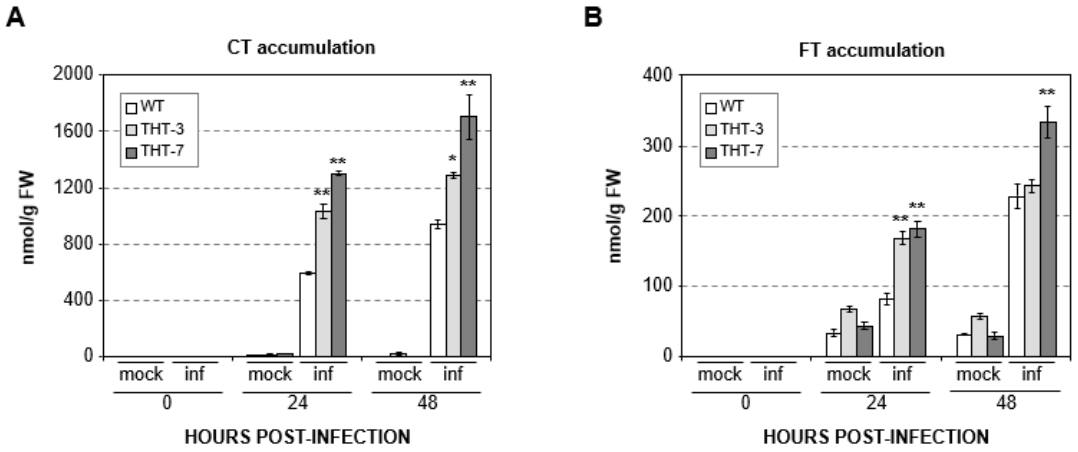


Figure 4. HCAA accumulation in leaves from THT transgenic tomato plants upon infection with *Pseudomonas syringae* pv. *tomato*. (A) Levels of coumaroyltyramine (CT) and (B) feruloyltyramine (FT) in infected (inf) and mock-inoculated (mock) leaves from wild type (WT) plants and transgenic lines THT-3 and THT-7 at 0, 24 and 48 hours after *P. syringae* pv. *tomato* infiltration. Samples were analyzed by UPLC-PDA-Micromass Q-TOF. Results correspond to means ± SE of three independent plants from a representative experiment. A *t*-test analysis was performed with the data coming from three independent experiments. Statistical significance is shown for infected transgenic plants with respect to infected WT plants. Asterisks (*) indicate statistical significance with p-value < 0.05 with respect to WT plants. Double asterisks (**) indicate statistical significance with p-value < 0.01 with respect to WT plants.

Figure 4



876

877 **Figure 5. Accumulation of HCAA of octopamine and noradrenaline in THT**
878 **transgenic tomato plants upon infection with *Pseudomonas syringae* pv. *tomato*.** (A)
879 Levels of coumaroyloctopamine (CO) and feruloyloctopamine (FO) in leaves from wild
880 type (WT) plants and transgenic lines THT-3 and THT-7 at 48 hours after *P. syringae*
881 pv. *tomato* infiltration. (B) Levels of coumaroylnoradrenaline (CNA) and
882 feruloylnoradrenaline (FNA) in leaves from wild type (WT) plants and transgenic lines
883 THT-3 and THT-7 tomato plants at 48 hours after *P. syringae* pv. *tomato* infiltration.
884 Samples were analyzed by UPLC-PDA-Micromass Q-TOF. Results correspond to
885 means ± SE of three independent plants from a representative experiment. A *t*-test
886 analysis was performed with the data coming from three independent experiments.
887 Asterisks (*) indicate statistical significance with p-value < 0.05 with respect to WT
888 plants. Double asterisks (**) indicate statistical significance with p-value < 0.01 with
889 respect to WT plants.

Figure 5

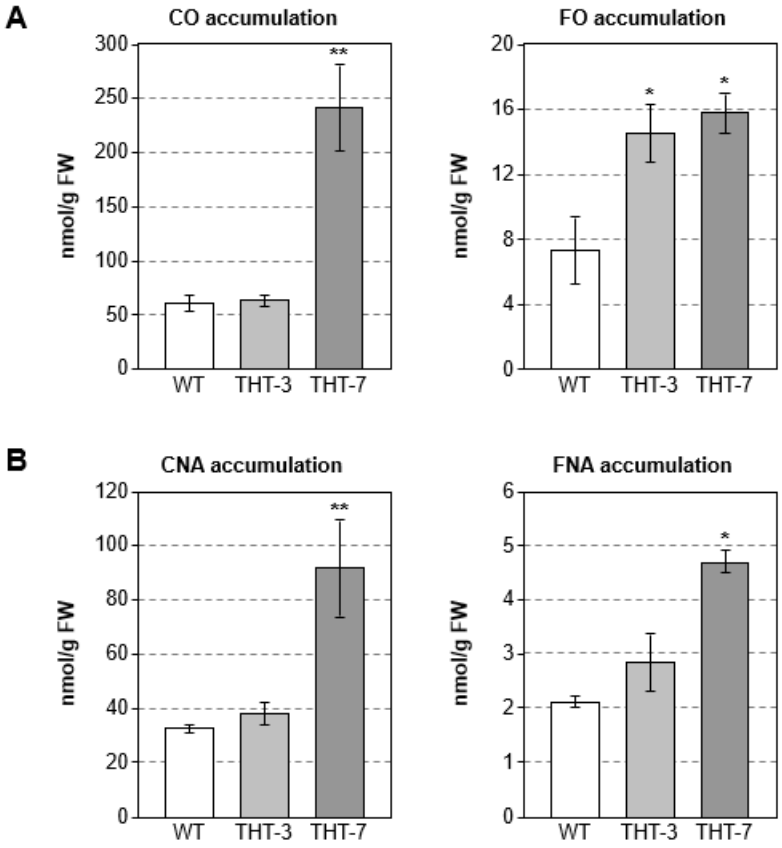
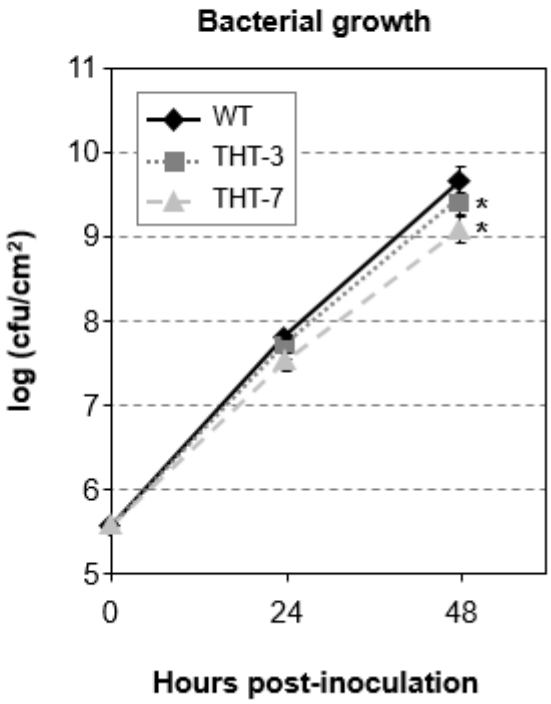


Figure 6. Growth of *Pseudomonas syringae* pv. *tomato* on leaves of THT transgenic tomato plants. Leaf colony-forming units (CFU) of bacteria were measured as described in Materials and Methods in wild type (WT) plants and transgenic lines THT-3 and THT-7. Bacterial growth was measured 24 and 48 hours after inoculation. Results correspond to means ± SE of five independent plants from a representative experiment. A *t*-test analysis was performed with the data coming from three independent experiments. Asterisks (*) indicate statistical significance with p-value < 0.05 with respect to WT plants.

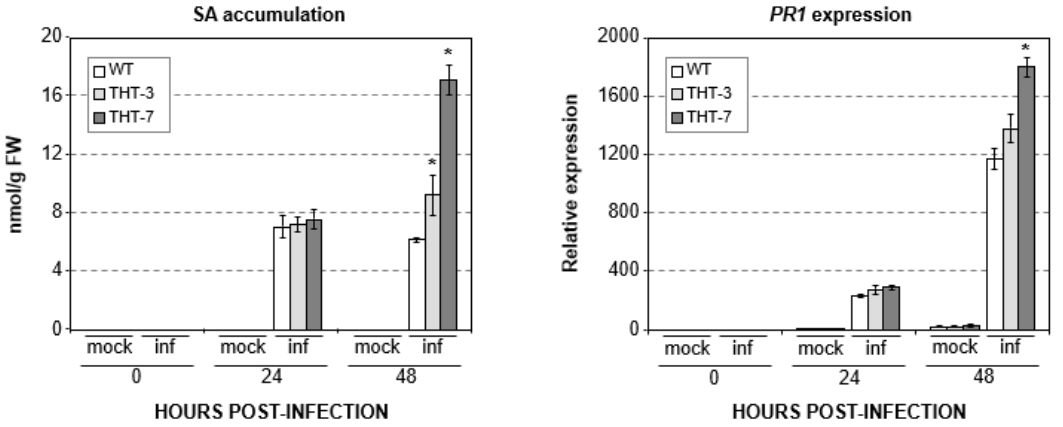
Figure 6



899

900 **Figure 7. Accumulation of salicylic acid and expression of *PR-1* gene in THT**
901 **transgenic tomato plants upon infection with *Pseudomonas syringae* pv. *tomato*.** (A)
902 Levels of salicylic acid (SA) in infected (inf) and mock-inoculated (mock) third and
903 fourth leaves from wild type (WT) plants and transgenic lines THT-3 and THT-7
904 harvested at 0, 24 and 48 hours after *P. syringae* pv. *tomato* infiltration. (B)
905 Quantitative RT-PCR analysis of *PR-1* gene in infected and mock inoculated leaves
906 from the plants shown in (A). Results correspond to means ± SE of three independent
907 plants from a representative experiment. A *t*-test analysis was performed with the data
908 coming from three independent experiments. Statistical significance is shown for
909 infected transgenic plants with respect to infected WT plants. Asterisks (*) indicate
910 statistical significance with p-value < 0.05 with respect to WT plants.

Figure 7



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912

913 **Table X1. Primer sequences used for quantitative RT-PCR analysis of *THT1-3*,**
914 ***PR-1* and *eEF1α* tomato genes.**

Table X1

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
THT1-3	CCCCTTTTGACGAACCTAAA	TTTGGATCGGAATTCCTCAA
PR-1	ACTCAAGTAGTCTGGCGCAACTCA	AGTAAGGACGTTGTCCGATCCAGT
eEF1α	CCACCTCGAGATCCTAATGG	ACCCTCACGTATGCTTCCAG

Table X1. Primer sequences used for quantitative RT-PCR analysis of tomato *THT1-3*, *PR-1* and *eEF1α* genes.

915

916 **Table X2. *P*-values from *t*-test statistical analyses used in the Figures**. *P*-values
917 correspond to experiments where at least one transgenic line was significantly different
918 when compared to WT.

Table X2

	p-values		
	WT-THT3	WT-THT7	WT-THT4
Figure 1. Leaves			
THT mRNA levels	0,0285	0,0081	0,0476
CT content	0,0137	< 0,0001	0,0439
Figure 2. Flowers			
THT mRNA levels	0,0470	0,0005	
CT content	0,1331	0,1219	
FT content	0,0447	0,0110	
Figure 3. Fruits			
THT mRNA levels	0,0331	0,0007	
CT content	0,0044	0,0009	
FT content	0,1530	0,0033	
Figure 4. *Ps* infection			
CT content @ 24 hpi	< 0,0001	< 0,0001	
FT content @ 24 hpi	0,0003	0,0002	
CT content @ 48 hpi	0,0181	0,0032	
FT content @ 48 hpi	0,2594	0,0096	
Figure 5. *Ps* infection			
CO content	0,5369	0,0036	
FO content	0,0463	0,0123	
CNA content	0,1049	0,0047	
FNA content	0,0799	0,0485	
Figure 6. Bacterial growth			
Growth @ 48 hpi	0,0230	0,0176	
Figure 7. SA and PR1			
SA content @ 48 hpi	0,0472	0,0225	
PR1 levels @ 48 hpi	0,1297	0,0136	
Figure S1. Wounding			
CT content	< 0,0001	< 0,0001	

Table X2. *P*-values from *t*-test statistical analyses used in the Figures. *P*-values correspond to experiments where at least one transgenic line was significantly different when compared to WT.

Figure X1. HCAA accumulation in THT transgenic tomato plants upon wounding.

Levels of coumaroyltyramine (CT) and feruloyltyramine (FT) in third and fourth leaves

922 from wild type (WT) plants and transgenic lines THT-3 and THT-7 harvested 24 hours
923 after wounding. Samples were analyzed by UPLC-PDA-Micromass Q-TOF. Results
924 correspond to means ± SE of three independent plants from a representative experiment.
925 A *t*-test analysis was performed with the data coming from three independent
926 experiments. Asterisks (*) indicate statistical significance with *p*-value < 0.05 with
927 respect to WT plants. Double asterisks (**) indicate statistical significance with *p*-value
928 < 0.01 with respect to WT plants.

Figure X1

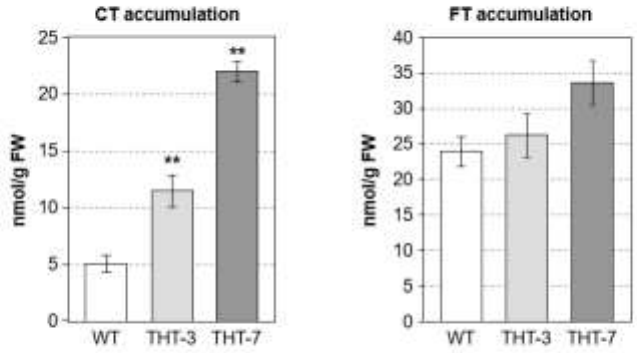

Figure X1. HCAA accumulation in THT transgenic tomato plants upon wounding. Levels of coumaroyltyramine (CT) and feruloyltyramine (FT) in third and fourth leaves from wild type (WT) plants and transgenic lines THT-3 and THT-7 harvested 24 hours after wounding. Samples were analyzed by UPLC-PDA-Micromass Q-TOF. Results correspond to means ± SE of three independent plants from a representative experiment. A *t*-test analysis was performed with the data coming from three independent experiments. Asterisks (*) indicate statistical significance with *p*-value < 0.05 with respect to WT plants. Double asterisks (**) indicate statistical significance with *p*-value < 0.01 with respect to WT plants.

929