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Pathogen-Induced Accumulation of an Ellagitannin Elicits Plant Defense Response

Alicia Mamaní,[1] María Paula Filippone,[2] Carlos Grellet,[1] Björn Welin,[2] Atilio Pedro Castagnaro,[2] and Juan Carlos Díaz Ricci[1]

[1]Instituto Superior de Investigaciones Biológicas (INSIBIO; CONICET- UNT) e Instituto de Química Biológica "Dr. Bernabé Bloj", Universidad Nacional de Tucumán, Chacabuco 461, 4000, San Miguel de Tucumán, Argentina; [2]Sección Biotecnología de la Estación Experimental Agroindustrial Obispo Colombres (EEAOC)-Unidad Asociada al INSIBIO, Av. William Cross 3150, Las Talitas, 4101, Tucumán, Argentina

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In an incompatible interaction between *Colletotrichum fragariae* and strawberry plants, the accumulation of phenolic compounds in plant leaves was observed. A particularly abundant penta-esterified ellagitannin that accumulated in response to pathogen attack was identified as 1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose (HeT) by mass spectroscopy and nuclear magnetic resonance. Foliar application of purified HeT prior to inoculation with a virulent pathogen was shown to increase resistance toward *C. acutatum* in strawberry plants and to *Xanthomonas citri* subsp. *citri* in lemon plants. The induced resistance in strawberry was associated with a rapid oxidative burst, callose deposition, a transient increase of salicylic acid in phloem, and induction of gene expression responsive to salicylic acid. Results obtained suggested that HeT could be a common plant defense response molecule capable of inducing pathogen resistance in different plant species.

Production of plant secondary metabolites can be developmentally regulated and they can be differentially produced in various tissues and organs. Among numerous metabolic and physiological functions, secondary metabolites are also involved in plant–environment interactions. Secondary metabolites can serve to attract animals for pollination and seed dispersal, as protection against UV, and as key components of active and potent defense mechanisms that help plants to protect themselves against pathogens (viruses, bacteria, and fungi) and herbivores (arthropods and vertebrates) (Wink 2003; Wu and Baldwin 2009). Although many secondary metabolites involved in plant defense are constitutive (Bednarek and Osbourn 2009; Filippone et al. 1999), some are specifically induced upon challenge by a hostile organism (Van Etten et al. 1994). Secondary metabolites may act directly by exerting toxic effects on the aggressor (Dixon 2001) or indirectly as elicitors of plant defense (Dangl and Jones 2001).

Defense responses may be specifically induced as in gene-for-gene-type interactions, leading to what is called effector triggered immunity (ETI). This type of response often but not always involves a form of localized cell death termed hypersensitive response which can effectively prevent pathogen spread. In addition, defense responses can also be triggered upon recognition of conserved microbial elicitors called pathogen-associated molecular patterns (PAMPs), leading to PAMP-triggered immunity (Dodds and Rathjen 2010). Despite differences in activation mechanisms, molecules, and signaling pathways involved in response to distinct elicitors within different plant species, a number of physiological reactions and accumulation of specific compounds are common features associated with defense responses. These include, among others, a rapid influx of calcium ions from external stores, production of reactive oxygen species (ROS), reinforcement of the cell wall by deposition of callose and lignin at sites of attempted infection, accumulation of soluble and cell-wall-bound phenolic compounds, and activation of gene expression, in both local and distant parts of the plant. A multitude of molecules of biotic, abiotic or synthetic origin have been reported to induce resistance in plants, including 2,6-dichloroisonicotinic acid, salicylic acid (SA), jasmonic acid (JA), benzothiadiazole (BTH), aminobutyric acid, and inorganic salts (Cohen 2001; Kessman et al. 1994; Ton et al. 2005). The SA and JA-ethylene (ET) hormone pathways have been shown to be important regulators of defense-gene expression (Bari and Jones 2009). Whereas SA is considered to be involved in resistance to biotrophic pathogens, the JA-ET pathway is activated in response to necrotrophic pathogens and insects. Although there is considerable crosstalk between these different signaling pathways, several genes have been shown to act as specific markers for the activation of each of the pathways (Bostock 2005; Kunkel and Brooks 2002).

Strawberry fruit is exceptionally rich in phenolic compounds such as flavonols (kaempferol and quercetin derivatives), anthocyanidins (cyanidin and pelargonidin derivatives), proanthocyanidins, hydrolyzable tannins (galloylglucoses and ellagitannins), and phenolic acids, some of which (i.e., flavonoids) contribute to plant defense (Halbwirth et al. 2006; Hanhineva et al. 2009; Määttä-Riihinen et al. 2004; Terry et al. 2004). Although accumulation of phenolic compounds has been correlated with resistance against several pathogens in strawberry, potential mechanisms involved are poorly understood (Amil-Ruiz and associates [2011] provide an extensive and comprehensive review). Yamamoto and associates (2000) reported that (+)-catechin, which is a normal component of strawberry leaves, was accumulated in response to inoculation with nonpathogenic spores of *Alternaria alternata* inhibiting the formation

A. Mamaní and M. P. Filippone contributed equally to this work.

Corresponding authors: A. P. Castagnaro; Telephone: +54-381-4521000 ext. 162; Fax: +54-381-4521000 int. 604; E-mail: atilio@eeaoc.org.ar; and J. C. Díaz Ricci; E-mail: juan@fbqf.unt.edu.ar; Telephone: +54-381-4248921.

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of infectious hyphae from fungal appressoria. Hukkanen and associates (2007) reported that BTH induced the accumulation of phenolic compounds, particularly ellagitannins, in leaves and fruit of 'Jonsok' strawberry, improving resistance toward powdery mildew infection under greenhouse conditions. However, most studies in strawberry have been done principally in the fruit, and relatively little is known about phytochemical accumulation in other organs such as root and flower (Hanhineva et al. 2008; Nemec 1976) or leaves (Himanen et al. 2005; Hukkanen et al. 2007), which have more relevance in the health of the whole plant.

We have previously reported that the inoculation of *Fragaria* × *ananassa* 'Pájaro' with an avirulent isolate (F7) of the pathogen *Colletotrichum fragariae* 72 h prior to inoculation with a virulent isolate (M11) of *C. acutatum* (responsible for the anthracnose disease in 'Pájaro') prevented the growth of the latter and disease development (Salazar et al. 2007). In this work, we describe that an ellagitannin is accumulated in leaves during the incompatible interaction with the avirulent isolate F7. The biosynthesis of hydrolyzable tannins (ellagitannin and gallotannin) has been extensively reviewed by Niemetz and Gross (2005) and by Feldman (2005). We characterized this compound as 1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose (HeT) and obtained evidence that treating plants with HeT not only protected the strawberry plants against the infection of the virulent pathogen M11 but also lemon plants against *Xanthomonas citri* subsp. *citri,* the causal agent of citrus bacterial canker disease. This effect is accompanied in the strawberry plants by a rapid oxidative burst, callose deposition, stomatal closure, increased levels of SA, and changes in gene expression typically associated with plant defense.

RESULTS

An ellagitannin is the major phenolic compound induced in the incompatible interaction between strawberry and F7 isolate of *C. fragariae.*

Leaves of strawberry 'Pájaro' plants were inoculated with conidia of the avirulent isolate F7 or the virulent isolate M11 of *Colletotrichum* spp. Leaf methanolic extracts were obtained and total soluble phenolic compounds were analyzed during the first 9 days after inoculation (dai). Total soluble phenolic content in leaves of plants inoculated with the avirulent F7 isolate transiently increased approximately fourfold 2 dai, compared with leaves of plants inoculated with the virulent M11 isolate or water-treated controls (Fig. 1A). A high-performance liquid chromatography (HPLC) elution profile of the leaf phenolic fraction showed one major peak, which was noticeably increased in the incompatible interaction (F7) (Fig. 1B). Electrospray ionization (ESI) and atmospheric pressure chemical

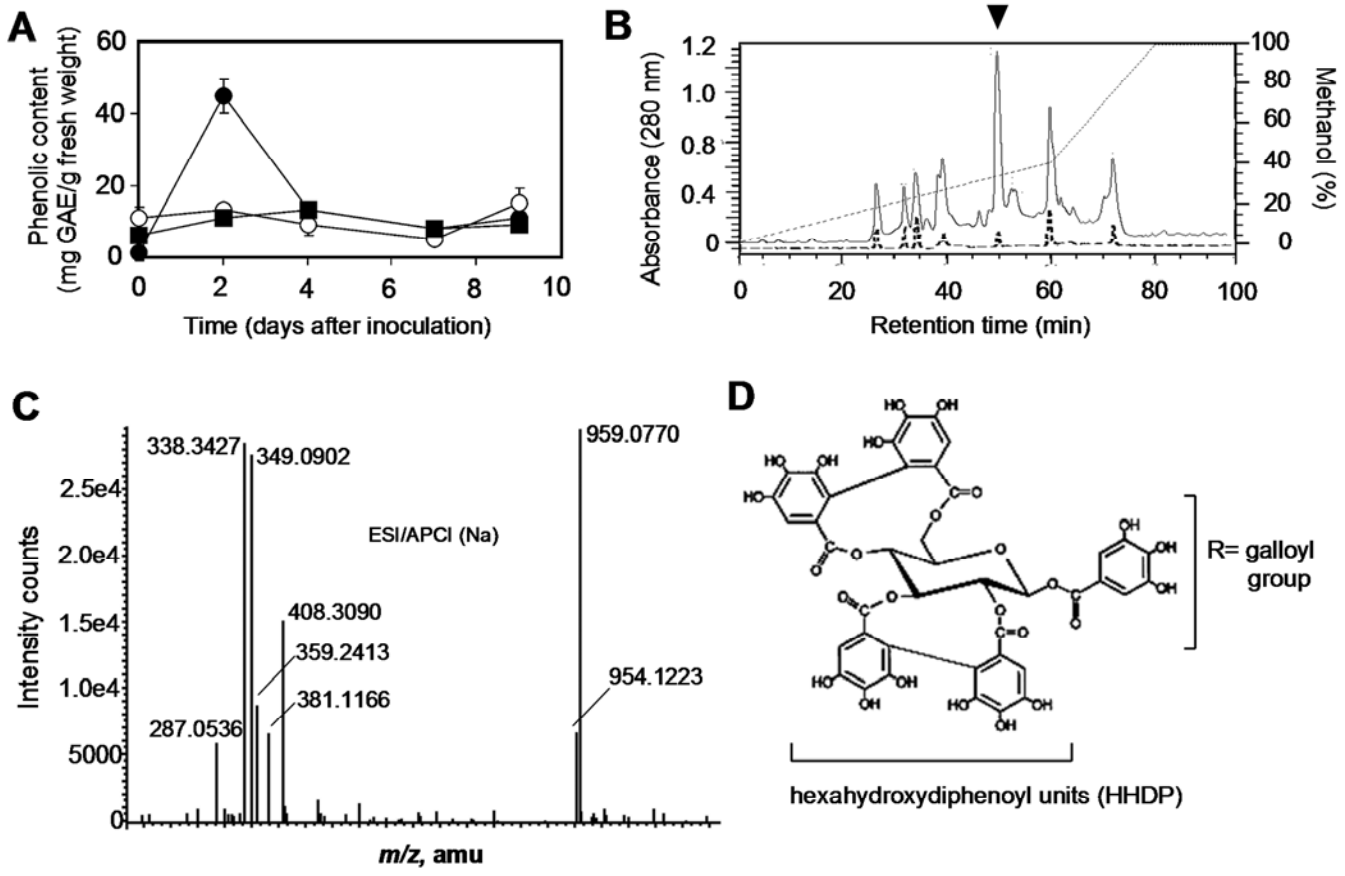

Fig. 1. Purification of phenolic compounds accumulated during an incompatible interaction between strawberry and *Colletotrichum* spp. **A,** Total phenolic content from leaf methanolic extracts from plants inoculated with an avirulent isolate F7 of *Colletotrichum fragariae* (filled circle), a virulent isolate M11 of *C. acutatum* (empty circle), and water-treated control plants (filled square), determined by the Folin-Ciocalteu method. Determinations were carried out until 9 days after inoculation, when disease symptoms were evident. **B,** Reverse-phase high-performance liquid chromatography analysis of strawberry leaf phenolic fraction 2 days after inoculation with the avirulent isolate F7 of *C. fragariae.* Compounds were detected by UV light absorbance at 280 nm. Chromatography was performed using a gradient methanol/water. The major peak (arrow) eluted at 48 min. A chromatogram of a leaf fraction from noninoculated plants assayed under the same experimental conditions is shown in dotted line. **C,** Electrospray ionization–mass spectrometry analysis of the pure chromatographically majority compound exhibited a prominent (MNa+) signal at *m/z* 959.0770. **D,** Chemical structure of the pure compound based on ^{1}H-nuclear magnetic resonance (NMR) (500 MHz) and ^{13}C-NMR (125 MHz) analysis. This compound corresponds to an ellagitannin named 1-*O*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose.

ionization (APCI) (Na^+)–mass spectrometric detection (MS) analysis of the pure compound exhibited a peak at *m/z* of 959.0770, which corresponded to a molecular formula of $C_{41}H_{28}O_{26}$ Na (Fig. 1C). UV spectral analysis of the compound in methanol indicated absorption peaks at 220 and 275 nm, suggesting a molecule with an ellagitannin skeleton. The ^{13}C and ^{1}H-nuclear magnetic resonance (NMR) spectra showed signals corresponding to a glucosyl group which was identified as β-D-glucopyranose by the coupling constant *J*H-H (8.5 to 10 Hz). Further heteronuclear multiple bond correlation (HMBC) NMR analyses showed that the glucosyl group was fully esterified with galloyl groups (R). The correlations among the C-carbonilic groups of each R unit with the four aromatic H (H6) enabled us to establish two hexahydroxydiphenoyl (HHDP) units at 2,3 and 4,6 positions. Thus, the pure compound corresponded to an 1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose (Fig. 1D) of the ellagitannin group, named as HeT in this work. Two isomers, casuarictin and potentillin (Gupta et al. 1982; Haddock et al. 1982; Okuda et al. 1981, 1982, 1983), which respond to the same molecular formula and chemical structure as HeT have previously been reported in strawberry (Fait et al. 2008; Hukkanen et al. 2007; Oertel et al. 2001).

HeT protects strawberry plants against a virulent pathogen by induction of defense responses.

In order to study the potential role of HeT in the resistance of strawberry to *Colletotrichum* spp. infection, plants were treated with the purified compound by foliar application prior to infection with the virulent pathogen. An aqueous solution of 20 μM HeT was sprayed only onto the oldest trifoliated leaf of strawberry plants (Fig. 2A) and, in order to rule out any possible direct effects of HeT on the pathogen, evolution of symptoms was evaluated in the rest of the plant. Seven days after HeT treatment, the entire canopy of plants was sprayed with a conidia suspension of the virulent isolate of *C. acutatum* (M11). Plants treated with HeT showed high levels of protection 2 weeks after inoculation (Fig. 2B), compared with water-treated plants that exhibited severe disease symptoms. Control experiments carried out with plants treated with SA prior to inoculation with isolate M11 showed some degree of protection, indicating that SA can activate defense responses in strawberry plants, as previously was reported in strawberry fruit, which enhanced resistance to pathogens such as *Botrytis cinerea* (Asghari and Aghdam 2010; Babalar et al. 2007). The degree of protection obtained with the different treatments was quantified as percentage of healthy leaves (Fig. 2C). Pretreatment with HeT gave the highest protection levels, resulting in plants showing 80% healthy leaves, whereas SA-treated plants only showed approximately 40% of nonaffected leaves. Non-inoculated control plants pretreated with water were considered as 100% protection. Additionally, when plants were treated with a crude methanolic extract (0.5% wt/vol) containing phenolic compounds, a protection level similar to that for plants treated with purified HeT was obtained (data not shown). The extract concentration was determined based on the actual content of HeT in the crude methanolic extract, in order not to exceed the concentration of purified HeT used in plant treatments.

Cellular and molecular markers related to plant defense responses were investigated in distal young leaves of strawberry

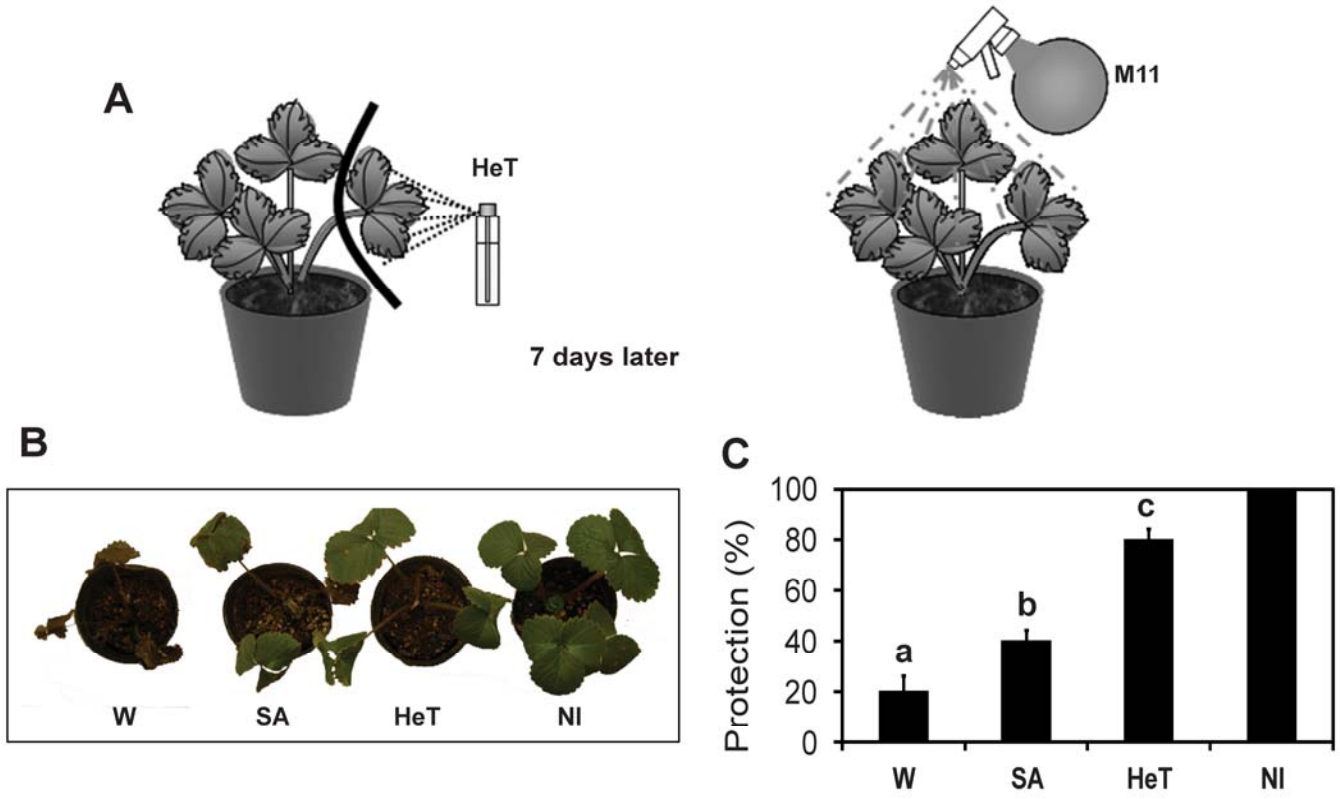

Fig. 2. Effect of 1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose (HeT) protecting strawberry plants against a virulent isolate of *Colletotrichum acutatum* (M11). **A,** One trifoliated leaf of a 16-week-old strawberry plant was pretreated by spraying with a 20 μM HeT aqueous solution 7 days before whole-plant inoculation with a 1.5×10^6 conidia ml^{-1} suspension of the virulent M11 strain. **B,** Appearance of plants pretreated with water (W), salicylic acid (SA), and HeT and later inoculated with the M11 pathogen. NI = not inoculated, plant pretreated with water. The image shows disease symptoms at 20 days after pathogen inoculation. **C,** Plant protection obtained by different treatments expressed as percentage of healthy plants. Bar graphs represent mean ± standard deviation from three independent experiments using six plants for each treatment. Different letters indicate statistically different values (Tukey's test, $P < 0.05$).

plants treated with HeT, in order to study the induced defense mechanism. The production of ROS has been shown to precede or accompany the development of defense responses (Apel and Hirt 2004; Doke 1983). Thus, accumulation of hydrogen peroxide and anion superoxide was determined in strawberry leaves at different times after spray treatment with 20 µM HeT. High amounts of ROS in localized areas of mesophyll and vascular bundles of leaves were detected within the first hours after treatment, with maximum production at 4 h after treatment (hat) and returning to basal levels at 8 hat (Fig. 3A). No ROS were produced at detectable levels in water-treated control plants or M11-inoculated plants (data not shown).

Callose is a β-(1,3)-glucan with (1,6) modifications, whose biosynthesis is enhanced by mechanical wounding and physiological stress and as part of the defense response against some pathogens (Donofrio and Delaney 2001; Hamiduzzaman et al. 2005; Luna et al. 2011). Therefore, to test whether callose synthesis is associated with the protection observed in strawberry plants treated with HeT, leaves were stained with aniline blue to detect callose deposition. Cytological observations were performed using ultraviolet fluorescence microscopy. In leaves of plants treated with HeT, brilliant points corresponding to callose deposits were observed compared with leaves of water-treated control plants. When plants were inoculated with the virulent isolate M11 7 days after HeT treatment (HeT+M11), a greater density of brilliant points was observed. In contrast, no staining was detected in M11-inoculated control plants (Fig. 3B).

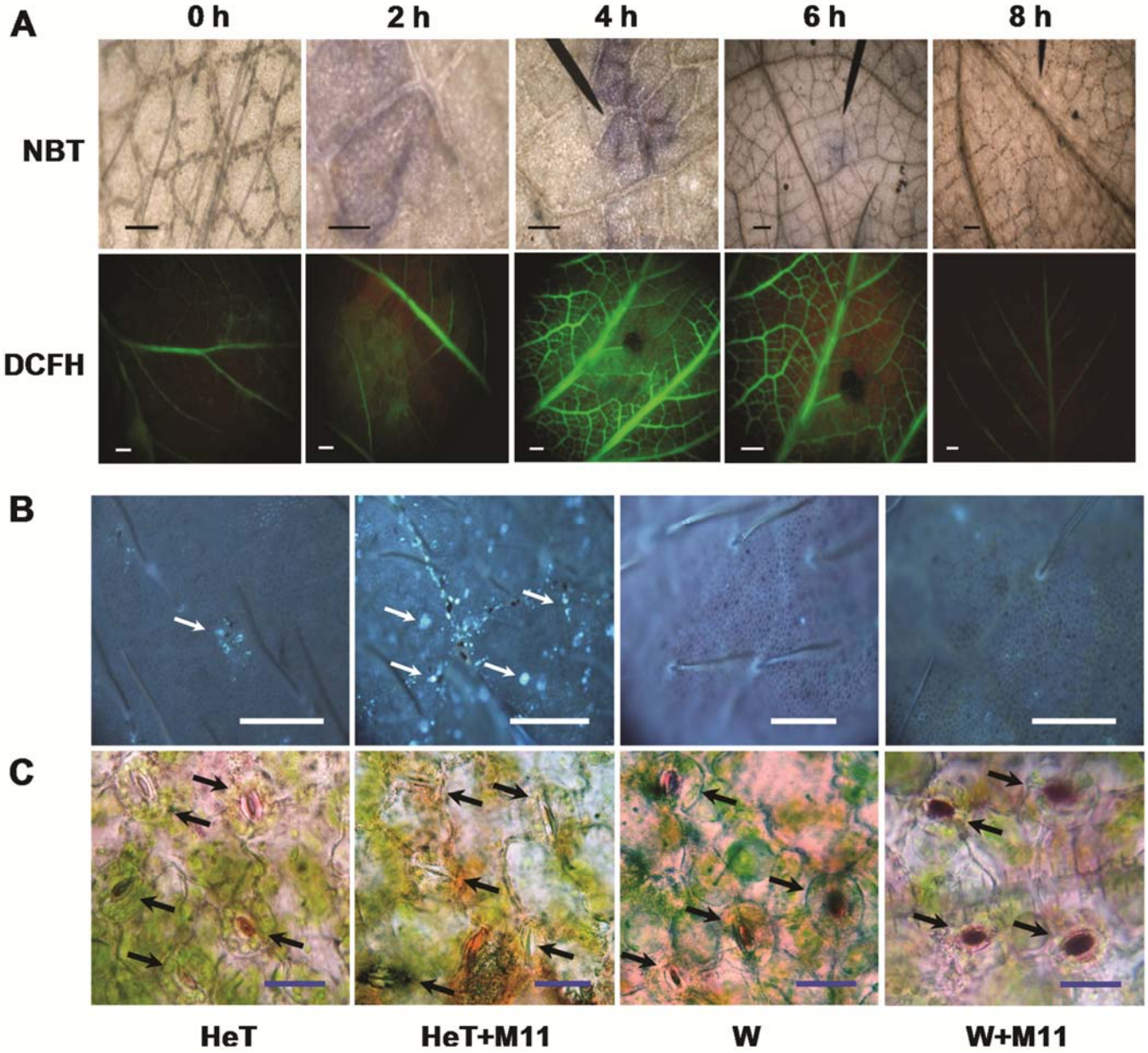

Fig. 3. Cellular and biochemical changes associated with 1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose (HeT)-induced pathogen resistance in strawberry. **A,** Histochemical detection of reactive oxygen species (ROS) induced in leaflets of strawberry 'Pájaro' treated with HeT. The temporal production of ROS was evaluated until 8 h after treatment. Superoxide anion was determined as blue spots after nitroblue tetrazolium (NBT) dying. The hydrogen peroxide appeared as a brilliant green area through 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) detection. In both cases, the increase of ROS was coincident, showing a maximum 4 h after HeT treatment. Scale bar equals 100 µm. **B,** Detection of callose deposits by staining with aniline blue, observed as brilliant dots by fluorescence microscopy (white arrows), was evaluated in strawberry plants pretreated with HeT or water and later inoculated with M11 strain (HeT+M11 and W+M11), and in plants pretreated with HeT or water but not inoculated with M11 (HeT and W). Scale bar corresponds to 100 µm. **C,** Stomata appearance (black arrows) captured by photography was taken after the same treatments as outlined in B. Scale bar corresponds to 10 µm.

In a plant–pathogen interaction study, it was reported that stomata can play an active role in limiting pathogen invasion as part of the plant defense and that the stomata status can be mediated by ROS and SA (Melotto et al. 2006). Because ROS levels increased in plants treated with HeT, the stomata status was investigated. Treatment with HeT results in stomata closure (Fig. 3C), an effect that was even more pronounced in plants treated with HeT and later inoculated with M11 (HeT+ M11). In water-treated control plants or M11-inoculated plants, most of the stomata were open.

It is well known that SA plays a central role in mediating systemic acquired resistance (SAR) (Ryals et al. 1996; Vlot et al. 2009). Therefore, we conducted experiments to determine whether SA was involved in HeT-mediated protection measuring free SA levels in phloem fluid from strawberry. SA increased in the phloem after treatment with HeT, reaching a maximum concentration at 48 hat, and water-treated or M11-inoculated plants did not accumulate SA (Fig. 4A). This result is consistent with a potential role for SA in HeT-induced resistance.

With the aim of gaining insight into SA-signaling pathways involved in the HeT-induced defense responses, expression of the SA-associated molecular marker Fa-*PR1* gene (Grellet-Bournonville et al. 2012) was evaluated. Fa-*PR1* exhibited a rapid and significant increase in expression during the first 48 hat, in concordance with the levels of free SA in the phloem, followed by a decrease of mRNA accumulation after that time point (Fig. 4B).

HeT protects lemon plants against *X. citri* subsp. *citri.*

To evaluate whether HeT could induce defense responses in plant species other than strawberry and generate resistance toward other plant pathogens, lemon (*Citrus limon)* plants were sprayed with a 20 µM HeT solution 15 days before inoculation with *X. citri* subsp. *citri*. *X. citri* subsp. *citri* is the etiological agent of citrus canker, a serious disease in many citrus species causing lesions on leaves, stems, and fruit. Bacterial inoculation was performed by spraying leaves with a suspension of 10^8 CFU/ml. Plants were maintained under controlled conditions for 35 dai and disease progression was monitored both phenotypically (Fig. 5A) and by measuring bacterial growth at different time points (Fig. 5B). A clear decrease of disease symptoms was observed in leaves of HeT-treated plants compared with water-treated ones (Fig. 5A). This result was consistent with the bacterial growth, determined as CFU per square centimeter of leaf. Thus, the population of *X. citri* subsp. *citri* in HeT-treated leaves never exceeded 10^7 bacteria at any time tested, whereas water-treated control plants showed bacterial growth above 10^9 at all times and, in most time points, reached growth levels as high as 10^{11} CFU/cm^2.

DISCUSSION

The induction of biosynthetic pathways leading to production of phenolic compounds is one of the reactions associated with plant defense responses (Dixon 2001; Dixon and Paiva 1995; Dixon et al. 2003). Phenolic compounds can participate directly in plant defense as induced or preformed antimicrobial agents (e.g., phytoalexins and phytoanticipins, respectively), whereas others participate indirectly as regulatory signals of the defense response (Bellés et al. 1999; Vlot et al. 2009). Transgenic plants with altered levels of phenolic compounds have provided direct evidence for their importance in mechanisms of plant defense (Hanhineva et al. 2009; Hipskind and Paiva 2000; Shadle et al. 2003, 2007).

We have previously reported that strawberry plants ('Pájaro') inoculated with an avirulent isolate of *Colletotrichum fragariae* (F7) prior to a second inoculation with a virulent isolate of *C. acutatum* (M11) acquires resistance to the virulent strain and shows no disease symptoms (Salazar et al. 2007). This response was correlated with the occurrence of a strong oxidative burst, accumulation of SA, callose deposition, and anatomical changes such as modification of parenchyma cells and accumulation of pigments and amorphous crystals, suggesting that this plant defense response was triggered by the avirulent isolate, resulting in an immunity associated with ETI.

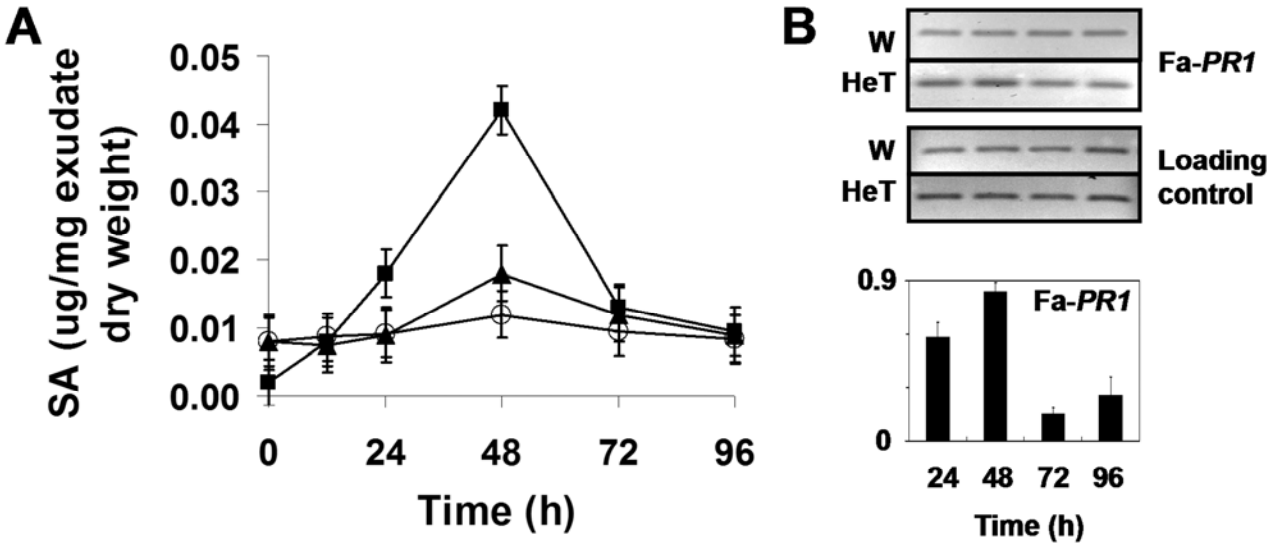

Fig. 4. Salicylic acid (SA) accumulation and Fa-*PR1* expression in strawberry plants treated with 1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose (HeT). **A,** Free SA accumulation in phloem of plants treated with HeT (filled square), treated with water (empty circle), or inoculated with M11 (filled triangle), determined by reverse-phase high-performance liquid chromatography. Levels of free SA were determined at 0, 12, 24, 48, 72, and 96 h after treatments by comparing the fluorescence with an SA standard solution. Each data point shows the average of three replicate samples using three plants per sample. **B,** Transcript accumulation of Fa-*PR1* was characterized after HeT or water (W) spraying on strawberry plants at time points indicated, visualized in agarose gel. To ensure equal loading of cDNA for each sample in polymerase chain reactions, the expression level of *GaPDH*1 gene was used as loading control. Fa-*PR1* expression levels were quantified by analyzing band intensities on gel digital images using Total Lab Quant software, and plotted in reference to the expression level of water-treated plants.

In this study, we demonstrate that, during the incompatible interaction between strawberry plants and the avirulent isolate F7, an increased production of total soluble phenolic compounds was detected in leaves of inoculated plants. By using MS and UV spectral analyses, and available literature, the compound that showed the most significant increase was identified as HeT, an ellagitannin with two units of HHDP. This result suggested a possible role for this phenolic compound in strawberry defense response.

Tannins are usually present in plants in much larger amounts than other phenolics, and are believed to be major components of plant chemical defenses. They have been shown to act as antioxidants; regulate the plant cell redox balance; provide protection against UV; and act as antimicrobials, insecticides, and feeding deterrents (Lattanzio et al. 2009; McAllister et al. 2005; Salminen and Karonen 2011). However, to our knowledge, there is no report showing that these compounds directly participate in the activation of plant defense as either direct inducers or mediators of the response. Instead, phenolic compounds other than tannins have been associated with induction of plant defense responses. One of the best-characterized compounds is SA, which plays a central role in mediating SAR (Ryals et al. 1996). Bellés and associates (1999) reported that, in tomato, in addition to SA, gentisic acid strongly accumulated upon non-necrotizing infections and that, when applied exogenously, induced the activation of antifungal pathogenesis-related (PR) proteins as part of a plant defense response.

Because HeT showed the most significant increase in the incompatible interaction, we directed our investigation to characterize the participation of this compound in the induction of strawberry defense responses. We show that foliar application of purified HeT on strawberry plants, without the intervention of the pathogen, elicits a defense response in a spatial-temporal way similar to those involved in the ETI response triggered by the avirulent pathogen, resulting in protection against the virulent pathogen. This response includes an early oxidative burst, callose deposition, an increase of free SA in the phloem, and induction of a defense gene associated with the SA-signaling pathway. Taking these results into account, we suggest that HeT plays an active role in the defense response in strawberry. Nevertheless, it remains to be tested whether the HeT is part of the signal transduction cascade or indirectly modulates some common compound or event such as the redox potential.

It has been reported that compounds that result from the hydrolysis of HeT, such as gallic acid, could induce ROS (Rudrappa et al. 2007) and, theoretically, a defense response. However, we suggest that the results presented in this study originate from the complete molecule of HeT because an aqueous solution of HeT is both light and heat stable (60°C) and HPLC analysis of HeT used in our experiments confirmed the absence of free gallic or ellagic acid. However, we cannot completely rule out the possibility that those products from hydrolysis of HeT could be released later than 2 dai with the avirulent pathogen or that a hydrolysis of HeT could occur inside the plant tissues (if HeT is incorporated once in the exogenous application), as reported for plants exogenously treated with aspirin, methyl JA, or methyl indole-3-acetic acid ester (Popova et al. 1997; Wu et al. 2008; Yang et al. 2008). In order to corroborate these two hypotheses, more experiments are required.

In addition, we showed that foliar application of SA, a simple phenolic compound with a similar structure of released phenolics after HeT hydrolysis, protects against virulent pathogens but gives a lesser protection compared with HeT treatment. Therefore, we could speculate that the defense response observed in strawberry when treated with HeT is at least partly different and more efficient compared with the effects observed in plants only treated with SA.

It is well known that hydrolyzable tannins can work as plant cell antioxidants that may function as free-radical scavengers to modulate redox balance (Yoshida et al. 1989). The high content of phenolic hydroxyl groups in the HeT molecule indicates that this compound could readily quench free radicals (Rice-Evans et al. 1997). Therefore, it can be speculated that the increase in HeT during the incompatible interaction could indicate a direct participation of HeT in the protection of the plant cell by modulating the redox balance. However, it has also been demonstrated that the anti-oxidative properties of

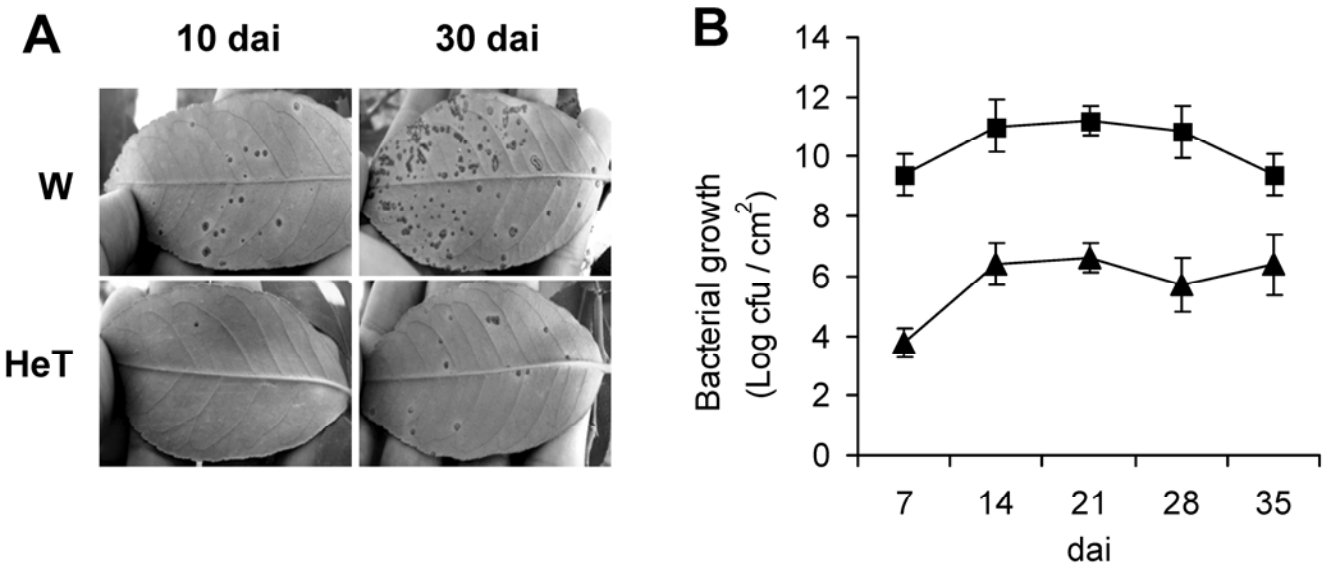

Fig. 5. 1-*0*-Galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose (HeT) protection of *Citrus limon* 'Eureka' plants against *Xanthomonas citri* subsp. *citri* infection. **A,** Macroscopic symptoms (canker) on lower surfaces of lemon leaves 10 and 30 days after inoculation (dai) with *X. citri* subsp. *citri.* Plants were treated by spraying leaf buds of lemon plants with a 20 µM HeT aqueous solution (HeT) or sterile water (control) 15 days before inoculation with a *X. citri* subsp. *citri* suspension of 10^8 CFU/ml. **B,** Bacterial population was determined over the course of 35 dai by homogenizing leaf tissues (1 cm^2) of plants pretreated with HeT (square) or sterile water (triangle) in 10 mM $MgCl_2$ solution followed by dilution plating. Values are expressed as means ± standard deviation from three separate measurements.

tannins may change to pro-oxidative activities at high concentrations and high oxygen partial pressures (Labieniec et al. 2003) or when the cellular pH is changed (Salminen and Karonen 2011). Thus, in the protection against the virulent pathogen observed in strawberry plants treated with HeT, this compound could act as a pro-oxidant, generating the early oxidative burst and leading to the activation of plant defense. In addition, the ROS production could be related to the ability of these compounds to dissociate the cell membrane potential (Choi and Gu 2001; Sirk et al. 2008), creating a proton gradient across the cell membrane. However, the exact mechanism of induction or mediation of the defense response through HeT remains to be determined.

It is noteworthy that, because the HeT pretreatment was applied to a single leaf and the pathogen protective effect was observed in the whole strawberry plant, the induced defense response can be considered systemic. Our results agree with previous reports of plants treated with SA or other compounds used as activators of a systemic defense (Bellés et al. 1999, 2006). The accumulation of free SA and the Fa-*PR1* induction, associated with ROS accumulation observed in leaves distant from the HeT treated leaf, indicates that SA is involved in the systemic defense induced by HeT.

Interestingly, the resistance induced in strawberry by foliarly applied HeT is not specific against *Colletotrichum* spp. because the HeT treatment also increased resistance against a bacterial pathogen in *Citrus limon.* It has been suggested that, although immunity generated in an ETI is induced by a specific effector produced by the pathogen, which is recognized by a host-specific receptor, the signal branches into multiple signaling pathways and, as a consequence, multiple signals enter from different points of the common defense network (Katagiri and Tsuda 2010). In concordance with this hypothesis, we propose that HeT is part of this multiple signal flow and operates as a common signal molecule among plant species.

Our results contribute directly to improving knowledge about mechanisms underlying plant defense responses in strawberry and will undoubtedly initiate increased interest in tannin compounds and their specific role in signaling of defense responses in other plant species. In addition, foliar application of HeT offers an interesting alternative to be used in sustainable crop disease management, because its nonpathogen specificity increases the possibility that HeT-induced resistance will not be readily overcome.

MATERIALS AND METHODS

Plant material.

Micropropagated plants of *Fragaria* × *ananassa* 'Pájaro' (14 to 16 weeks old) were maintained in cabinets at 28°C and 70% relative humidity (RH) with a light cycle of 16 h/day and a light irradiance of 150 μmol s^{-1} m^{-2}. Plants were watered every other day with distilled water. All senescent leaves and petioles were removed periodically until 20 days before the experiments, leaving only four to five young healthy leaves.

Buds were collected from pathogen-free lemon plants, *C. limon* (L.) Burm 'Eureka Frost Nucelar', grown at the Sanitation Center at Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina. Collected buds were grafted on *C. reshni* Hort. ex Tanaka 'Cleopatra Mandarin' seedlings and grown for 6 to 12 months in a greenhouse (18 to 27°C, 16-h light photoperiod, 70% RH). The resulting grafted lemon plants were then used for the experiments.

Plant pathogens.

Strain M11 of *Colletotrichum acutatum* and F7 of *C. fragariae* used in this work correspond to local isolates characterized in our laboratory. Isolates were single-spore propagated to obtain pure cultures on potato dextrose agar (PDA) supplemented with streptomycin (300 μg ml^{-1}) and maintained on PDA slants at 4°C.

The wild-type strain of *X. citri* subsp. *citri* was obtained from cankerous leaves of infected lemon trees in the province of Tucumán, Argentina. The *X. citri* subsp. *citri* strain was cultured at 28ºC in a peptone-yeast extract-malt extract nutrient medium (Vojnov et al. 2001), supplemented with D-glucose to a final concentration of 2% (wt/vol). Bacterial growth was monitored in a spectrophotometer by optical density measurements at 600 nm.

HeT extraction and purification.

Leaves of *Fragaria* × *ananassa* were frozen at –70°C, lyophilized, and ground by a mortar and pestle. The dried plant tissue powder was extracted three time with 80% methanol for 24 h with continuous agitation at 25°C. The homogenate was filtered through a Whatman number 1 paper (Springfield Hill, UK), and the resulting filtrate was thereafter concentrated in a rotary evaporator under reduced pressure at 50°C (Speed Vac Plus SC110A; SAVANT, Hobrook, NY, U.S.A.) The dry extract was resuspended in distilled water and named "crude polar-solvent extract" (CPE). Total phenolic content was determined by the Folin-Ciocalteu method (Singleton and Rossi 1965) and concentration was expressed as gallic acid equivalent (milligrams per gram fresh weight). The CPE was extracted sequentially with *n*-hexane, chloroform, and ethyl acetate. The soluble phenolic compounds present in the ethyl acetate subextract (EASE) were purified by HPLC (Analytical HPLC System, Gilson, Middleton, ID, U.S.A.). EASE was dried in a rotary evaporator and suspended in a final methanol concentration of 20%. Purification was carried out using a C18 column (Prodigy 5 ODS-2; Phenomenex, Torrance, CA, U.S.A.) and solvent A, water in 0.1% trifluoracetic acid (TFA); and solvent B, methanol in 0.1% TFA. Chromatography was performed initially in solvent A, then with a linear gradient of 0 to 40% solvent B over 60 min, followed by a second linear gradient of 40 to 100% B over 30 min. The elution was monitored spectrophotometrically at 280 nm. The major peak dissolved in methanol was analyzed with a Beckman DU 7500 spectrophotometer by measuring the absorbance at 200 to 400 nm.

HeT structure determination.

HeT structure was determined by LC with MS and ^{1}H- and ^{13}C-NMR, together with two-dimensional NMR spectroscopy analyses (correlation spectroscopy, heteronuclear single-quantum correlation, and HMBC). High-resolution mass spectra were recorded on a VG ZAB SE spectrometer. The molecular mass and elemental formula (C, H, and O) were determined with ESI and APCI techniques. Spectral assignment and structure identification was carried out by NMR spectroscopy using a Bruker AVANCE DRX 500 (Bruker BioSpin GmbH, Rheinstetten, Germany) spectrometer operating at 500.13 MHz for proton and 125.77 MHz for carbon, equipped with a 5-mm inverse triple-resonance probe. The samples obtained from preparative HPLC were dissolved in methanol-d4 (CD3OD, 99.96% D). Chemical shifts were referenced to the residual solvent peak at 3.31 ppm for proton and 49.00 ppm for carbon.

Inoculums, inoculations, and plant treatments.

For strawberry inoculations, fungal isolates were grown on PDA for 10 days under continuous fluorescent light at 28°C to induce conidia formation (Smith and Black 1990). The conidia were collected from the culture surface, suspended in sterile distilled water (SDW), diluted to a concentration of 1.5×10^{6} conidia ml^{-1} in SDW with 0.02% Silwet L-77, and sprayed

onto strawberry plants. Immediately after inoculation, plants were placed in an infection chamber at 100% RH, 28°C, and 48 h of darkness. Plants were then returned to a growth chamber with 70% RH, 28°C, and a photoperiod (white fluorescent, 450 μmol m^{-2} s^{-1}) of 16 h light day^{-1}. Treated plants were monitored for disease development for 30 dai, in which symptom appearance versus control of disease were reported as the relation between number of healthy leaves compared with total number of leaves. For experiments with HeT in strawberry, only the oldest leaves of plants were sprayed with 20 μM HeT in distilled water, 5 mM SA, or water, 7 days prior to inoculation with *C. acutatum*.

For assays with lemon, plants were treated with either a solution of 20 μM HeT or sterile water by spraying the leaf buds 15 days before the inoculation with *X. citri* subsp. *citri*. Inoculation was carried out by spraying a *X. citri* subsp. *citri* suspension of 10^8 CFU ml^{-1} in 10 mM $MgCl_2$ and 0.02% Silwet L-77 onto the lamina of younger lemon leaves. This inoculation method mimics the natural *X. citri* subsp. *citri* infection process, in which bacteria enter the leaf via stomata, followed by colonization of the apoplasm. Inoculated plants were maintained for 35 days in a growth cabinet, with temperatures of 25 to 28°C, high humidity, a photoperiod of 16 h of light, and a light intensity of 150 to 200 (μmol m^{-2} s^{-1}). Disease progression was monitored both phenotypically and by bacterial population growth analysis (Siciliano et al. 2006). To assess bacterial development in a plant, three 1-cm^2 leaf disks were selected randomly from the inoculated leaves at different days after inoculation and bacterial populations were monitored by triplicate as previously described (Yun et al. 2006). Ten young leaves per plant and three plants per treatment were inoculated. All experiments were carried out in triplicate.

Oxidative burst.

Determination of ROS was performed on plant leaves treated with 20 μM HeT, water, or *C. acutatum* (M11) as controls at 0, 2, 4, and 6 hat. Generation of superoxide anion was detected using a superoxide-dependent reduction of nitroblue tetrazolium according to Doke (1983). Microscopic observations and images were taken with an Olympus BH-2 microscope. Hydrogen peroxide was detected by a peroxidase-dependent in situ histochemical staining procedure using 2′,7′-dichlorodihyrofluorescein diacetate (H_2DCFDA). For determinations, leaves were submerged in loading buffer (Tris KCl at 10 and 50 mM, pH 7.2) and 50 μl of 10 mM H_2DCFDA, infiltrated by vacuum for 3 min, and maintained in the dark for 20 min. Leaves were then removed and placed in fresh loading buffer to wash off excess dye and examined under fluorescence microscopy using a Leica MZ FLIII fluorescence stereomicroscope (Leica, Rueil Malmaison, France) (excitation filter 470/40 nm, barrier filter 515 nm).

Determination of SA.

Phloem exudates were collected with 50-μl capillary pipettes from the cut ends of petioles of strawberry plants. Known volumes of exudates were collected into three volumes of cold absolute ethanol to precipitate proteins and other high molecular weight compounds, and the insoluble materials were removed by centrifugation (5 min at 10,000 × *g*). The ethanol extract was vacuum-dried and dissolved in methanol (100%). Free SA present in the phloem was determined by reverse-phase HPLC by comparison with a standard solution of known concentration, using a C18 column eluded with a linear water-methanol gradient of 20 to 100% in 0.1% TFA over 20 min. The elution was monitored at 302 nm. The peak corresponding to the elution time of the SA standard was collected, vacuum dried, and suspended in 30% methanol for subsequent quantification. Quantitative analysis was performed with a PC1 fluorometer (Photon Counting 1, ISS, Owingen, Germany) at excitation wavelength = 296 nm and emission wavelength = 406 nm. Each data point reported corresponds to the average value of three replicate samples (three plants per sample). The value of each replicate is the average of 20 fluorescent readings taken over 20 s. The detection limit of SA in a final volume of 1 ml was 0.2 nmol.

Callose staining and stomata status determination.

Strawberry plants pretreated with HeT or water were inoculated with *C. acutatum* (M11) strain 7 days after treatment, placed in an infection chamber (28°C, 100% RH without light) for 2 days, and thereafter transferred back to normal growth conditions (28°C, 70% RH, and a photoperiod of 16 h). Twenty-four hours later, callose and stomata status were analyzed. Plants pretreated with HeT or water but not inoculated with M11 were subdued to exactly the same conditions as described above. Callose staining was performed as described by Hauck and associates (2003). Chlorophyll was removed from leaves using ethanol and then placed in water before staining for 30 min with 0.01% aniline blue (Sigma-Aldrich, St. Louis) in 150 mM K_2HPO_4 (pH 9.5). Samples were mounted in 50% glycerol and examined with a Nikon Eclipse E800 photomicroscope with an A4 fluorescence cube. For the stomata status determination, epidermis strips from the underside of fully expanded young leaves were peeled off, stained with a neutral red dye, and immediately mounted in a drop of oil on a microscope slide and examined with an optical microscope (Leica DM IL, Wetzlar, Germany). For each determination, three strips were taken from three different leaves and 10 microscope fields chosen at random were analyzed. The experiment was repeated twice and images were captured with a digital camera (COOLPIX990; Nikon, Japan).

RNA isolation and evaluation of *Fa-PR-1* gene expression.

Total RNA from leaves of strawberry treated with HeT was isolated at 24, 48, 72, and 96 hat following the protocol described by Iandolino and associates (2004). Retrotranscription reactions were carried out using 5 μg of DNase-treated total RNA using SuperScript II RT (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. To evaluate the relative level of expression of genes, a reverse-transcriptase polymerase chain reaction (PCR) method was used (Tian et al. 2007). Expression of Fa-*PR1* (AB462752) was investigated using the glycealdehyde-3-phosphate dehydrogenase transcript (*GaPDH*1) gene (AF421492) as internal control to standardize amounts of cDNA loaded for each sample in PCR reactions.

Specific primers used were *GaPDH*1 sense (5′-CTACAGC AACACAGAAAACAG-3′) and antisense (5′-AACTAAGTGC TAATCCAGCC-3′) and Fa-*PR1* sense (5′-TGCTAATTCACA TTATGGCG-3′) and antisense (5′-GTTAGAGTTGTAATTAT AGTAGG-3′). PCR parameters were: initial hold, 7 min at 94°C; cycles (number of cycles depended on each primer) of 45 s at 94°C, 1 min of primer annealing (temperature depended on each primer), and 1.5 min at 72°C; and 10 min at 72°C for final extension. Annealing temperatures and cycle number of each primer pair were adjusted to obtain a specific band within the exponential phase of the PCR reaction. Results were analyzed in ethidium-bromide-stained (10 μg ml^{-1}) agarose gel (2%) and photographed; band intensities were calculated from digital images using Total Lab Quant software (Total Lab Ltd., version 11.5; Newcastle, U.K.). To ensure the absence of genomic DNA in each cDNA sample, *GaPDH*1 primer sequences were designed to encompass an intronic region, whose genomic amplification product would be longer. Plants treated

with distilled water were used as controls. Standard deviations were calculated from at least three independent experiments in which G*aPDH*1 was used as an internal control. Differences were considered significant when the P values were <0.05 (Student's t test).

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