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METABOLISM OF TYRAMINE AND FERULOYLTYRAMINE IN TMV INOCULATED LEAVES OF *NICOTIANA TABACUM*

JONATHAN NEGREL and PHILIPPE JEANDET

Station de Physiopathologie Végétale, I.N.R.A. B.V. 1540 21034, Dijon Cédex, France

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Key Word Index—*Nicotiana tabacum*, Solanaceae, metabolism, tyramine, cinnamic acid amides, phenolic conjugation, lignin like compounds, cell wall, hypersensitive reaction, tobacco mosaic virus.

Abstract—TMV inoculation is known to stimulate tyramine *N*-feruloyl-CoA transferase activity in *Nicotiana tabacum* cv Xanthi n.c. leaves during the hypersensitive reaction. When [2-^{14}C]-tyramine is fed for 2 hr to TMV inoculated leaf discs or detached leaves, *ca* 1% of the supplied radioactivity is integrated into cinnamoyl-, *p*-coumaroyl- and feruloyltyramine and up to 14% is integrated into the cell wall residue. [2-^{14}C]-tyramine can only be partially released from this residue by acid hydrolysis. After nitrobenzene oxidation, 97% of the radioactivity found in the cell walls is made soluble but only 13% is recovered in *p*-hydroxybenzaldehyde. Feruloyltyramine is very rapidly metabolised, *ca* 20% of the administrated radioactivity is found after 2 hr feeding in unindentified methanol soluble metabolites. Acid hydrolysis of the cell wall fraction, which hydrolyses the amide bond of feruloyltyramine, releases labelled tyramine, while radioactivity is still detected in the acid insoluble residue. Label from [^{14}C]-feruloyltyramine is integrated into this residue more quickly than from free [2-^{14}C]-tyramine.

INTRODUCTION

Although cinnamic acid amides are widespread in the plant kindom [1, 2], investigations on their metabolism are still scarce, especially when compared with the data available on cinnamic acid esters [3]. The characterization of the enzyme tyramine *N*-feruloyl-CoA transferase (TFT) in tobacco leaves [4] led us to investigate the metabolism of the amides of phenethylamines which are synthetised by TFT. In *Nicotiana tabacum* cv Xanthi n.c., TFT activity is increased *ca* 6 fold during the hypersensitive reaction to tobacco mosaic virus (TMV) [4]. This stimulation therefore provides a good model to study the metabolism of the amides *in vivo*.

In plant cell suspension cultures, phenethylamines are known to undergo conjugation and oxidative coupling reactions leading to the formation of insoluble polymers [5]. In the course of the study of the biosynthesis of cinnamic acid amides of tyramine in tobacco using [2-^{14}C]-tyramine as precursor, we noticed that considerable amounts of radioactivity remained associated with pellets obtained after extraction of leaves with methanol. The integration of tyramine into this residue increased after inoculation of the leaf by TMV. It was therefore interesting to study the possibility that cinnamic acid amides, which are good substrates of peroxidases *in vitro* [4], could be intermediates in the reactions involved in the insolubilization of tyramine. Moreover, after inoculation of potato tubers with *Phytophthora infestans* isolates, which leads to a hypersensitive reaction, cinnamic acid amides of tyramine and octopamine are synthesized and bind to the cell walls [6]. This observation together with the finding that TFT stimulation does not lead to an important accumulation of amides in tobacco [4], led us to study the fate of the amides, synthesized *in vivo* from [2-^{14}C]-tyramine, or fed externally after chemical synthesis.

We report here that in tobacco leaves, [2-^{14}C]-tyramine is integrated into cinnamoyl-, *p*-coumaroyl- and feruloyltyramine and that exogenously supplied [^{14}C]-feruloyltyramine is very rapidly metabolished. Furthermore, feruloyltyramine is integrated into cell walls more quickly that tyramine. It is however difficult to know whether the formation of the amides plays a part in the insolubilisation of exogenously administrated [^{14}C]-tyramine, which can probably undergo oxidative coupling before conjugation can take place.

RESULTS

Relative activity of tyramine analogues in the tyramine N-*feruloyl-CoA transferase assay*

Partially purified TFT preparations can catalyse *in vitro* the synthesis of a wide range of cinnamic acid amides of phenethylamines [4]. To study the fate of labelled tyramine *in vivo*, it was useful to find tyramine analogues which could not be rapidly conjugated *in vitro* by TFT to cinnamic acids. Figure 1 shows that substitution of the phenolic group of tyramine by a halogen, a nitro, or a methoxy group results in a nearly complete loss in transferase activity. Among these products, 4-methoxyphenethylamine (4) was the only analogue commercially available in radioactive form. We therefore used it as a control during feeding experiments *in vivo*. 4-Hydroxybenzylamine (8), which possesses the same functional groups as tyramine but differs in the length of the side chain is also inactive in the TFT assay. It was used in competition experiments to examine the role of amide formation in tyramine incorporation into the cell walls.

Labelling of cell walls by [2-^{14}C]-*tyramine.*

When [2-^{14}C]-tyramine is fed *in vivo* for 2 hr to tobacco leaf discs, up to 14% of the supplied radioactivity is found associated with cell walls. In the experimental conditions used, the incorporation rate was linear for up to 3 hr. After

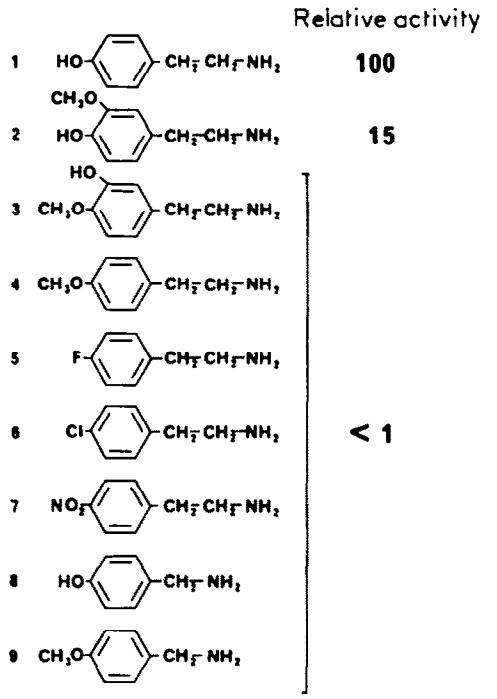

Fig. 1. Relative activity of tyramine analogues in the tyramine *N*-ferulolyl-CoA transferase assay. Compounds 1–9 are respectively: tyramine, 3-methoxy-tyramine, 4-methoxy-3-hydroxyphenethylamine, 4-methoxy-phenethylamine, 4-fluorophenethylamine, 4-chloro-phenethylamine, 4-nitrophenethylamine, 4-hydroxy-benzylamine, 4-methoxybenzylamine. See also ref [4]. < 1 means that the activity is below the sensitivity of the spectrophotometric assay.

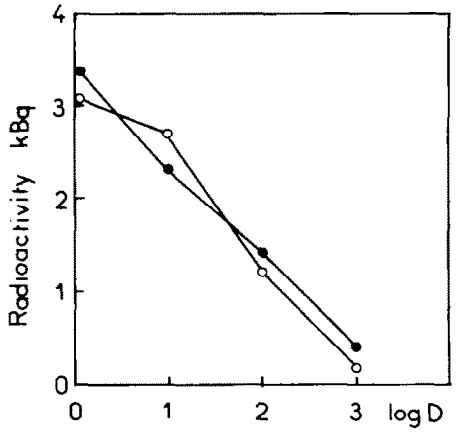

Fig. 2. Incorporation of [2-^{14}C]-tyramine in the cell wall fraction of TMV inoculated leaf discs after feeding 37 kBq tyramine diluted 1, 10, 100 or 1000 fold with cold tyramine (O) or *p*-hydroxybenzylamine (●) in a total volume of 200 μl. The cell wall material was combusted to CO_2 to quantify the total radioactivity integrated. D = dilution.

feeding 37 kBq tyramine for 2 hr, combustion of the cell walls to carbon dioxide showed that labelling was more rapid in TMV inoculated discs (5.31 kBq released) than in control discs (1.68 kBq). In both cases, *p*-methoxyphenethylamine was only very slowly incorporated (0.32 and 0.21 kBq respectively). This shows that tyramine insolubilisation is not the result of ionic interactions between the amino group and cell walls. However, replacement of the phenolic function of tyramine by a methoxy group prevents the conjugation of *p*-methoxyphenethylamine by TFT but also protects it from direct enzymatic oxidation. This led us to design competition experiments with *p*-hydroxybenzylamine, which was found to inhibit incorporation of [2-^{14}C]-tyramine (Fig. 2). Diluting labelled tyramine with 4-hydroxybenzylamine has the same effect as using tyramine of lower specific activity (Fig. 2). Although this does not prove that 4-hydroxybenzylamine competes with tyramine in oxidation reactions, it indicates that the mechanism leading to the incorporation of tyramine in the cell wall fraction is probably not entirely specific for the phenethylamine structure.

Metabolism of [2-^{14}C]-*tyramine.*

First attempts to detect alcohol soluble metabolites of [2-^{14}C]-tyramine in leaf discs indicated that, apart from labelled tyramine, only traces of radioactivity could be detected in compounds migrating with pigments after TLC. The experiment was therefore repeated on a larger scale with entire leaves. Leaves taken 72 hr after inoculation by TMV at 20° and which had absorbed 148 kBq of [2-^{14}C]-tyramine were extracted with methanol. After concentration, the crude methanol extract was analysed by thin layer electrophoresis followed by chromatography in the second dimension. In these conditions, label was still detected in compounds associated with pigments. After phase partition between water and ethyl acetate, the tyramine metabolites concentrated in the ethyl acetate layer, were identified as cinnamoyl-, *p*-coumaroyl- and feruloyl-tyramine by TLC in systems 1 and 2 and co-chromatography with the synthetic amides. The identification was confirmed by HPLC (Fig. 3) after prepurification of the ethyl acetate phase on silica columns. The same method was used to quantify the amides. Table 1 shows that their level is low (12 nmol/g fr. wt) but their specific activity is relatively high when compared with the administrated tyramine. One % of the absorbed radioactivity is found after 4 hr in the 3 amides.

Analysis of the [2-^{14}C]-*tyramine labelled cell wall fraction*

It was of interest to determine whether tyramine could be recovered from the cell walls after its integration. Incubation of the cell wall fraction with commercial preparations of cellulase or pronase failed to solubilize substantial amounts of radioactivity. This was however achieved by oxidation with sodium chlorite (data not shown), which is used as a delignification reagent [7]. Acid hydrolysis of the labelled cell wall fraction, purified from TMV inoculated leaves after feeding [2-^{14}C]-tyramine, releases 20 nmol tyramine per g fr. wt (Table 1). The specific activity of these tyramine residues is slightly higher than that of the free amides. Nevertheless, a large amount of radioactivity was still detected in insoluble form after filtration or centrifugation of the hydrolysate. After nitrobenzene oxidation of the purified cell wall fraction obtained after feeding [2-^{14}C]-tyramine (37 kBq) for 2 hr to TMV-inoculated leaf discs, the soluble oxidation products were partitioned into diethyl ether from

Table 1. *In vivo* labelling of cinnamic acid amides and cell wall bound tyramine residus by [2-^{14}C]-tyramine

	Amide or amine levels (nmols/g fr. wt)	Specific activity (MBq/mmol)	Total radioactivity (in % of the total radioactivity absorbed)
p-Coumaroyltyramine	5.5 ± 2.6	20 ± 12	
Feruloyltyramine	3.2 ± 2.8	24.7 ± 14	1.48 kBq (1%)
Cinnamoyltyramine	3.3 ± 1.4	18.5 ± 8	
Wall-bound tyramine residues	20 ± 11	37 ± 15	5.18 kBq (3,5%)

Tyramine (148 kBq, 1.86 GBq/mmol) was fed for 4 hr by petiolar uptake to TMV inoculated leaves taken 72 hr after inoculation at 20°. After absorption of the radioactive solution (200 μl) the leaf was placed on water. Different leaves were used to extract the amides and to purify the cell walls. After pre-purification, the amides were analysed by HPLC. The cell walls were hydrolysed in 6 M HCl. Tyramine in the hydrolysate was analysed by HPLC after dansylation. Peaks were collected and counted by liquid scintillation.

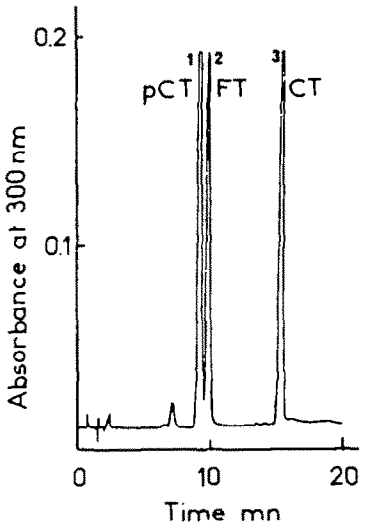

Fig. 3. HPLC analysis of cinnamic acid amides of tyramine. Chromatographic conditions: linear gradient elution within 20 min from 40 to 60% methanol in water at a flow rate of 2 ml/min. Detection was at 0.2 absorbance units full scale. The separation was performed on a Waters Radialpack C18 Column (8 × 100 mm, 10 μm). 1: *p*-Coumaroyltyramine (pCT) 10 min 50 sec, 2: feruloyltyramine (FT) 11 min 22 sec, 3: cinnamoyltyramine (CT) 16 min 19 sec.

water 61.5% of the radioactivity present in the cell walls was recovered in the aqueous phase, 36% in the organic phase whereas 2.5% remained associated with the cell wall residue. Only 13% of the radioactivity found in the cell walls was recovered in labelled *p*-hydroxybenzaldehyde which was analysed in the diethyl ether phase by thin-layer and high pressure liquid chromatography. The specific activity of *p*-hydroxybenzaldehyde was not determined as it is known that it can be released by oxidation of tyrosine residues of proteins, and of lignin [8].

Metabolism of [^{14}C]-*feruloyltyramine.*

[^{14}C]-Feruloyltyramine was synthesized chemically from [2-^{14}C]-tyramine and had therefore the same specific activity. It was very rapidly metabolized in leaf discs. Its transformation could be monitored by TLC in system 7 after extraction of the discs in methanol. The decrease in label in feruloyltyramine was already notable after 30 min when label could be detected in 3 spots of low R_f and on the origin (see Experimental for R_f values). After 2 hr, 50% of the radioactivity of the methanol extract remained on the origin whereas considerable amounts of radioactivity was detected in the pellets obtained after centrifugation of the tissues homogenates and in the purified cell walls. The transformation products of feruloyltyramine were not identified.

Analysis of the [^{14}C]-*feruloyltyramine labelled cell wall fraction.*

The amide bond of feruloyltyramine is hydrolysed in the conditions used to hydrolyse peptides (6 M HCl, 16 hr at 110°). In these conditions the [^{14}C]-feruloyltyramine labelled cell wall fraction released labelled tyramine which was identified by TLC in systems 3 and 4 after dansylation. Radioactivity incorporated into the acid insoluble material could be monitored rapidly by suspension counting. Figure 4 shows the relative incorporation rates of tyramine, *p*-methoxyphenethylamine and feruloyltyramine. TMV inoculated tissue had higher incorporation rates of three precursors but incorporation of *p*-methoxyphenethylamine was insignificant. Surprisingly, radioactivity from tyramine conjugated to ferulic acid was integrated more quickly in the acid insoluble fraction than free tyramine, and TMV inoculation induced a very marked increase in the incorporation rate of tyramine. This confirms results obtained after combustion of the cell wall fraction. The observed differences in incorporation rates were not due to differences in uptake of the precursors. After 2 hr feeding, 45% of the supplied radioactivity was absorbed by the leaf discs.

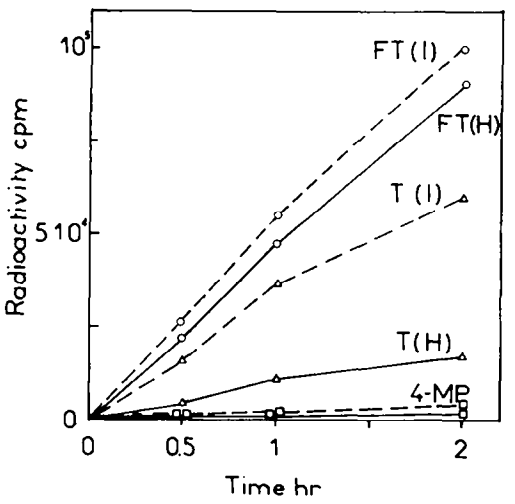

Fig. 4. Comparison of the integration rates of [2-^{14}C]-tyramine (Δ, T), [2-^{14}C]-4-methoxyphenethylamine (□, 4MP) and [^{14}C]-feruloytyramine (O, FT) in the HCl insoluble residue of the cell wall fraction of healthy (———) or TMV inoculated (- - -) leaf discs. After purification, the cell wall fraction was refluxed in 10 ml 6 M HCl for 16 hr at 110°. The hydrolysates were then centrifuged in conical tubes. After washing with water, the pellet was resuspended in 1 ml H_2O and transfered to a liquid scintillation flask for suspension counting. The counting efficiency was not determined.

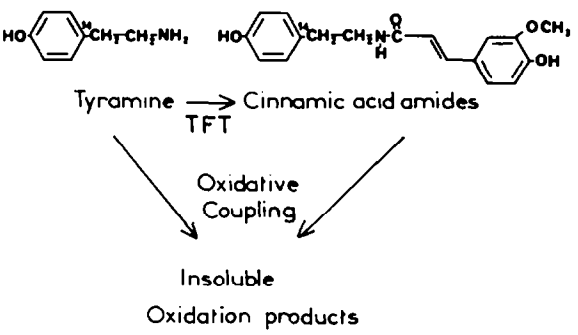

Fig. 5. Proposed mechanisms of integration of exogenous tyramine in the cell wall fraction of tobacco leaves.

DISCUSSION

Plant phenolics are generally metabolically reactive products and are often subject to polymerisation reactions [9]. Tyrosine, for example, is integrated in insoluble polyphenols in tobacco cell cultures and callus [8, 10]. In plant cell suspension cultures, phenethylamines are also subject to conjugation and oxidative polymerisation. The nature of the conjugates formed during these reactions is however unknown [5].

We have shown that in tobacco [2-^{14}C]-tyramine can be conjugated to cinnamic acids *in vivo* and that both tyramine and feruloyltyramine are rapidly incorporated into cell walls when administrated externally. This therefore strongly supports Clarke's observations [6, 11] and confirms that cinnamic acid amides of hydroxyphenethylamines have the chemical reactivity required to be integrated into wall bound phenolics. From the results presented here, it is however not possible to know whether part of the tyramine integrated into the cell walls is conjugated to cinnamic acids prior to its insolubilisation. Two results nevertheless indicate that this mechanism may function *in vivo* (Fig. 5). (i) The specific activities of cinnamoyl-, *p*-coumaroyl- and feruloyl-tyramine are comparable with the specific activity of tyramine residues which are released by acid hydrolysis. Although some contamination of the cell walls by free [2-^{14}C]-tyramine cannot be completely excluded, even after the drastic extraction conditions used to purify them, dilution of the exogenous precursor (80 nmol) with endogenous tyramine (*ca* 120 nmol/g fr. wt [21]) cannot explain the 50-fold dilution between the specific activities of the precursor and wall bound tyramine residues. The possible occurrence of such residues had already been mentioned in tobacco [12]. (ii) Label from [2-^{14}C]-tyramine conjugated to ferulic acid is incorporated into cell walls more quickly than free tyramine. It may be misleading to compare integration rates of compounds with very different solubility in water and which could therefore penetrate preferentially in different cell compartments. Even so, tyramine and feruloyltyramine, which are both substrates of peroxidases *in vitro* [5, 13] display a different reactivity *in vivo*.

During the hypersensitive reaction to TMV in tobacco, both peroxidases [14] and polyphenol oxidase [15] activities are stimulated. Although [^{14}C]-dopamine, which can be formed by hydroxylation of tyramine in plants, was not detected in our experiments, intervention of polyphenol oxidase cannot be ruled out. From a chemical point of view, it is possible for cinnamic acid amides of hydroxyphenethylamines to undergo oxidative coupling on both side of the molecule, thus preventing tyramine release by hydrolysis of the amide bond. Aryl–aryl ether and biphenyl bonds, which are commonly formed during oxidative coupling of phenolics are stable in the conditions used to hydrolyse amide bonds. The presence of tyramine residues released during acid hydrolysis may then result from the occurrence in the cell walls of some tyramine molecules linked by amide bonds but still having free phenol groups.

The synthesis of cinnamic acid amides of tyramine requires precursors which are also used during lignin formation. It occurs during the hypersensitive reaction when the phenylpropanoid pathway is stimulated, leading to a rapid lignification of the resistant tissues [16]. The amides could form lignin like polymers or could simply be linked to other constituents of the cell walls. Wall bound phenolics have been found associated with pectins [17] and hemicelluloses [18]. Some of them are thought to play a role as cross linking agents [19, 20]. Synthesis of phenethylamine derivatives has been found to be stimulated in a variety of physiological conditions, including resistance to pathogens [4, 6, 21], herbicide treatment [22], cell proliferation and crown gall infection [23]. It would be therefore very interesting to know precisely both their fate and their functions in plant cells. Chemical degradation of insoluble polyphenols is difficult, but valuable informations might be obtained on the fate of endogeneous tyramine by comparison of the metabolism of (U-^{14}C]- and carboxy-labelled tyrosine. Moreover integration of nitrogen into cell walls is generally entirely attributed to proteins. Confirmation of the incorporation

of nitrogen containing phenolics into lignin like compounds could therefore disclose a new aspect of plant cell wall chemistry.

EXPERIMENTAL

Plant material. Nicotiana tabacum cv Xanthi n.c. were grown and inoculated by TMV as previously described [4]. Control leaves were inoculated with H_2O using carborundum as abrasive. TLC, unless otherwise stated, was on Kieselgel using the following solvents: (1) $CHCl_3$–MeOH (24:1) [4]; (2) $CHCl_3$–EtOAc–AcOH (8:1:1) [22]; (3) $CHCl_3$ [28]; (4) cyclohexane–EtOAc (4:5) [28]; (5) hexane–*iso*amyl alcohol–AcOH (400:64:1) [18]; (6) *iso*propylether–H_2O (1:1, organic layer), R_f *p*-hydroxybenzaldehyde 0.63, vanillin 0.45, siryngaldehyde 0.23, (7) $CHCl_3$–MeOH (22:3) R_f feruloyltyramine 0.3, feruloyltyramine metabolites 0.2; 0.14 and 0.1.2D-chromatoelectrophoresis was performed as described in ref [21].

Chemicals and radiochemicals. [2-^{14}C]-Tyramine acetate (1.8×10^9 Bq/mmol) and [2-^{14}C]-*p*-methoxyphenethylamine acetate (1.8×10^9 Bq/mmol) were obtained from CEA (France), *trans*-[^{14}C]-feruloyltyramine was synthesized from [2-^{14}C]-tyramine and feruloyl-*N*-hydroxysuccinimide ester as described in [4]. After purification by TLC in system 1 it was dissolved in water adjusted to pH 7.5 with KOH. Radiochemical purity of *trans*-feruloyltyramine was checked by TLC in systems 1 and 2 and by HPLC (see Fig. 3). Tyramine analogues were commercially available, except 4-hydroxybenzylamine which was synthesized from 4-methoxybenzylamine (Aldrich) by hydrolysis in HI 57% (16 hr, 130°). The hydrochloride was recovered by ion exchange on Amberlite IR 120. After elution with 6 M HCl, the hydrochloride was evaporated to dryness and crystallised from EtOH–Et_2O (1/5) (mp 234–236°). The product was further characterized by reaction with ninhydrin and diazotized sulphanilic acid after TLC [24] on cellulose (BuOH–EtOH–H_2O, 4:1:2, R_f 0.5).

TFT activity was estimated spectrophotometrically as described in [4] using the partially purified enzyme obtained after $(NH_4)_2SO_4$ fractionation and dialysis.

Feeding experiments. (a) With leaf discs: 5 discs (1.5 cm diameter) were cut in the blade of an inoculated leaf 72 hr after inoculation with TMV at 20° (*ca* 5 lesions /cm^2) and placed in a Petri dish. Droplets of the radioactive soln (total vol. 200 μl, 185 kBq/ml) were applied at the periphery of each disc with a syringe. (b) With entire leaves: the radioactive soln (148 kBq, 200 μl) was fed by petiolar uptake in 1 ml conical tubes. After *ca* 30 min, the soln was completely absorbed and the leaf transferred on water.

Cell wall purification. The cell wall fraction was purified by a modification of the method of ref. [25]. Leaf discs were extracted in a mortar in 1% sodium deoxycholate. After centrifugation (10 min, 5000 *g*), the pellet was re-extracted in the centrifuge tube successively with sodium deoxycholate, H_2O, PhOH–HOAc–H_2O (twice), H_2O, EtOH and Et_2O. Yield of cell wall residue was 2.7–4.3% of fr. wt. As cell walls are very difficult to purify from EtOH or MeOH extracted tissues [25], parallel experiments were run to monitor radioactivity integration into cell walls.

The cell walls were hydrolysed by refluxing in 6 M HCl for 16 hr at 110°. In these conditions feruloyltyramine was completely hydrolysed and [2-^{14}C]-tyramine was stable (95% recovery). 3-Methoxytyramine (5 μmol) was added to the cell walls before hydrolysis in experiments designed to identify and quantify labelled tyramine residues linked to the cell walls. It was used as an internal standard and to protect tyramine from degradation during the hydrolysis. Recovery of 3-methoxytyramine after hydrolysis in the presence of cell walls and after dansylation was of 90%.

Radioactivity measurements. Radioactivity in compounds separated by HPLC was determined by liquid scintillation after collection of the peaks. Radioactivity in compounds separated by TLC was detected and quantified using a TLC radioactivity detector. The acid insoluble residue obtained after hydrolysis of the cell walls was recovered from the hydrolysate by centrifugation, washed in conical tubes with H_2O, centrifuged, resuspended in 1 ml H_2O and counted in suspension in 15 ml scintillation fluid containing 33% Triton. The counting efficiency was not determined.

The cell walls were combusted to CO_2, according to the method of ref. [26].

Nitrobenzene oxidation of the cell wall residue was done according to the method of ref. [27]. The soluble oxidation products were partitioned into Et_2O from the aq. phase. The Et_2O phase was analysed by TLC in systems 5 and 6, aldehydes were detected with dinitrophenylhydrazine. Identification of *p*-hydroxybenzaldehyde was confirmed by HPLC. The aldehyde was prepurified by preparative TLC in system 6 and analysed within 15 min on a C18 column (Ultrosphere ODS, 4.6 × 25 mm, 5 μm), at a flow rate of 0.5 ml/min: linear gradient elution from 40 to 45% MeOH in H_2O. Detection was at 290 nm. Retention time of *p*-hydroxybenzaldehyde 12.5 min.

Tyramine in cell wall hydrolysates was dansylated according to the method of ref. [28]. *O-N*-bis-Dans-tyramine was analysed by TLC in systems 3 and 4 and by HPLC. The chromatography was performed within 15 min on a C18 column (Ultrosphere ODS, 4.6 × 25 mm, 5 μm) at a flow rate of 1 ml/min in Me_2CO–H_2O (70-30). *O-N*-bis-Dans-tyramine was detected by fluorimetry. Retention times *O-N*-bis-Dans-tyramine 11.46 min, *O-N*-bis-dans-3-methoxytyramine 10.81 min.

Cinnamic acid amides of tyramine were extracted in MeOH as described in [4]. After concn, the crude extract was either directly analysed by TLC in systems 1 and 2 or purified by phase partition [4]. The EtOAc phase was evaporated to dryness and redissolved in 1 ml EtOAc. It was then prepurified on small (5 g) Kieselgel 60 columns (Merck, 70–230 mesh). After washing the column with $CHCl_3$, the amides were eluted with MeOH. After evaporation, the residue was redissolved in 1 ml MeOH and aliquots (50 μl) analysed by HPLC. For chromatographic conditions see Fig. 3.

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