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ACCUMULATION OF FERULOYLTYRAMINE AND *p*-COUMAROYLTYRAMINE IN TOMATO LEAVES IN RESPONSE TO WOUNDING

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Key Word Index—*Lycopersicon esculentum*; Solanaceae, feruloyltyramine, *p*-coumaroyltyramine, wounding, tomato.

Abstract—The amounts of two phenolic compounds, *E*-feruloyltyramine and *E*-*p*-coumaroyltyramine, increased 10-fold in tomato leaves in response to mechanical wounding, and 25-fold in response to the oligosaccharide elicitor chitosan. Using fluorescence detection, feruloyltyramine was shown to accumulate in response to wounding, but not to jasmonic acid or systemin, two elicitors of systemic wound-inducible defense responses in tomato. Feruloyltyramine also accumulated in leaves of mutant tomato plants deficient in the octadecanoid signaling pathway in response to wounding, indicating that its synthesis is not regulated through this pathway. These data support a role for hydroxycinnamate-tyramine conjugates as part of the array of defense chemicals and protective biopolymers induced in leaves and other plant tissues by wounding, to protect the plants against pathogen and herbivore attacks.

INTRODUCTION

Plants can defend themselves in response to herbivore attacks by producing an array of defense chemicals and protective biopolymers. In tomato leaves over 15 genes are activated both locally and systemically in response to signals released at the sites of herbivore attacks [1]. Chemical signals derived from pathogen attacks that activate localized responses include oligogalacturonides, released from plant cell walls, and chitosan, released from fungal cell walls. A primary candidate for the systemic signal is an 18 amino acid polypeptide, called systemin. Systemin is a mobile, powerful inducer that is released from wound sites by attacking herbivores [2,3]. Other chemical and physical signals have been associated with signalling events, but do not fulfill the criteria required for systemic signalling [4]. Among the inducible defensive proteins are inhibitors of serine, cysteine and aspartyl proteinases, and the enzyme polyphenol oxidase (PPO) [1,5,6]. These proteins reduce the digestibility of leaves by herbivores by either inhibiting their proteolytic enzymes (i.e. proteinase inhibitors) or by cross-linking the dietary proteins in their guts (i.e. PPO), as well as by inducing the synthesis of additional cross-linking of the plant phenolic cell wall matrix in areas adjacent to wound sites [7,8].

We report herin, that wounding results in the accumulation of two phenolic compounds, *E*-feruloyltyramine (FT) and *E*-*p*-coumaroyltyramine (*p*-CT), that are localized to cells at the wound sites where they may also function as components of the chemical and physical defenses against herbivores and pathogens.

RESULTS AND DISCUSSION

Methanol extracts of leaves of unwounded and wounded tomato (*Lycopersicon esculentum*) plants were subjected to HPLC analysis 24 hr following wounding as part of our studies to assay the small M_r components that might be synthesized in leaves in response to herbivore attacks. The effluents were monitored for the presence of components that fluoresced at 410 nm when excited at 298 nm, including salicylic acid and other related phenolics, that have been associated with defense against both pathogen and herbivore attacks. The extract from wounded leaves contained one predominant fluorescing peak eluting at 15.6 ml that exhibited a small shoulder at its leading edge [Fig. 1(B)], suggesting the presence of more than one component. By contrast, this peak was

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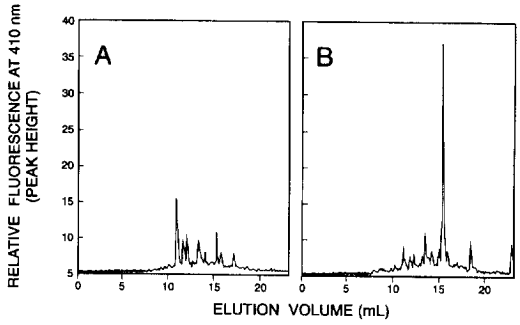


Fig. 1. Accumulation of wound-inducible fluorescence in methanol extracts of tomato leaves. Twenty-four hours following wounding, 1 g of leaves from 14-day-old tomato plants was extracted and analysed by HPLC as described in Experimental. Fluorescence was monitored at 410 nm, with excitation at 298 nm. Panel A; extract from unwounded tomato leaves: Panel B; extract from wounded tomato leaves.

relatively small in extracts of leaves of unwounded plants [Fig. 1(A)]. Moreover, the elution profiles of extracts from unwounded leaves of wounded plants did not show any increase in the region of the peak that accumulated in wounded leaves, suggesting that the induction was not systemic (data not shown).

The time course of the accumulation of the fluorescent components following wounding is shown in Fig. 2. Fluorescence increased steadily for about 10 hr, and then began to plateau until 72 hr (data not shown) when the experiments were terminated.

Several elicitors and inducers of the octadecanoid pathway, including oligouronides [9], chitosan [9], Pmg elicitor [10], jasmonic acid (JA) [11] and systemin [1], were assayed for their ability to induce the accumulation of the fluorescing component(s) in leaves of young excised tomato plants (Fig. 3). The elicitors and inducers were supplied at levels known to maximally induce defense-related proteins in various plant species, including tomato. Supplying the plants with NaPi buffer alone through the cut stem caused about a three-fold increase in their fluorescence when compared to leaves from unwounded intact plants, whose extracts exhibited a constitutively low level (i.e. Fig. 2). When plants were supplied with NaPi buffer through the cut stems, about a two- to three-fold increase in the fluorescence of the extracts occurred. Pmg elicitor induced fluorescence to increase moderately, whereas chitosan induced double the peak-height as did the Pmg elicitor. Oligouronides caused a weak increase in fluorescence, but fluorescence in extracts of leaves supplied with systemin or JA was essentially the same as the buffer control.

Because chitosan caused a greater increase in overall fluorescence than wounding alone, excised tomato plants were supplied with chitosan in order to obtain the component(s) of interest in sufficient amount for detailed characterization. Extracts of leaves of plants supplied with chitosan (25 μg plant^{-1}) were next subjected to semi-preparative HPLC (see Experimental) and the UV absorbing peak that also fluoresced at 410 nm, eluting at approximately 73 ml [Fig. 4(A)], was recovered. The solution was lyophilized and subjected to further HPLC separation on an analytical C_{18} column (see Experimental). Under these conditions, two UV absorbing peaks closely eluting at about 32 ml, were cleanly separated from abscisic acid (data not shown), and were pooled and lyophi-

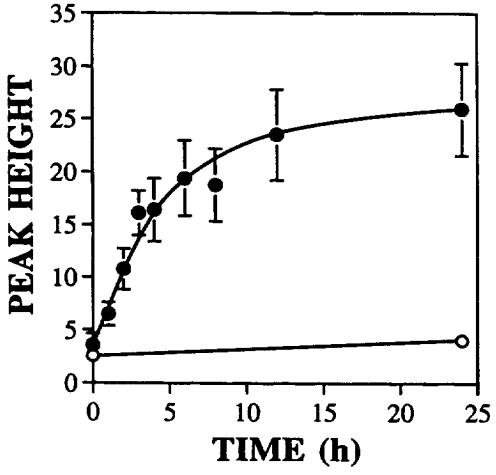


Fig. 2. Time course of accumulation of the major fluorescing component(s) shown in Fig. 1, Panel B following wounding. Each point represents the mean of the peak-height from extracts of at least 4 different 1 g samples. Tomato plants were wounded to initiate the experiments, as described in Experimental. Closed circles represent peak-heights from leaves of wounded plants, and open circles from leaves of unwounded plants.

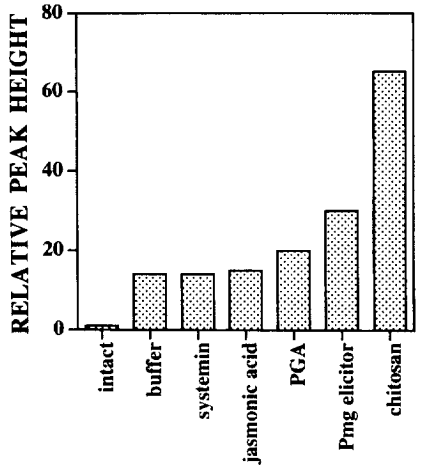


Fig. 3. A comparison of the wound-induction of the major fluorescing component(s) shown in Fig. 1, Panel B with the induction by various known elicitors of plant defensive genes. Fourteen-day-old tomato plants were excised and supplied with buffer (15 mM Na-Pi, pH 6.5, 50 μl), and with elicitors in buffer for 45 min. The plants were then transferred to distilled H_2O and incubated for 24 hr under constant light. 1) intact plants; 2) buffer; 3) systemin, 2.5 pmoles per plant; 4) jasmonic acid, 40 μM; 5) polygalacturonic acid (PGA), 250 μg ml^{-1}; 6) *Phytopthera megasperma* (Pmg) elicitor, 100 μg ml^{-1}; 7) chitosan, 100 μg ml^{-1}.

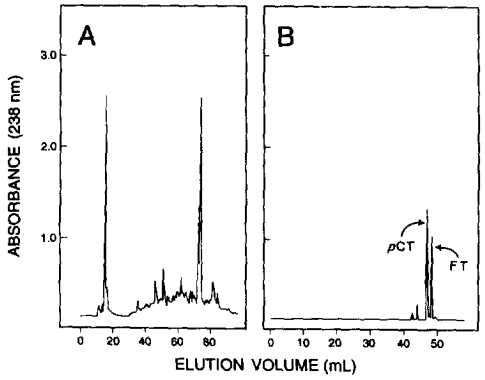
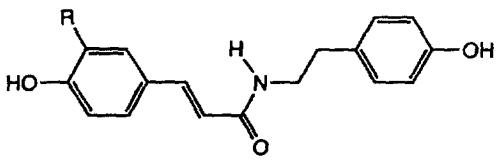


Fig. 4. Purification of the components of the major fluorescing peak following treatment of tomato leaves with chitosan. A) Semi-preparative C_{18} HPLC was employed as described in Experimental and monitored at 238 nm. B) The fluorescing components eluting at 73 ml were pooled and subjected to analytical HPLC using KPi buffer (see Experimental). The resulting overlapping components were further sepd and recovered using an analytical C_{18} column employing a shallow, acidic, linear gradient as described in the text. Peaks identified as *p*-CT and FT eluted at 47.2 ml and at 48.7 ml, respectively.

lized. Subsequent separation of the two components was achieved by HPLC, as shown in Fig. 4(B). The first of the two major peaks to elute exhibited little fluorescence, whereas the second major peak strongly fluoresced (data not shown).

Utilizing ^{1}H NMR and mass spectroscopy techniques, the identity of the lead component was unequivocally established as *E*-*p*-coumaroyltyramine (*p*-CT) and the later eluting peak as *E*-feruloyltyramine (FT). The structures of these compounds are shown in Fig. 5. Purified FT and *p*-CT exhibit identical UV, mass and proton NMR spectra as authentic, synthetic compounds, and were identical with properties reported in the literature [13–15]. FT exhibits strong fluorescence at 410 nm, due to the methoxy group in the feruloyl moiety, while *p*-CT is only weakly fluorescent. To establish that the compounds were not a result of the extraction and purification, LC mass spectral analyses were performed at each step of purification. Throughout the process, the two peaks exhibited the same relative retention times, and UV and mass spectral properties.

Because FT could be readily identified in methanol extracts of tissues by fluorescence spectroscopy of



R = H: *E*-*p*-Coumaroyltyramine (*p*-CT)
R = OCH_3: *E*-Feruloyltyramine (FT)

Fig. 5. *E*-*p*-Coumaroyltyramine (*p*-CT) and *E*-feruloyltyramine (FT).

HPLC eluates, we could estimate the levels of FT in the areas of the leaf surrounding the wound site. A small (3 mm diameter) circular wound was produced at the centre of the terminal leaflets of intact plants and the plants were incubated in continuous light for 48 hr to accumulate FT and proteinase inhibitor II. Concentric circular sections around the would site were excised and the tissues were quantified for FT levels, as shown in Fig. 6. The circular sections were also assayed for inducible proteinase inhibitor II levels to compare the FT increases with those of a known systemically inducible defense protein. The FT was found to be confined to the cells including and just surrounding the wound site (3 mm diameter). The level of FT in this region was 15.4 nmoles g^{-1}, based on the spectral properties of pure FT. In contrast, low levels of inhibitor II accumulated in this region, but increased in leaf tissue with increasing distance from the wound, as previously reported [16].

In a mutant tomato line, called def 1, the octadecanoid signalling pathway is severely impaired, and neither wounding nor elicitors can effectively induce the synthesis of systemic wound response proteins [16, 17] (Table 1). This mutant is deficient in the conversion of 13(*S*)-hydroperoxylinolenic acid to 12-oxy-phytodienoic acid, thus interfering with the signalling cascade to jasmonic acid and to defense protein synthesis. Wounding of leaves of the def 1 mutant resulted in the accumulation of FT to levels similar to levels found in wild-type plants (Table 1). Even though both wounding and chitosan induce the accumulation of FT and proteinase inhibitors, the overall data, including the

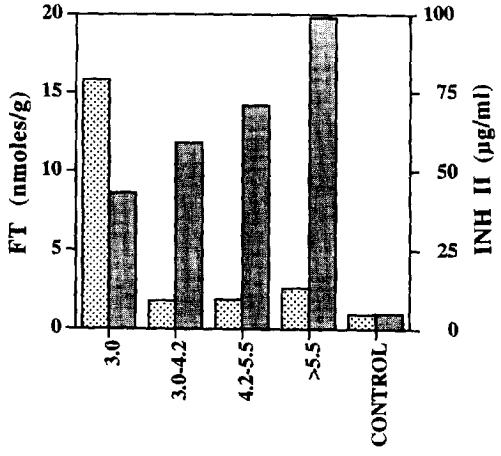


Fig. 6. Localization of FT and inhibitor II to wound sites. Circular wounds of 3 mm diameter were made on leaves of 14-day-old plants by crushing between a dowel and a flat file. The plants were incubated for 48 hr under light to accumulate FT and proteinase inhibitor II. Concentric sections were excised around the wound sites using increasing sized cork borers and the levels of FT and inhibitor II were assayed. FT, dotted bars; inhibitor II, shaded bars. Columns show levels of FT and inhibitor II within different zones (cm diameter) from point of wounding. As can be seen, FT drops dramatically beyond the 3 cm diameter zone, whereas inhibitor II steadily increases.

Table 1. Accumulation of feruloyltyramine by wounding and chitosan treatment in wild-type tomato plants (Castlemart) and a mutant tomato line (def 1) with a defective octadecanoid signaling pathway

Treatment*	Tomato plants† Castlemart	def 1 Mutant
Intact plants	0.6±0.1	0.8±0.2
Intact, wounded‡	6.1±0.6	8.9±1.2
Excised§	2.8±0.5	2.9±0.3
Excised, chitosan§	15.2±0.7	15.6±1.0

*Plants were incubated for 24 hr under light following treatments.

†nmoles FT g^{-1} fresh wt.

‡Several wounds were made on each leaf to induce accumulation of FT in the unwounded surrounding cells.

§After excision, or excision and supplying chitosan (100 μg ml^{-1} in 50 μl 15 mM Na-Pi buffer, pH 6.5, for 45 min through the cut stem). Plants were supplied with distilled H_2O for 24 hr under light.

lack of systemic response and the inactivity of systemin and JA as inducers of FT synthesis, indicate that the synthesis of FT is likely mediated by a pathway distinct from the octadecanoid pathway. The biochemical basis for the localized accumulation of FT by wounding and chitosan remains to be investigated.

FT levels were also estimated in leaves of potato, tobacco, nightshade and pepper, in response to wounding (Table 2). The response to tomato is included for comparative purposes. In each case, leaves were extracted with methanol 6 hr after wounding and FT was quantified by HPLC. FT levels increased in potato and tobacco in response to wounding, similar to tomato. While FT levels increased from 0.39 to 2.42 nmoles g^{-1} in tomato, FT in potato increased from 1.75 to 4.39 nmoles g^{-1}. No FT was detected in unwounded tobacco plants, but 6 hr after wounding, FT levels rose to 0.86 nmoles g^{-1}. A time course of induction of FT in tobacco (data not shown) indicated that FT was induced at wound sites to levels comparable to tomato within 24 hr after wounding, but

Table 2. Accumulation of feruloyltyramine by wounding in leaves of several members of the Solanaceae family

Family Member	Feruloyltyramine in leaves (nmoles g^{-1} fresh wt.) Unwounded	Wounded
Tomato	0.39	2.42
Potato	1.75	4.39
Tobacco	0	0.86
Pepper	0	0
Nightshade	0	0

Leaves from each plant family member were collected from unwounded plants and from wounded plants 6 hr after wounding, when feruloyltyramine was extracted with MeOH and separated on HPLC for quantification. Values are the average of two 1 g samples.

unlike tomato, FT decreased to background levels by 72 hr. On the other hand, neither nightshade nor pepper resulted in accumulation of FT, suggesting that the response can vary within a plant family.

Metabolites such as *p*-CT and FT may also have antifeedant activities, since they can be presumed to act as substrates for polyphenol oxidases (PPOs) [18]. In this context, PPO has been shown recently to be synthesized *in planta* in response to herbivore attacks [6], although, the specificity of inducible PPO or other phenol oxidases in tomato leaves has not yet been examined. Nevertheless, it can be envisaged that these compounds may act as substrates for such enzymes in herbivore guts, thereby helping to cross-link the ingested leaf proteins and reduce their nutritional value [5], thus resulting in decreased growth and development of the herbivores.

FT and *p*-CT had been found previously to be inducible by elicitors in cell cultures of *Solanum khasianum* [19] by *Phytophthora infestans* infection, also in leaves and cell cultures of *Solanum tuberosum* and in wounded potato tuber discs after 5 days [21]. Both compounds have recently been shown to be major components of the aromatic domain of suberin [7,8], the wound periderm that is formed as a barrier to pathogens in response to wounding. The final steps of synthesis of both *p*-CT and FT are catalysed from tyramine (from tyrosine decarboxylation) and the appropriate CoA derivatives (from the phenylpropanoid pathway) by the enzyme tyramine hydroxycinnamoyl transferase (THT) [22]. The kinetics and specificities of this enzyme from various plants have been extensively studied [21–24]. Interestingly, THT has been reported to be induced in suspension cultures [24] and by TMV infection of tobacco plants [23] and in resistance reactions of potato against pathogen attacks [25]. The products of the enzymes were found to be incorporated into cell walls [21, 23, 24], apparently as part of a barrier against pathogens. However, free FT can also strongly inhibit hyphal growth [26], and, therefore, in plants in which phenolic-tyramine adducts accumulate at the wound sites, they may act as antibiotics as well as cell wall components to protect the freshly wounded tissues against pathogen attacks.

EXPERIMENTAL

Plant Materials. Tomato plants (*Lycopersicon esculentum* var. Castlemart) were grown to a two leaf stage (12–14 days) in growth chambers with a 17 hr day at 28° and 7 hr night at 18° under light at 300 μeinsteins m^{-2} sec^{-1}. Potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), nightshade (*Solanum nigrum*) and pepper (*Capsicum annum*) were grown under the same conditions. To initiate time-course experiments, three separate wounds were applied across the leaves of each plant with a haemostat, perpendicular to the main veins. At specific intervals, the leaves were excised, and four 1 g leaf tissue samples were collected,

frozen in liquid N_2, and stored at $-80°$ until assayed. To determine the distance of the response from the wound site, a single wound was produced on each leaf by pressing it between a flat file and a 3 mm dowel. Forty-eight hours after wounding, circular sections of increasing diameter were removed from around each wound site with stainless steel cork borers of increasing diameter, and 1 g samples of each diameter were collected, frozen in liquid N_2, and stored at $-80°$ until used for experiments.

Administration of Elicitors. Plants were excised at the base of the stem with a razor blade and supplied through their cut stems with 50 μl of the elicitor solns in 15 mM Na-Pi buffer, pH 6.5, (about 45 min were required to take up the soln). The plants were then transferred to 20 ml vials containing distilled H_2O and incubated in a closed plexiglass box containing a CO_2 trap for 24 hr at 28° under 300 μeinsteins m^{-2} s^{-1}. Proteinase inhibitor II was assayed immunologically in leaf juice [27,28].

Extraction of Plant Material. Plant material was extracted with MeOH by a modification of the method developed by Raskin *et al.* The frozen 1 g samples were homogenized in 2.5 ml of 90% aq. MeOH for 1 min in an Omni Mixer with a micro attachment (Sorvall, Newton, Conn.) and centrifuged for 10 min at 12000 *g* in an SS34 rotor (Sorvall). The supernatants were collected and the pellets were re-extracted in 2.5 ml MeOH and centrifuged as above. The supernatants were pooled, frozen in liquid N_2 and freeze-dried. Samples were mixed in 2.5 ml of 50 mM TCA, vortexed and centrifuged at 12000 *g* for 10 min. The supernatant was added to 5 ml of a mix. containing 1% *iso*-ProH in 1:1 EtOAc–pentane. After vortexing, the organic phase was removed and evapd to dryness under a stream of N_2. Each sample was dissolved in 1 ml 0.1% TFA, filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA) and the filtrate was subjected to analysis by reversed-phage HPLC.

HPLC Analysis. HPLC sepns were performed on a Vydac analytical C_{18} column (5 μm, 4.6 × 250 mm, 218TP54, Western Analytical Products, Inc. Temecula, CA.) using a Gilson 45NC Gradient Analytical System equipped with a Model 117 variable wavelength detector with detection at 238 and 280 nm. Elution details were: Solvent A, 0.1% TFA–H_2O, and Solvent B, 0.1% TFA–CH_3CN. For analytical sepns, a linear gradient was employed from 100% Solvent A to 100% Solvent B over 30 min with a flow rate of 1 ml min^{-1}. The same solvent system as described above was employed for prep. sepns, using a gradient from 0–50%B over 45 min. In this case, a Vydac semi-prep. C_{18} column (5 μm, 10 × 250 mm 218TP510) was used, with a flow rate of 2 ml min^{-1}. To specifically detect feruloyltyramine, the eluant was also analysed in a flow cell attached to a fluorescence spectrophotometer (Perkin–Elmer Model LS-50B, Norwalk CT) equipped with a 16 μl capacity flow cell, with the excitation wavelength set at 298 nm (5 nm slit width) and the emission of the fluroescence monitored at 410 (5 nm slit width). To separate the components, the analytical C_{18} column described above was employed using Solvent C (10 mM K-Pi buffer, pH 6) and a 45 min gradient from 0% to 100% Solvent D (Solvent C in 50% CH_3CN). This procedure sepd abscisic acid from the fluorescing components. A further sepn of the components remaining in the fluorescing peak was achieved with the same column using a shallower gradient as described for the sepns in Fig. 2(A). After 2 min at 0% Solvent B, a linear gradient from 0% B to 50% B was applied over 90 min [Fig. 2(B)].

Synthesis of E-p-*Coumaroyltyramine and* E-*Feruloyltyramine. E*-Feruloyltyramine (FT), and *E-p*-coumaroyltyramine (*p*-CT), were synthesized by condensation of tyramine with ferulic and *p*-coumaric acid, respectively, as described [30]. Synthetic FT was employed to quantify the extraction procedure and as a standard for assays of FT in plant tissues. When 9.6 nmoles of synthetic FT was added to 1 g samples of tomato tissue and extracted and analysed as above; the recovery was approximately 55%. The reported values of FT are adjusted to account for losses during extraction, based on this recovery.

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