Transgenic Tomato Plants Overexpressing Tyramine *N*-Hydroxycinnamoyltransferase Exhibit Elevated Hydroxycinnamic Acid Amide Levels and Enhanced Resistance to *Pseudomonas syringae*

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Hydroxycinnamic acid amides (HCAA) are secondary metabolites involved in plant development and defense that 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 with wild type (WT) 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 WT 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 defense of tomato plants against *P. syringae* infection.

Hydroxycinnamic acid amides (HCAA) are low molecular-weight phenolic compounds that 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 shown to form part of HCAA (Facchini et al. 2002; Strack 1997). 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; Handrick et al. 2010; Luo et al. 2009). These phenolic compounds, showing strong antioxidant and chemotherapeutic effects, are often considered as nutraceuticals (Kawashima et al. 1998; Nagatsu et al. 2000; Park and Schoene 2002; Roh et al. 2004; Zhang et al. 1997), and 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 defense, 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). In this respect, tomato plants challenged with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* 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 defense. 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 are 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 either by reducing the digestibility of the cell wall, by directly inhibiting the growth of fungal hyphae, or both (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 reported to present antifungal activity (Fattorusso et al. 1999). Moreover, it has

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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 defense 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 (Facchini et al. 2002; Negrel and Javelle 1997; Negrel and Martin 1984; Schmidt et al. 1999) and is induced in response to elicitor treatment, wounding, and pathogens (Negrel et al. 1993; Schmidt et al. 1998; Villegas and Brodelius 1990). This suggests a general role for THT in plant defense 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* homologs have been identified, one being *tomTHT1-3*, which is 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 previously. 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). Also, 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 accumula-

tion 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 in 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* gene. 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 (WT) tomato (*S. lycopersicum* cv. MoneyMaker) plants were transformed with the binary vector pBI-THT via *Agrobacterium tumefaciens* (discussed below). Different independent transgenic lines were regenerated in vitro in the presence of kanamycin. Plants from all these lines were first assayed for β -glucuronidase (GUS) activity and were then tested by reverse transcription-polymerase chain reaction (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 WT plants were used to perform quantitative RT-PCR, using specific primers for the tomato *THT* gene. All the transgenic lines ana-

Fig. 1. Tyramine *N*-hydroxycinnamoyl transferase (*THT*) gene expression and hydroxycinnamic acid amides content in leaves of *THT* transgenic tomato plants. **A**, Quantitative reverse transcription-polymerase chain reaction analysis of *THT* mRNA accumulation in leaves from different transgenic tomato lines (3, 4, and 7), and wild-type (WT) plants. The *Elongation Factor 1 alpha* (*eEF1 α*) gene was used as endogenous reference. **B**, Accumulation of coumaroyl-tyramine (CT) in leaves from the plants shown in A, analyzed by ultra-performance liquid chromatographic-photodiode array-Micromass quadrupole-time-of-flight. Results correspond to means $\pm$ standard error 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.

lyzed presented higher constitutive *THT* gene expression when compared with the parental plants (Fig. 1A). The *THT* gene-expression levels largely varied among the different transgenic lines, with line 4 showing 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 than that observed in WT 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 ultra-performance liquid chromatographic-photodiode array (UPLC-PDA)-Micromass quadrupole-time-of-flight (Q-TOF) analysis to measure the HCAA content (discussed below). 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 coumaric acids in noninfected transgenic tomato plants. Although FT was barely detectable (data not shown), all the transgenic lines presented CT levels higher than those found in WT plants (Fig. 1B). These CT accumulation levels correlated well with the observed *THT* mRNA levels (Fig. 1A). Line 7 presented the highest levels of CT accumulation, containing up to 10 times the content found in WT plants. Statistical analysis showed that the differences observed in the three lines with respect to WT plants were statistically significant, with lines 3 and 7 exhibiting the highest levels. Therefore, these two independent lines were selected for further assays.

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

To better characterize the overexpressing transgenic plants, we used quantitative RT-PCR to analyze the *THT* mRNA accumulation in flowers at the anthesis stage for transgenic tomato and WT plants. As shown in Figure 2A, both transgenic lines constitutively expressed elevated *THT* mRNA levels. In line 7, *THT* mRNA levels were around threefold higher than those observed in WT flowers. On the other hand, to determine whether the *THT* expression levels in flowers were correlated

with HCAA accumulation, flowers at the anthesis stage from WT and transgenic tomato plants were subjected to UPLC-PDA-Micromass Q-TOF analysis (discussed below). Unlike what was found in leaves, accumulation of both CT and FT was detected in tomato flowers. The level of CT was quite similar in transgenic and WT flowers, showing no statistical differences. However, the accumulation of FT was significantly higher in both transgenic lines as compared with WT plants. Line 7 presented FT levels up to fourfold higher than the levels detected in WT flowers.

***THT* gene expression and HCAA accumulation in fruits of transgenic tomato.**

In a manner similar to that 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 WT plants were analyzed by quantitative RT-PCR, using specific primers for tomato *THT* gene expression. As observed in leaves and flowers, the level of *THT* gene expression in transgenic fruits was higher (approximately sixfold in line 7) than that observed in the WT fruits (Fig. 3A).

HCAA levels were also examined in WT and transgenic tomato fruits. For this analysis, mature fruits from transgenic tomato and WT plants were collected and processed as described below. Samples were then subjected to UPLC-PDA-Micromass Q-TOF analysis to measure the content of HCAA. Similar to observations in flowers, levels of CT and FT were higher in transgenic plants than in the WT (Fig. 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 fourfold higher than those observed in the WT 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 pos-

Fig. 2. Tyramine *N*-hydroxycinnamoyl transferase (*THT*) gene expression and hydroxycinnamic acid amides (HCAA) content in flowers of *THT* transgenic tomato plants. **A**, Quantitative reverse transcription-polymerase chain reaction analysis of *THT* mRNA accumulation in flowers harvested from the flower stalk at the anthesis stage from wild-type (WT) plants and transgenic lines THT-3 and THT-7. The *Elongation Factor 1 alpha* (*eEF1α*) gene was used as endogenous reference. **B**, Accumulation of coumaroyltyramine (CT) and feruloyltyramine (FT) in flowers from the plants shown in A, analyzed by ultra-performance liquid chromatographic-photodiode array-Micromass quadrupole-time-of-flight. Results correspond to means $\pm$ standard error of three independent plants from a representative experiment. A *t*-test analysis was performed with the data coming from three independent experiments. Asterisks (*) or (**) indicate statistical significance with *P* values <0.05 or <0.01 with respect to WT plants.

sible role for this gene in plant defense (von Roepenack-Lahaye et al. 2003). Moreover, it has been reported that 'Rutgers' tomato leaves infected with *Pseudomonas syringae* pv. *tomato* showed a rapid *THT* activation, followed by HCAA accumulation (López-Gresa et al. 2011; Zacarés et al. 2007). 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 below, infected and mock-inoculated leaves from WT and THT tomato plants were harvested at 0, 24 and 48 h 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. syrin-*

gae 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 with WT plants. Differences between line 3 and WT plants were statistically significant for CT levels at both 24 and 48 h postinoculation (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

Fig. 3. Tyramine *N*-hydroxycinnamoyl transferase (*THT*) gene expression and hydroxycinnamic acid amides (HCAA) content in fruits of THT transgenic tomato plants. **A**, Quantitative reverse transcription-polymerase chain reaction analysis of *THT* mRNA accumulation in mature fruits from wild-type (WT) plants and transgenic lines THT-3 and THT-7. The *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 ultra-performance liquid chromatographic-photodiode array-Micromass quadrupole-time-of-flight. Results correspond to means $\pm$ standard error of three independent plants from a representative experiment. A *t*-test analysis was performed with the data coming from three independent experiments. Asterisks (* or **) indicate statistical significance with *P* values <0.05 or <0.01 with respect to WT plants.

Fig. 4. Hydroxycinnamic acid amides (HCAA) accumulation in leaves from tyramine *N*-hydroxycinnamoyl transferase (*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 h after *P. syringae* pv. *tomato* infiltration. Samples were analyzed by ultra-performance liquid chromatographic-photodiode array-Micromass quadrupole-time-of-flight. Results correspond to means $\pm$ standard error 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 (* or **) indicate statistical significance with *P* values <0.05 or <0.01 with respect to WT plants.

upon bacterial infection was approximately twofold higher when compared with that observed in WT plants. CO and FO 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), in which the same amides were found to accumulate in ‘Rutgers’ tomato leaves at 24 and 48 h 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 h after *P. syringae* challenge. We observed that plants from line 3 exhibited statistical differences in the content of FO when

compared with WT 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 threefold higher upon *Pseudomonas syringae* infection as compared with that observed in WT plants. In contrast with HCAA of tyramine, HCAA of octopamine and noradrenaline were not detected in mock-inoculated leaves during 48-h 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 defense, we studied the response of transgenic tomato plants to infection with the bacterial pathogen *P. syringae* pv. *tomato*. To test bacterial growth, WT and transgenic tomato plants were infiltrated with *P. syringae* and were harvested at 24 and 48 hpi (dis-

Fig. 5. Accumulation of hydroxycinnamic acid amides (HCAA) of octopamine and noradrenaline in tyramine *N*-hydroxycinnamoyl transferase (*THT*) transgenic tomato plants upon infection with *Pseudomonas syringae* pv. *tomato*. **A**, Levels of coumaroyloctopamine (CO) and feruloyloctopamine (FO) in leaves from wild-type (WT) plants and transgenic lines THT-3 and THT-7 at 48 h after *P. syringae* pv. *tomato* infiltration. **B**, Levels of coumaroylnoradrenaline (CNA) and feruloylnoradrenaline (FNA) in leaves from WT plants and transgenic line THT-3 and THT-7 tomato plants at 48 h after *P. syringae* pv. *tomato* infiltration. Samples were analyzed by ultra-performance liquid chromatographic-photodiode array-Micromass quadrupole-time-of-flight. Results correspond to means $\pm$ standard error of three independent plants from a representative experiment. A *t*-test analysis was performed with the data coming from three independent experiments. Asterisks (*) or (**) indicate statistical significance with *P* value <0.05 or <0.01 with respect to WT plants.

cussed below). Results of the time-course analysis corresponding to bacterial growth in leaves from transgenic (THT-3 and THT-7) and WT tomato plants upon *P. syringae* challenge are presented in Figure 6. Bacterial growth was significantly re-

Fig. 6. Growth of *Pseudomonas syringae* pv. *tomato* on leaves of tyramine *N*-hydroxycinnamoyl transferase (*THT*) transgenic tomato plants. Leaf colony-forming units (CFU) of bacteria were measured in wild-type (WT) plants and transgenic lines THT-3 and THT-7. Bacterial growth was measured 24 and 48 h after inoculation. Results correspond to means $\pm$ standard error 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 a *P* value <0.05 with respect to WT plants.

duced 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) as compared with WT plants at 48 hpi. Comparison of bacterial growth between lines 3 and 7 reveals a dosage effect, thus correlating resistance with HCAA accumulation (Figs. 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 SA plays a central role in plant defense (Dempsey et al. 1999; Métraux and Raskin 1993). 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 signaling, we followed SA levels in transgenic and WT plants along the course of infection with *P. syringae* (Fig. 7A). We observed no significant differences in SA levels between WT and transgenic lines at 24 hpi. Nevertheless, at 48 hpi, 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, in which significant differences in bacterial growth occurred at 48 hpi between transgenic lines and WT. Among them, SA levels in line 7 were threefold higher in *P. syringae*-inoculated plants as compared with the corresponding WT plants.

Since SA is a major regulator of PR protein gene induction, total RNA is isolated at different timepoints after the onset of bacterial infiltration and was subjected to quantitative RT-PCR analysis in order to determine the expression levels of *PR-1* mRNA (Fig. 7B). Correlating with the observed SA accumulation, the expression of *PR-1* increased as a consequence of the infection, being higher in both THT lines. Expression of the *PR-1* gene was approximately 1.5-fold higher in line 7 when compared with that observed in WT plants at 48 hpi, a statistically significant difference.

Fig. 7. Accumulation of salicylic acid and expression of *PR-1* gene in tyramine *N*-hydroxycinnamoyl transferase (*THT*) transgenic tomato plants upon infection with *Pseudomonas syringae* pv. *tomato*. **A**, Levels of salicylic acid (SA) in infected (inf) and mock-inoculated (mock) third and fourth leaves from wild-type (WT) plants and transgenic lines THT-3 and THT-7 harvested at 0, 24, and 48 h after *P. syringae* pv. *tomato* infiltration. **B**, Quantitative reverse transcription-polymerase chain reaction analysis of *PR-1* gene in infected and mock inoculated leaves from the plants shown in A. Results correspond to means $\pm$ standard error 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 a *P* value <0.05 with respect to WT plants.

DISCUSSION

THT is the key enzyme in HCAA synthesis, catalyzing the condensation of hydroxycinnamic acids with different amines. The resulting amides have been described as 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). In addition, *THT* induction and the consequent HCAA accumulation occurs upon pathogen infection or wounding, all pointing to their role in plant defense (Grandmaison et al. 1993; Hahlbrock and Scheel 1989; Keller et al. 1996; Lee et al. 2004; Newman et al. 2001; Pearce et al. 1998; Schmidt et al. 1998; Tebayashi et al. 2007). In this study, to better understand the biological function of this accumulation, we generated and characterized transgenic tomato plants overexpressing *THT*. In addition, we studied their response to the bacterial pathogen *P. syringae* pv. *tomato* as compared with the response displayed by the WT plants.

Several overexpressing *THT* transgenic tomato lines have been generated, showing abundant *THT* gene expression levels in the leaves. Specifically, the best line presented a *THT* mRNA accumulation approximately 12-fold higher than the WT levels. This overexpression was directly coupled with a high HCAA accumulation. This line showed the highest CT accumulation as well, reaching levels 10-fold higher than that observed in the WT plants.

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

Interestingly, we have observed that CT constitutively accumulated in leaves of transgenic tomato plants overexpressing *THT*, indicating that tyramine content is high enough in this species, as described by Ly and associates (2008), and does not play a substrate rate-limiting role in HCAA synthesis. Basal levels of tyramine of WT and *THT* transgenic tomato plants were measured and no clear differences were 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 with the WT, while FT levels remained practically unchanged (Supplementary Fig. S1).

We further characterized the transgenic tomato plants by studying *THT* gene expression in flowers. Similar to what was observed in the leaves, flowers showed an increase in *THT* mRNA accumulation as compared with the WT 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 fourfold higher in flowers from transgenic line 7 compared with 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.

Similar to our observations, pepper flowers have been reported to accumulate high levels of HCAA. In addition, flowers proved 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 observed in transgenic tomato flowers may be due to the basal content of tyramine present in these organs. This HCAA accumulation suggests an important function for these compounds, acting as antimicrobial defense against pathogens, reinforcing the cell wall during anthesis, or controlling sexual organogenesis (Hahlbrock and Scheel 1989; Martin-Tanguy et al. 1987; Negrel and Lherminier 1987; Liyama et al. 1994).

THT gene expression was also tested in mature (red) tomato fruits from transgenic plants, showing approximately sixfold higher levels in transgenic fruits in line 7 compared with WT. This elevated *THT* expression was accompanied by a higher FT and CT content. A high HCAA accumulation in fruits has been proposed to contribute to the defense response. In this respect, Kang and associates (2010) have recently reported the accumulation of the HCAA caffeoylserotonin (CaS) in pepper fruits challenged with the anthracnose fungus *Colletotrichum gloeosporoides*. These authors suggested that CaS, which is induced by pathogen infection and exhibits antifungal activity, plays a key role in pepper plant defense. In agreement with these results, the overaccumulation of CT and FT found in *THT* tomato fruits may act as a constitutive defense against possible infections. In fact, CT and FT have been long implicated in plant defense against pathogen attack, and several studies showed that their synthesis in plants is activated in response to infection by fungi, viruses, or bacteria. For instance, potato plants infected with *Phytophthora infestans* have been reported to increase the synthesis of CT and FT (Keller et al. 1996) and FT biosynthesis was induced in tobacco upon *Tobacco mosaic virus* infection (Negrel and Jeandet 1987).

In order to study the response of *THT*-overexpressing plants to pathogen attack, transgenic tomato plants were infected with the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. We observed that CT and FT accumulated in a time-dependent manner along the course of infection, the highest values corresponding to CT. The accumulation of CT and FT was up to twofold higher in *P. syringae*-inoculated transgenic plants from line 7 compared with the corresponding infected WT plants. In addition, accumulation of other HCAA of octopamine (CO and FO) and noradrenaline (CNA and FNA) was also detected, with levels approximately threefold higher in infected transgenic tomato plants from line 7 as compared with WT. 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 defense response against pathogens (López-Gresa et al. 2011; Zacarés et al. 2007). 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 hpi. 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 were higher as 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, than the concentration found in the incompatible interactions described in tomato by von Roepenack-Lahaye and associates (2003).

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

SA plays a central role in plant defense signaling and it has been reported to accumulate in tomato plants upon challenge with *P. syringae* (Bellés et al. 1999; Dempsey et al. 1999). Thus, levels of SA were measured in THT transgenic and WT plants infected with *P. syringae*. SA levels were approximately threefold higher in THT plants from line 7. Considering that SA is a major regulator of *PR* gene induction (van Loon et al. 2006), the expression level of *PR-1* was also tested at different timepoints after the onset of bacterial infiltration. We have observed that levels of *PR-1* increased along the course of the infection, being higher in THT plants as than WT plants. These results correlated well with the SA accumulation levels observed in the *THT* overexpressing plants.

HCAA promote resistance by creating a barrier against pathogens or directly acting as antimicrobial agents (Clarke 1982; Fattorusso et al. 1999; Negrel and Martin 1984; Newman et al. 2001). Our results show that the accumulation of these compounds is accompanied by the SA synthesis, leading to the activation of plant defense. 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 defense system in order to prevent possible infections would be of particular interest.

To summarize, this study shows that transgenic tomato plants overexpressing *THT* display 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. In addition, the accumulation of these two amides, together with CO, FO, CNA, and FNA, was higher in *Pseudomonas*-infected THT tomato plants as compared with WT 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 engi-

neered transgenic plants as a tool to provide new evidence of the HCAA role in this and further plant-pathogen interactions.

MATERIALS AND METHODS

Vector construction and tomato transformation.

The full-length cDNA of *tomTHT1-3* (von Roepenack-Lahaye et al. 2003) was amplified by RT-PCR, using total RNA obtained from tomato leaves infected by the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. Primers used were 5'-CCGGATCCTCTAGAATGGCTCCTGCTCTTGAA CA-3' as the forward primer and 5'-CCGGATCCCTAACAGC TCCCTTTCGCCGT-3' as the reverse primer, both oligonucleotides containing the *Bam*HI restriction site. The resulting PCR product (THT) was cloned into the pGEM-T Easy vector (Stratagene, La Jolla, CA, U.S.A.) and was sequenced. The generation of the binary vector for plant transformation was carried out in two steps. First, the DNA band obtained by digestion of pGEM-THT with the restriction enzyme *Bam*HI was cloned into a pBluescript modified vector containing a double region of the *Cauliflower mosaic virus* 35S and a *nos* termination sequence. Then, the [2XCaMV35S::THT::nos] cassette was released by digestion with the restriction enzyme *Hind*III and was inserted into pBI121 vector. This vector carries the neomycin phosphotransferase gene (*NPTII*) as a transgenic selectable marker. The final construction (pBI-THT) was used to transform *Agrobacterium tumefaciens* LBA4404. This *Agrobacterium tumefaciens* was then used for coculture with tomato (*Lycopersicon esculentum* cv. Moneymaker) cotyledons. Explant preparation, selection, and regeneration followed the methods described by Ellul and associates (2003). Transformation frequencies were around 15%. Transformants were selected in kanamycin-containing medium and were propagated in soil, for obtaining T₁ seeds. WT tomato seeds used in all the studies came from in vitro-regenerated plants, which were obtained in parallel with the transgenic plants.

GUS activity.

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

Plant material and treatments.

Seeds from tomato (*Lycopersicon esculentum* cv. Money-maker, *Tm*², *pto*), and transgenic tomato plants overexpressing the *THT* gene were used in this study. Tomato 'Money-maker' seeds were provided by J. D. G. Jones (John Innes Centre, Norwich, U.K.). The *Tm*² gene confers resistance to *Tomato mosaic virus*. These plants lack the *Pto* resistance gene that recognizes the AvrPto effector from *Pseudomonas syringae* pv. *tomato* DC3000, and therefore, they are susceptible to this pathogen.

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

Three different types of tissue were analyzed for 'Money-maker' control and T₁ THT transgenic tomato plants: leaves, flowers, and fruits. For leaf tissues, the third and fourth leaves from 4-week-old tomato plants at the five- to six-leaf stage

were harvested. Flowers were harvested from the flower stalk in the anthesis stage. Tomato fruits were collected when ripe (red). Each fruit was washed and cut, and seeds and placentas were removed, so that the tissue sample consisted of the fleshy part of the fruit (mesocarp and endocarp).

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

Plant material was harvested in liquid nitrogen at the indicated timepoints and were immediately reduced to a fine powder with mortar and pestle or stored frozen at -80°C .

Bacterial inoculation and CFU determination.

The third and fourth leaves from 4-week-old tomato plants ('MoneyMaker' *pto* control and T₁ THT transgenic plants) were infected with a bacterial suspension of *Pseudomonas syringae* pv. *tomato* DC3000 Δ avrPto (provided by S. Giménez, Centro Nacional de Biotecnología, Madrid). There was no *AvrPto*-*Pto*-mediated gene-for-gene interaction; consequently, a compatible interaction was established.

Bacteria were grown overnight at 28°C in 20-ml petri dishes with King's B agar medium supplemented with 100 μl of rifampicin (50 mg/ml). Then, bacterial colonies were transferred to 15 ml of King's B medium and were grown overnight at 28°C . Bacteria were pelleted by centrifugation and were resuspended in 10 mM MgCl_2 to an optical density of 0.1 at 600 nm. Dilution plating was used to determine the final inoculum concentration, which averaged 1×10^5 CFU/ml. One hundred microliters of this bacterial suspension were injected into the abaxial side of each leaflet with a 1-ml sterilized plastic syringe without a needle, as described by Collinge and associates (1987). Equivalent control leaflets were mock-inoculated with a sterile solution of 10 mM MgCl_2 .

For determining 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 timepoints (24 and 48 h 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, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. After extraction, RNA was further precipitated by adding one volume of 6 M LiCl, and then, the pellet was washed with 3 M LiCl and dissolved in RNase-free water. Finally, 2 U of TURBO DNase (Ambion, Austin, TX, U.S.A.) were added per microliter of RNA to remove contaminating genomic DNA. Quantitative RT-PCR analysis was performed as previously described by Campos and associates (2014). One microgram of 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 Bio Inc, Otsu, Shiga, Japan) 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 Biosystems, Foster City, CA, U.S.A.), and amplification was monitored in real time with the 7500 FAST Real-Time PCR system (Life Technologies). A housekeeping gene transcript, *Elongation Factor 1 alpha* (eEF1 α), was used as endogenous reference. The PCR primers were designed using the pcrEfficiency software (Mallona et al. 2011) (Supplementary Table S1).

Extraction procedure and

UPLC-PDA-micromass Q-TOF analysis of HCAA.

Extraction of methanol-soluble HCAA from tomato leaves, flowers, and fruits was done according to López-Gresa and associates (2011). An aliquot of 0.5 g of frozen powdered tissue from each sample was transferred to a mortar and was homogenized with 1.5 ml of methanol. The extraction mixture was vortexed for 1 min, and was then sonicated for 10 min and centrifuged at $14,000 \times g$ for 15 min. The pellet was resuspended in 0.5 ml of methanol, and the same steps were repeated as above. Both supernatants (total volume 2 ml) were transferred to 5-ml glass tubes and were dried under a flow of nitrogen at 40°C . The residue was dissolved in 300 μl of methanol and were filtered through 13-mm Nylon 0.45 μm Minispike filters (Waters Corp., Milford, MA, U.S.A.). The solvent was evaporated and the residue dissolved again in 200 μl of methanol. All steps of the extraction were performed under dark conditions to avoid *cis/trans* light-induced isomerization of phenylpropanoid double bonds (Muhlenbeck et al. 1996).

A 5- μl aliquot from the final 200- μl sample was analyzed by UPLC-mass spectrometry (MS) using an ACQUITY UPLC-PDA system coupled to a Q-ToF Micromass spectrometer (Waters Corp.). Separation was performed on an Acquity BEH C18 column (2.1 mm $\times$ 150 mm i.d., 1.7 μm). The mobile phase consisted of formic acid/ultrapure water (1:1,000 vol/vol; phase A) and formic acid/acetonitrile (1:1,000 vol/vol; phase B). Gradient conditions were as follows: 95 to 90% A in 14 min, 90 to 80% A in 15 min, 80 to 65% A in 10 min, 65 to 57% A in 1 min, 57 to 0% A in 1 min, held at 100% B for 3 min, returned to 95% A in 1 min, and equilibrated for 4 min before the next injection. The flow rate was 0.4 ml/min. The column and sample temperatures were kept at 40 and 4°C , respectively. UV spectra were acquired between 210 and 600 nm with a 1.2-nm resolution and 20 points per second sampling rate. MS analysis was performed by electrospray ionization in negative mode. The mass spectrometer was calibrated with sodium formate (10 ng/ μl in 90:10 propan-2-ol/water). Analysis conditions were as follows: capillary voltage, 3.0 kV; cone voltage, 45 eV; desolvation temperature, 300°C ; source temperature, 120°C ; cone gas flow, 50 liters/h; desolvation gas flow, 500 liters/h; collision energy, 5 eV. MS data were acquired in centroid mode in the mass-to-charge ratio scan range 100 to 1,500 with a scan time of 0.52 s and an interscan time of 0.1 s. Leu-enkephalin was used as the lockmass using a Lock-Spray exact mass ionization source. All data were acquired using Masslynx NT4.1 software (Waters Corp.). HCAA were quantified using synthesized HCAA as standard. HCAA synthesis was performed as described by Zacarés and associates (2007) and López-Gresa and associates (2011).

Extraction and HPLC analysis of SA.

Extraction of SA from tomato leaflets was done according to previously published protocols (Bellés et al. 2008). Briefly, leaves (0.3 to 0.5 g fresh weight of tissue) were ground with a pestle in a mortar using liquid nitrogen and were then homogenized in 1.5 ml of methanol. The extracts were vortexed vigorously, were sonicated for 10 min, and were then centrifuged at $14,000 \times g$ 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 was dried under nitrogen at 40°C using 5-ml glass tubes. 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 U/mg 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, to stop the reaction, perchloric acid (70%) was added to

all of them until a final concentration of 5%. The supernatant was extracted with 2.5 ml of 1:1 cyclopentane/ethyl acetate using 5-ml glass tubes, 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 was filtered through 13-mm nylon 0.45 µm Minispike filters (Waters Corp.).

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 Corp.) 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 Millennium³² software using authentic SA as standard. Data were corrected for losses in the extraction procedure, and recovery of metabolites ranged between 50 and 80%.

Statistical analysis.

Results shown in Figures 1 to 5 and 7 correspond to means $\pm$ standard error of three independent plants from a representative experiment. For the statistical analysis, data coming from three independent growth experiments were used, which corresponds to a total of nine individuals (three from each experiment). For the bacterial growth analysis shown in Figure 6, five individual plants per time were inoculated, and the corresponding statistical analysis was performed for a total of 15 individuals.

Comparison between WT and each transgenic line were performed by a *t*-test analysis, using Excel from Microsoft's Office Suite. A *P* value < 0.05 was considered as significant. The *P* values coming from the *t*-test analysis are presented in Supplementary Table S2.

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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.

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

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

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

Clarke, D. D. 1982. Active defence mechanisms in plants. Pages 321-322 in: *The Accumulation of Cinnamic Acid Amides in the Cell Walls of Potato Tissue as an Early Response to Fungal Attack*. R. K. S. Wood, ed. Plenum Press, New York.

Coego, A., Ramirez, V., Ellul, P., Mayda, E., and Vera, P. 2005. The H₂O₂-regulated Ep5C gene encodes a peroxidase required for bacterial speck susceptibility in tomato. *Plant J.* 42:283-293.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Kawashima, S., Hayashi, M., Takii, T., Kimura, H., Zhang, H. L., Nagatsu, A., Sakakibara, J., Murata, K., Oomoto, Y., and Onozaki, K. 1998. Serotonin derivative, *N*-(*p*-coumaroyl) serotonin, inhibits the production of TNF- α , IL-1 α , IL-1 β , and IL-6 by endotoxin-stimulated human blood monocytes. *J. Interferon Cytokine Res.* 18:423-428.

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

- Lee, D. E., Kang, K., Lee, S. G., and Back, K. 2007. Enhanced synthesis of feruloyltyramine and 4-coumaroyltyramine is associated with tyramine availability in transgenic rice expressing pepper tyramine *N*-hydroxycinnamoyltransferase. *Plant Sci.* 172:57-63.
- Lee, D. G., Park, Y., Kim, M. R., Jung, H. J., Seu, Y. B., Hahm, K. S., and Woo, E. R. 2004. Antifungal effects of phenolic amides isolated from the root bark of *Lycium chinense*. *Biotechnol. Lett.* 26:1125-1130.
- Lisón, P., Rodrigo, I., and Conejero, V. 2006. A novel function for the cathepsin D inhibitor in tomato. *Plant Physiol.* 142:1329-1339.
- Liyama, K., Lam, T. B. T., and Stone, B. A. 1994. Covalent cross links in the cell wall. *Plant Physiol.* 104:315-320.
- López-Gresa, M. P., Torres, C., Campos, L., Lisón, P., Rodrigo, I., Bellés, J. M., and Conejero, V. 2011. Identification of defence metabolites in tomato plants infected by the bacterial pathogen *Pseudomonas syringae*. *Environ. Exp. Bot.* 74:216-228.
- Luo, J., Fuell, C., Parr, A., Hill, L., Bailey, P., Elliott, K., Fairhurst, S. A., Martin, C., and Michael, A. J. 2009. A novel polyamine acyltransferase responsible for the accumulation of spermidine conjugates in Arabidopsis seed. *Plant Cell* 21:318-333.
- Ly, D., Kang, K., Choi, J. Y., Ishihara, A., Back, K., and Lee, S. G. 2008. HPLC analysis of serotonin, tryptamine, tyramine, and the hydroxycinnamic acid amides of serotonin and tyramine in food vegetables. *J. Med. Food* 11:385-389.
- Mallona, I., Weiss, J., and Marcos, E. C. 2011. pcrEfficiency: A Web tool for PCR amplification efficiency prediction. *BMC Bioinformatics* 12:404.
- Martin-Tanguy, J., Cabanne, F., Perdrizet, E., and Martin, C. 1978. Distribution of hydroxycinnamic acid-amides in flowering plants. *Phytochemistry* 17:1927-1928.
- Martin-Tanguy, J., Negrel, J., Paynot, M., and Martin, C. 1987. Hydroxycinnamic acid amides, hypersensitivity, flowering and sexual organogenesis in plants. New York.
- Métraux, J. P., and Raskin, I. 1993. Role of phenolics in plant disease resistance. Pages 191-209 in: *Biotechnology in Plant Disease Control*. Wiley-Liss, New York.
- Muhlenbeck, U., Kortenbusch, A., and Barz, W. 1996. Formation of hydroxycinnamoylamides and α -hydroxyacetovanillone in cell cultures of *Solanum khasianum*. *Phytochemistry* 42:1573-1579.
- Muroi, A., Ishihara, A., Tanaka, C., Ishizuka, A., Takabayashi, J., Miyoshi, H., and 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 Javelle, F. 1997. Purification, characterization and partial amino acid sequencing of hydroxycinnamoyl-CoA: Tyramine *N*-(hydroxycinnamoyl) transferase from tobacco cell-suspension cultures. *Eur. J. Biochem.* 247:1127-1135.
- 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 Martin, C. 1984. The biosynthesis of feruloyltyramine in *Nicotiana tabacum*. *Phytochemistry* 23:2797-2801.
- Negrel, J., Javelle, F., and Paynot, M. 1993. Wound induced tyramine hydroxycinnamoyl transferase in potato (*Solanum tuberosum*) tuber disks. *J. Plant Physiol.* 142:518-524.
- Newman, M. A., von Roepenack-Lahaye, E., Parr, A., Daniels, M. J., and Dow, J. M. 2001. Induction of hydroxycinnamoyl-tyramine conjugates in pepper by *Xanthomonas campestris*, a plant defense response activated by *hrp* gene-dependent and *hrp* gene-independent mechanisms. *Mol. Plant-Microbe Interact.* 14:785-792.
- Park, J. B., and Schoene, N. 2002. Synthesis and characterization of *N*-coumaroyltyramine as a potent phytochemical which arrests human transformed cells via inhibiting protein tyrosine kinases. *Biochem. Biophys. Res. Commun.* 292:1104-1110.
- Pearce, G., Marchand, P. A., Griswold, J., Lewis, N. G., and Ryan, C. A. 1998. Accumulation of feruloyltyramine and *p*-coumaroyltyramine in tomato leaves in response to wounding. *Phytochemistry* 47:659-664.
- Roh, J. S., Han, J. Y., Kim, J. H., and Hwang, J. K. 2004. Inhibitory effects of active compounds isolated from safflower (*Carthamus tinctorius* L.) seeds for melanogenesis. *Biol. Pharm. Bull.* 27:1976-1978.
- Schmidt, A., Scheel, D., and Strack, D. 1998. Elicitor-stimulated biosynthesis of hydroxycinnamoyltyramines in cell suspension cultures of *Solanum tuberosum*. *Planta* 205:51-55.
- Schmidt, A., Grimm, R., Schmidt, J., Scheel, D., Strack, D., Rosahl, S. 1999. Cloning and expression of a potato cDNA encoding hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl) transferase. *J. Biol. Chem.* 274:4273-80.
- Strack, D. 1997. Phenolic metabolism. Pages 387-416 in: *Plant Biochemistry*, P. M. Dey and J. B. Harborne, eds. Academic Press, London.
- Tebayashi, S. I., Horibata, Y., Mikagi, E., Kashiwagi, T., Mekuria, D. B., Dekebo, A., Ishihara, A., and Kim, C. S. 2007. Induction of resistance against the leafminer, *Liriomyza trifolii*, by jasmonic acid in sweet pepper. *Biosci. Biotechnol. Biochem.* 71:1521-1526.
- Tornero, P., Gadea, J., Conejero, V., and Vera, P. 1997. Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. *Mol. Plant Microbe Interact.* 10:624-634.
- van Loon, L. C., Rep, M., and Pieterse, C. M. J. 2006. Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* 44:135-162.
- Villegas, M., and Brodelius, P. E. 1990. Elicitor induced hydroxycinnamoyl-CoA: Tyramine hydroxycinnamoyltransferase in plant-cell suspension cultures. *Physiol. Plant.* 78:414-420.
- von Roepenack-Lahaye, E., Newman, M. A., Schornack, S., Hammond-Kosack, K. E., Lahaye, T., Jones, J. D. G., Daniels, M. J., and Dow, J. M. 2003. *p*-coumaroylnoradrenaline, a novel plant metabolite implicated in tomato defense against pathogens. *J. Biol. Chem.* 278:43373-43383.
- Yu, M., and Facchini, P. J. 1999. Purification, characterization, and immunolocalization of hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase from opium poppy. *Planta* 209:33-44.
- Zacarés, L., López-Gresa, M. P., Fayos, J., Primo, J., Bellés, J. M., and Conejero, V. 2007. Induction of *p*-coumaroyldopamine and feruloyldopamine, two novel metabolites, in tomato by the bacterial pathogen *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 20:1439-1448.
- Zhang, H. L., Nagatsu, A., Watanabe, T., Sakakibara, J., and Okuyama, H. 1997. Antioxidative compounds isolated from safflower (*Carthamus tinctorius* L.) oil cake. *Chem. Pharm. Bull.* 45:1910-1914.