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FORMATION OF HYDROXYCINNAMOYLAMIDES AND α-HYDROXYACETOVANILLONE IN CELL CULTURES OF *SOLANUM KHASIANUM*

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Key Word Index—*Solanum khasianum*; Solanaceae; cell suspension cultures; elicitation; *cis*/*trans*-isomerization; hydroxycinnamoylamides; α-hydroxyacetovanillone.

Abstract—In elicitor-treated photomixotrophic *Solanum khasianum* cell cultures, nine phenolic compounds accumulated in the cell culture medium. They were isolated and structurally identified as the *cis*- and *trans*-isomers of *N*-*p*-coumaroyloctopamine, *N*-feruloyloctopamine, *N*-*p*-coumaroyltyramine and *N*-feruloyltyramine as well as α-hydroxyacetovanillone. The *cis*-isomers of the hydroxycinnamoyl moieties were only formed during illumination of the cultures. The hydroxycinnamoylamides showed a transient accumulation with maximum values between 6 and 24 hr after elicitation, depending on the compound. The decrease of the compounds is very likely a peroxidase-mediated reaction.

INTRODUCTION

Hydroxycinnamoylamides of various amines are widespread in higher plants, where they have been preferentially found in the reproductive organs [1, 2]. The amides of ferulic and *p*-coumaric acids formed with tyramine and octopamine were identified as soluble constituents in roots of eggplant and bell pepper [3, 4]. In recent years these amides have especially been found associated with cell walls in some species within the Solanaceae [5–8], where they seem to play an important role in plant defence against pathogens. It is believed that the incorporation of the amides leads to a reinforcement of the cell wall and a reduced digestibility by microbial enzymes [9], which will prevent the pathogen from invading the plant cell. Furthermore, recent investigations also showed that *N*-feruloyltyramine directly inhibits hyphal growth of an endomycorrhizal fungus [10]. Moreover, antiviral effects of hydroxycinnamoylamides have been discussed [11].

In our investigations of cell cultures of *Solanum khasianum*, a widespread plant in Asia often studied due to its high content of the steroidal alkaloid solasodine, we also analysed elicitor-induced effects on secondary constituents. Photomixotrophic cell cultures were chosen as the experimental system and they were treated with an elicitor preparation from yeast. In this report we describe the elicitor-induced accumulation of the *trans*- and *cis*-isomers of the hydroxycinnamoylamides *N*-feruloyl- and *N*-*p*-coumaroyltyramine as well as the corresponding octopamine conjugates. Furthermore, the formation of α-hydroxyacetovanillone, a compound which has recently been isolated from exudates of wounded cells of some Solanaceae species [12], is described.

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RESULTS

The photomixotrophic cell suspension cultures of *S. khasianum* were established from stem-derived calli and first cultured with 3% and finally with 1.5% sucrose in the culture medium. The cells form aggregates of about 5 mm diameter. Attempts to reduce aggregation of the cells by changing the concentrations of phytohormones were unsuccessful. During a growth cycle of 14 days, the cultures showed a 2.5-fold increase when a 5 g inoculum was used. The chlorophyll content of the cells reached values between 11 and 15 $\mu g\, g^{-1}$ fr. wt. Meanwhile the cultures have been cultivated for more than three years with more or less constant growth.

Identification of the compounds

Elicitation of the cell cultures with a yeast polysaccharide elicitor led to an accumulation of nine phenolic compounds in the cell culture medium (Fig. 1B). In the non-elicited control cultures only three of these compounds were found (Fig. 1A). The nine compounds

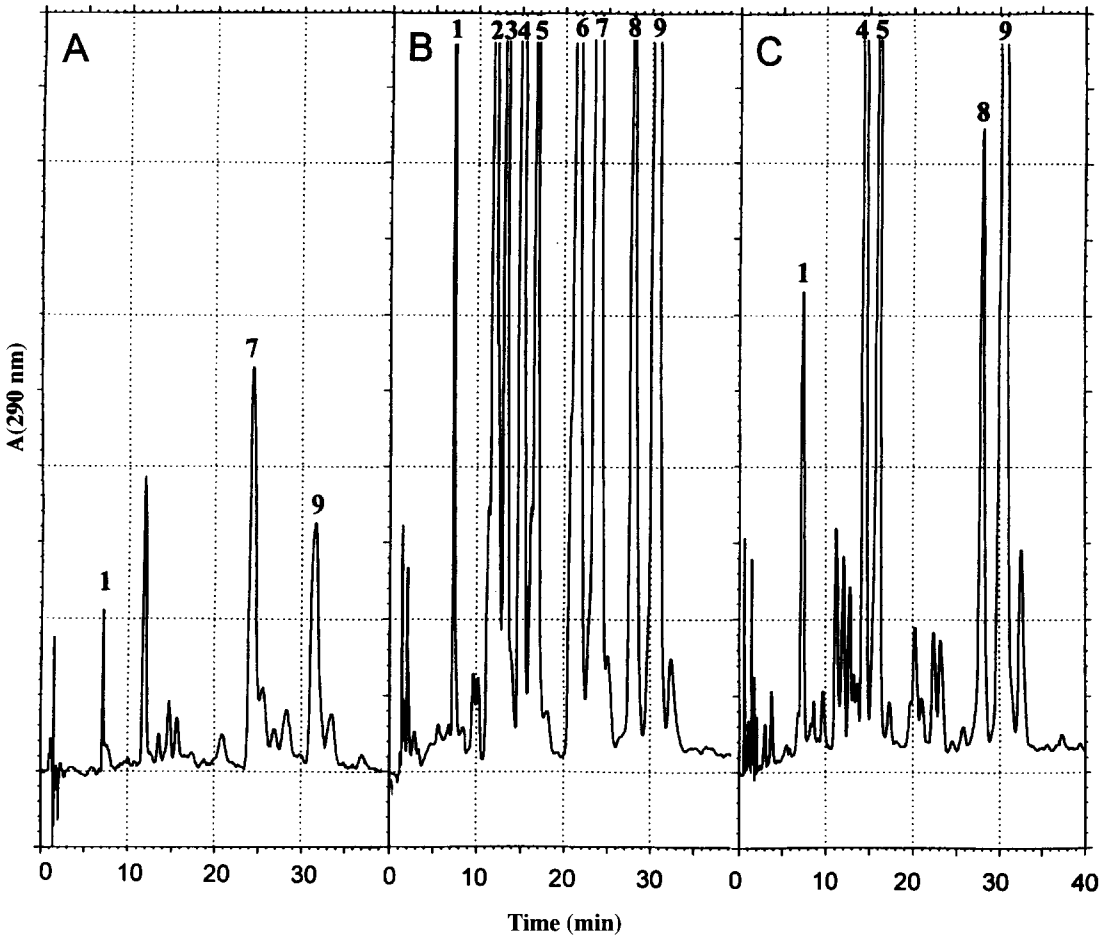
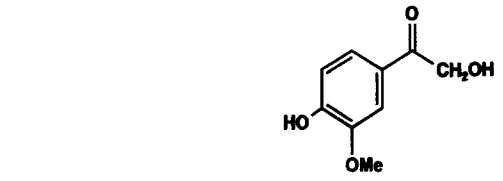


Fig. 1. HPLC chromatograms of phenolic compounds accumulating in the medium of *S. khasianum* cell cultures: (A) a control extract; (B) an extract 16 hr after elicitation under light; (C) a medium extract of cultures elicited in the dark. 1 = HO-AV; 2 = *cis*-*p*CO; 3 = *cis*-FO; 4 = *trans*-*p*CO; 5 = *trans*-FO; 6 = *cis*-*p*CT; 7 = *cis*-FT; 8 = *trans*-*p*CT; 9 = *trans*-FT.

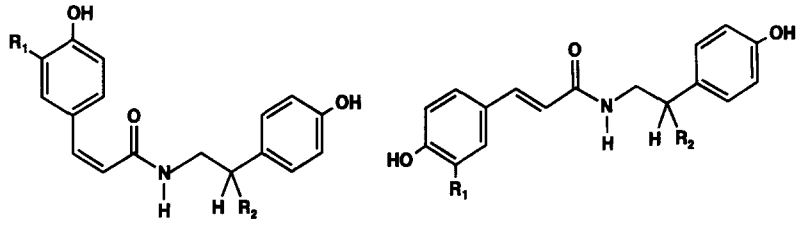
were isolated by extraction and HPLC. The structures of **2–9** were elucidated by UV and GC-mass spectroscopy analyses as well as by comparison with reference compounds, whereas **1** was identified by means of EI- and GC-mass spectroscopy and NMR spectroscopy, respectively. The spectroscopic data are all listed in the Experimental section.

The UV spectrum of **1** showed similarity to that of vanillin. Comparison of the molecular $[M]^+$ ion (*m/z* 182) and the $[M]^+$ ion of the silylated compound (*m/z* 326) suggested that the molecule had two hydroxyl groups which had been silylated. The strong $[M-103]^+$ fragment ion of the silylated substance most likely resulted from the cleavage of a $[CH_2\text{-OTMSi}]^+$ moiety from the molecular ion. The ^1H NMR spectrum showed the signals of a 1,3,4-substituted phenyl ring (H-2; 7.52 ppm; H-5: 6.96 ppm; H-6: 7.43 ppm). In addition, this spectrum exhibited a methoxyl signal (3.95 ppm) and a CH_2 signal (4.81 ppm). In the ^{13}C NMR with its nine peaks, the signal at 197.0 ppm can be attributed to a carbonyl group. From these data the structure of **1** was elucidated as α-hydroxy-3-methoxy-4-hydroxyacetophenone (α-hydroxyacetovanillone, HO-AV).

The compounds **4**, **5**, **8** and **9** were identified as the *trans*-isomers of the hydroxycinnamoylamides *N*-*p*-coumaroyloctopamine (*p*CO), *N*-feruloyloctopamine (FO), *N*-*p*-coumaroyltyramine (*p*CT) and *N*-feruloyltyramine (FT) by co-chromatography with synthesized references and comparison of their GC–mass spectral data with the values obtained for the references. Compounds **2** and **4**, **3** and **5**, **6** and **8** as well as **7** and **9** showed for each pair an identical fragmentation pattern after GC-mass spectroscopy, but they exhibited different UV spectra (see Experimental section). These data suggested that **2**, **3**, **6** and **7** were the *cis*-isomers of the aforementioned hydroxycinnamoylamides [13]. To corroborate this assumption, methanolic solutions of the reference substances, which clearly are the *trans*-isomers, were incubated for 24 hr under the same light conditions as the cell cultures. Subsequent HPLC analyses always revealed two signals representing the



1



2	$R_1 = H$	$R_2 = OH$	**4**	$R_1 = H$	$R_2 = OH$
3	$R_1 = OMe$	$R_2 = OH$	**5**	$R_1 = OMe$	$R_2 = OH$
6	$R_1 = H$	$R_2 = H$	**8**	$R_1 = H$	$R_2 = H$
7	$R_1 = OMe$	$R_2 = H$	**9**	$R_1 = OMe$	$R_2 = H$

cis- and the *trans*- isomers. After co-chromatography of these *cis*/*trans*-isomer mixtures with the isolated products, compounds **2**, **3**, **6** and **7** could be identified as the *cis*-isomers of *p*CO, FO, *p*CT and FT.

Formation of the cis-*isomers*

Since *cis*-isomers of cinnamates are very limited in plants, we investigated the formation of these *cis*-isomers by the cell cultures. It was assumed that the plant cells themselves are not able to synthesize these isomers, but that they were formed by a light-induced isomerization process in the culture medium of the light-grown photomixotrophic cells. Therefore, the cell cultures were transferred into darkness for subsequent elicitation. HPLC analyses of the elicited products accumulating under these conditions revealed that almost exclusively the *trans*-isomers had been formed (Fig. 1C). In general, these cultures produced lower amounts of hydroxycinnamoylamides than the cultures treated under light conditions (Table 1). However, it is evident that the content of the *cis*-isomers is very low in the cultures kept in darkness, in contrast to the cells which had been elicited in the light where the content of the *cis*-isomers is constantly higher than the level of the *trans*-isomers. To prove further that the isomerization of the cinnamoyl moieties is a light-driven process, the medium of cultures, which had been elicited in the dark, was again transferred to light. Analyses of the accumulated constituents showed that in this case the *cis*-isomers were again formed, whereas in the medium continuously incubated in the dark only low amounts of the *cis*-isomers could be found (Table 1).

Time course of the accumulation

The accumulation of compounds **2**–**9** after treatment of the photomixotrophic cell cultures with the yeast

Table 1. Quantitative comparison of *cis*- and *trans*-isomers of hydroxycinnamoylamides in the culture medium formed after elicitation under light and in darkness

	Content (nmol g^{-1} fr. wt)			
	16 hr elicitation under light	16 hr elicitation in the dark	16 hr elicitation in the dark	
Compound			5 hr light*	5 hr dark*
cis-*p*CO	92	0	32	0
trans-*p*CO	42	34	9	32
cis-FO	66	6	31	8
trans-FO	40	35	10	27
cis-*p*CT	109	0	16	0
trans-*p*CT	28	18	6	17
cis-FT	228	12	85	9
trans-FT	92	112	27	84

*After elicitation in darkness the cell-free medium of the cultures was further incubated under light or in the dark.

elicitor was measured over a period of 96 hr. All hydroxycinnamoylamides showed a transient accumulation, but pronounced differences between the compounds with regard to the time of maximum accumulation were observed. As an example, Fig. 2 depicts the accumulation curves of the tyramine conjugates. *trans*-*p*CT already reached its maximum amount after 6 hr and *cis*-*p*CT after 12 hr (Fig. 2A), whereas the maximum content of FT was only observed after 12 and 24 hr for the *trans*- and *cis*-isomers, respectively. The decrease of *trans*-*p*CT was more rapid than found for the *cis*-compound. After 24 hr only a small residual amount was left, but after 96 hr neither *trans*- nor *cis*-*p*CT could be found. In contrast to *p*CT, *cis*- and *trans*-FT were constitutively found in the cell culture medium. In the control medium maximum values of 100 nmol g^{-1} fr. wt (*cis*) and 40 nmol g^{-1} fr. wt (*trans*) were observed (data not shown). Elicitation caused a two-fold increase of the FT-isomers. However, the constitutively present FT-isomers at 0 hr disappeared after 3 hr of elicitation and only then the accumulation phase started (Fig. 2B).

The accumulation curves of the octopamine conjugates showed a very similar course as shown for the tyramine conjugates. Like FT, FO also occurred constitutively in very low amounts (2–8 nmol g^{-1} fr. wt). At the present time, the ratio between the octopamine and the tyramine conjugates is 1:1, but during earlier stages of the cell culture cultivation it was found to be 1:5 in favour of the tyramine conjugates.

HO-AV, essentially as the feruloylamides, also occurs constitutively in the cell culture medium at a level of *ca* 8 nmol g^{-1} fr. wt (Fig. 1A). Elicitation led to a substantial increase with a maximum content after 72 hr (57 nmol g^{-1} fr. wt) and after a total of 96 hr a slight decrease was observed.

Metabolism of the hydroxycinnamoylamides

The decrease in the level of the hydroxycinnamoylamides (Fig. 2) was thought to result from a peroxidase-mediated reaction. In fact, after elicitation, peroxidase activity could be detected in the cell culture medium (4 nkat g^{-1} fr. wt). In order to further prove this hypothesis, H_2O_2 was added to the cell-free medium of elicited cultures. Even after only 10 min the feruloylamides could no longer be detected in the medium, whereas the content of the *p*-coumaroylamides was only reduced to 30% of the initial concentration. In control media supplemented either with water or after boiling of the medium with H_2O_2 no reduction in the level of the compounds was measured. Application of a commercial horseradish peroxidase to the boiled medium led to a complete disappearance of the hydroxycinnamoylamides. Furthermore, addition of the H_2O_2 consuming enzyme catalase to elicited cell cultures had a stimulatory effect on the accumulation of the hydroxycinnamoylamides. As an example, Fig. 3 shows the amounts of *cis*-*p*CT and *trans*-FT after the addition of yeast elicitor and either catalase or dena-

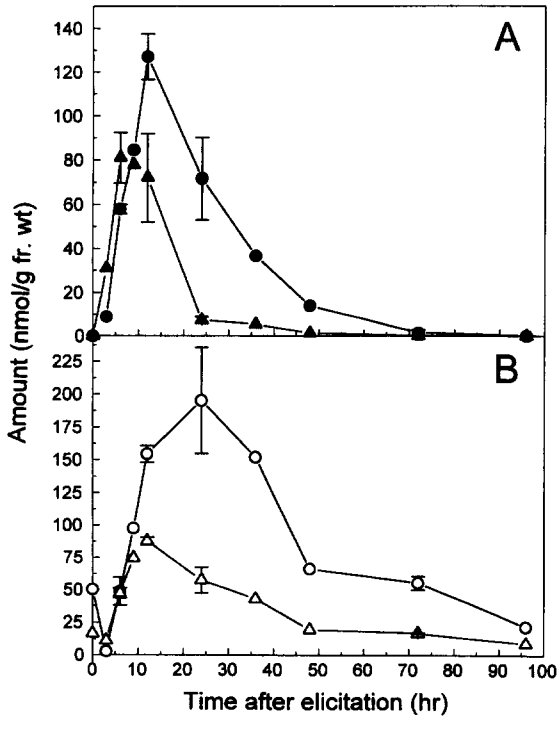


Fig. 2. (A) Time course of the accumulation of (●) *cis*-*p*CT and (▲) *trans*-*p*CT after application of yeast elicitor. (B) Time course of the accumulation of (○) *cis*-FT and (△) *trans*-FT after application of yeast elicitor. In the medium of non-elicited cells no *p*CT was found. In the case of FT a maximum value of 100 nmol g^{-1} fr. wt was reached after 24 hr in non-elicited cells.

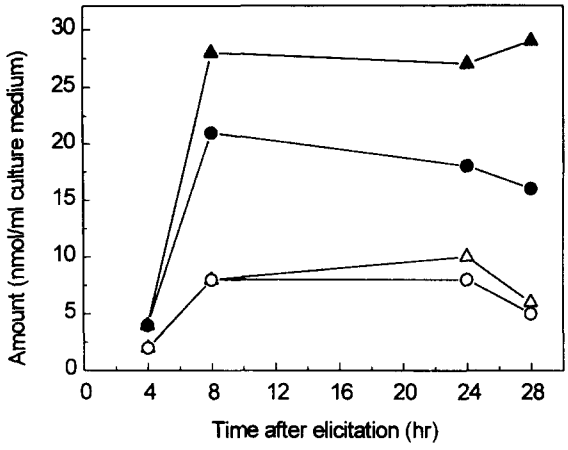


Fig. 3. Effect of catalase addition on the amounts of *cis*-*p*CT and *trans*-FT. Catalase was added 4 hr after the application of yeast elicitor to the cell cultures. Control cultures were treated with denaturated (boiled) catalase. (●) *cis*-*p*CT + catalase; (○) *cis*-*p*CT + boiled catalase; (▲) *trans*-FT + catalase; (△) *trans*-FT + boiled catalase.

tured (boiled) catalase. In the cultures supplemented with catalase a two- to four-fold higher content of *cis*-*p*CT and *trans*-FT was observed in contrast to the cultures supplemented with denatured catalase. These results suggest that peroxidases are involved in the observed metabolism of the amides, with the feruloyl conjugates being the preferential substrates.

DISCUSSION

Amides of cinnamates appear to play an essential role in the expression of antimicrobial defence reactions in species of Solanaceae. In potato tuber discs inoculated with *Phytophthora infestans* the *p*-coumaroylamides of tyramine and octopamine have been found [5]. These amides accumulated in cell walls near the infection site. In tobacco infected with tobacco mosaic virus, exogenously applied FT was rapidly incorporated into the cell walls [6]. Hydroxycinnamoylamides have also been isolated from cell cultures after elicitor treatment. In elicited *Lycopersicon peruvianum* cell cultures, both FT and *p*CT have been detected as cell wall constituents [7]. In general, the incorporation of the amides into cell walls is thought to render a reinforced barrier against the attacking pathogens.

In photomixotrophic cell cultures of *S. khasianum* the *N*-feruloyl- and *N*-*p*-coumaroylamides of octopamine and tyramine were found to accumulate in the cell culture medium in contrast to cell cultures of *Nicotiana glutinosa*, where no hydroxycinnamoyltyramines were detected in the medium, although the enzymes involved in the biosynthesis of these amides were induced by elicitation [14]. Another interesting feature of the *S. khasianum* cell cultures was the massive occurrence of the *cis*-isomers of the hydroxycinnamoylamides. The *trans*-isomers are the more stable compounds and normally they exclusively occur in plants. However, several reports describe the occurrence of *cis*-cinnamoyl conjugates in plants. Thus, in bell pepper, *cis*-isomers of FT have been found [4] and from cell cultures of hornwort, grown under light, *cis*-*N*-cinnamoylglutamic acids have been isolated [15]. In the case of our photomixotrophic *S. khasianum* cell cultures, the cinnamoylamides secreted into the medium are exposed to light. Since under dark conditions *S. khasianum* cultures only accumulated the *trans*-isomers (Fig. 1C, Table 1), the formation of the *cis*-isomers results from a light-driven isomerization process in the culture medium.

With regard to the biosynthetic pathways leading to the elicitor-induced *N*-cinnamoyltyramines and octopamines in *S. khasianum* several reports may be taken as suitable examples [14, 16]. However, the stereochemistry of the octopamines **2–5** remains to be elucidated.

The transient accumulation of the amides (Fig. 2) was partially clarified in terms of a polymerization process. In bell pepper, for example, dimers of FT have been identified [4] and in barley dimers of *N*-*p*-coumaroylagmatine were shown to occur [17, 18]. Furthermore, hydroxycinnamoylamides are good substrates for peroxidases *in vitro* [4]. Therefore, the disappearance of the amides from the *S. khasianum* culture medium may be regarded as a peroxidase-mediated reaction, which is supported by the detection of peroxidase activity in the medium and the data on the assays involving addition of H_2O_2 and catalase. In these experiments the feruloylamides were found to be the better substrates in comparison with the *p*-coumaroylamides. However, it remains to be clarified whether under cell culture conditions the amides are only subject to oxidative polymerization or whether degradative processes or uptake reactions by the cells may also be involved.

In addition to the hydroxycinnamoylamides, HO-AV has been found as an elicitor-stimulated compound. This compound has recently been identified in the exudates of wounded cells of different species of the Solanaceae [12]. Acetophenones and their hydroxyl

derivatives are further known to be wound-induced plant compounds involved in the activation of the *vir*-genes of *Agrobacterium tumefaciens*. Furthermore, such compounds have been identified in other cell cultures of the Solanaceae after elicitation [19, 20]. It is feasible that elicitation of cells leads to stress symptoms which are comparable with wounding. The occurrence of HO-AV in the unelicited *S. khasianum* cells is possibly due to mechanical or osmotic stress exerted on the cells during transfer into fresh medium. On the other hand, an acetophenone with a similar substitution pattern in the aromatic ring as HO-AV has recently been isolated as a novel phytoalexin from *Sanguisorba minor* [21]. The high elicitor-induced accumulation HO-AV in our *S. khasianum* cultures may be used as a suitable system to clarify hitherto unknown steps in HO-AV biosynthesis.

EXPERIMENTAL

Cell cultures and elicitation. Cell suspension cultures were established from stem-derived calli of *S. khasianum* CB Clarke. The cultures were cultivated in MS medium [22] supplemented with 1 mg l^{-1} 2,4-D, 1 mg l^{-1} kinetin and 15 g l^{-1} sucrose on rotary shakers (120 rpm) at 25° under continuous white light at 100–120 μEinstein m^{-2} sec^{-1}. Every 14 days *ca* 5 g of the cells were transferred into 40 ml fresh medium. Upon transfer the cell aggregates were reduced to small pieces with a sterile spoon. For elicitation, 80 mg yeast elicitor [23] was dissolved in 2 ml H_2O and the autoclaved soln was added to 7-day-old cultures. The control cultures were treated with 2 ml H_2O. For isolation of the compounds the culture medium was harvested 16 or 24 hr after elicitation.

Extraction and isolation of the metabolites. Cells were sepd from the culture medium by filtration and the medium was extracted ×2 with 1 vol. EtOAc. The combined EtOAc phases were dried at a rotary evaporator and the residue was resuspended in small vols of MeOH. The isolation of the compounds was performed by means of semi-prep. HPLC using a Waters chromatograph attached to a photodiode array detector for recording UV spectra. The compounds were sepd using a LiChrosorb RP-18 column (Merck, 10 × 250 mm, 7 μm) and a flow rule of 4 ml min^{-1}. The solvents were an MeCN azeotrope containing 15% H_2O (A) and H_2O (B). For elution of the compounds a linear gradient of 10% A–20% A in 5 min and 20% A–30% B in 35 min was applied. Frs containing the compounds were collected and evapd to dryness for further identification.

For analyt. sepns, HPLC was performed using a LiChrospher RP-18 (Merck, 4 × 125, 5 μm) with a flow rate of 0.8 ml min^{-1} and the same gradient as aforementioned.

Synthesis of trans-*hydroxycinnamoylamides.* The *N*-hydroxysuccinimide esters of the hydroxycinnamic acids were synthesized as described in ref. [24]. Then, 4 mmol tyramine or octopamine, respectively, were dissolved in 20 ml H_2O, which had been adjusted to pH 8 with $NaHCO_3$, and the aq. soln was then mixed with 20 ml Me_2CO, in which 4 nmol of the synthesized acyl *N*-hydroxysuccinimide-ester had been dissolved. The mixt. was stirred for 24 hr in the dark. After acidification to pH 2 with H_3PO_4, the Me_2CO was evapd and the aq. phase was partitioned ×2 with Et_2O. After evaporating the Et_2O phase, the residue was dissolved in MeOH and purified by semi-prep. HPLC as described above. The structures were confirmed by GC-MS analyses.

Isomerization experiments. 30 nmol of the synthesized *trans*-cinammoylamides were dissolved in MeOH and then incubated under light for 24 hr. The mixt. of isomers was then analysed by HPLC and used for co-chromatography. For investigations on cellular formation of the *cis*-isomers, the cultures, which had been grown normally under light, were transferred into darkness, incubated for 2 hr and then elicited with 80 mg yeast elicitor. After 16 hr the cells were sepd from the culture medium and 5 ml of the medium was extracted as described above. One-half of the remaining medium was further incubated in the dark and the other half was transferred into light again. After 5 hr the medium of both samples was extracted. The ratio of the *cis*/*trans*-isomers was analysed by HPLC.

Identification of the compounds. UV spectra were recorded in MeOH using a Kontron Uvicon 810 spectrophotometer. GC-MS measurements were carried out on a Varian GC 3400 with a capillary column (HPU2, 25 m). The compounds were silylated with *N*-trimethylsilyl-*N*-methyl-triflouroacetamide (*ca* 200 μg compound/30 μl MSTFA) for 15 min at 85° and 1 μl samples were sepd by GC. For **1** a temp. gradient from 150° to 300° at 10° min^{-1} was used. For analysis of hydroxycinnamoylamides a gradient from 280° to 300° at 8° min^{-1} was applied and the temp. was held at 300° for 30 min. The injector temp. was 320°. The column was coupled with a Finnigan Mat 8230 MS (70 eV). EIMS was performed on a Finnigan Mat 312 (70 eV). NMR spectroscopy was carried out on a Bruker WM 300 MHz instrument.

α-Hydroxyacetovanillone (**1**). UV λ_{max} nm: 227, 276, 304. EIMS *m/z* (rel. int.): 182 $[M]^+$ (15), 151 (100), 123 (47), 108 (27). GC-MS (TMSi) *m/z* (rel. int.): 326 $[M]^+$ (5), 311 $[M-Me]^+$ (23), 223 $[M\text{-}CH_2OTMS]^+$ (100), 209 (2), 193 (7). ^1H NMR (300 MHz, $CDCl_3$); δ 7.52 (*d*, 1H), 7.43 (*dd*, 1H), 6.96 (*d*, 1H), 4.81 (*s*, 2H), 3.95 (*s*, 3H). ^{13}C NMR (75 MHz, $CDCl_3$); δ 197.0 (C=0), 151.4, 146.9, 126.4, 123.0, 114.3, 109.8, 65.0 (CH_2), 56.3 (Me).

cis-N-p-*Coumaroyloctopamine* (**2**). UV λ_{max} nm: 275. GC-MS (TMSi) *m/z* (rel. int.): 515 $[M]^+$ (not detected), 500 $[M-Me]^+$ (2.2), 424 (3), 321 (4), 281 (5), 267 (100), 219 (11), 193 (5).

cis-N-*Feruloyloctopamine* (**3**). UV λ_{max} nm: 276, 310 sh. GC-MS (TMSi) *m/z* (rel. int.): 545 $[M]^+$ (not

detected), 530 $[M - Me]^+$ (2.5), 454 (4), 351 (15), 267 (100), 249 (15), 193 (8).

trans-N-p-*Coumaroyloctopamine* (**4**). UV λ_{max} nm: 292, 310. GC-MS (TMSi) *m/z* (rel. int.): 515 $[M]^+$ (not detected), 500 $[M - Me]^+$ (4.3), 424 (4.7), 321 (19), 280 (6), 267 (100), 219 (14), 193 (7).

trans-N-*Feruloyloctopamine* (**5**). UV λ_{max} nm: 290, 318. GC-MS (TMSi) *m/z* (rel. int.): 545 $[M]^+$ (not detected), 530 $[M - Me]^+$ (2.8), 454 (3), 351 (14), 280 (5), 267 (100), 249 (12), 193 (5).

cis-N-p-*Coumaroyltyramine* (**6**). UV λ_{max} nm: 276. GC-MS (TMSi) *m/z* (rel. int.): 427 $[M]^+$ (2.3), 412 $[M - Me]^+$ (4.8), 308 (5.5), 234 (17), 219 (22), 192 (100), 149 (2), 177 (13).

cis-N-*Feruloyltyramine* (**7**). UV λ_{max} nm: 276, 300 sh. GC-MS (TMSi) *m/z* (rel. int.): 457 $[M]^+$ (12), 442 $[M - Me]^+$ (6), 338 (1.4), 264 (100), 249 (35), 234 (10), 219 (8), 192 (62), 177 (14), 145 (6), 117 (5).

trans-N-p-*Coumaroyltyramine* (**8**). UV λ_{max} nm: 290, 310. GC-MS (TMSi) *m/z* (rel. int.): 427 $[M]^+$ (3.2), 412 $[M - Me]^+$ (5), 308 (5), 234 (16), 219 (27), 192 (100), 177 (14), 149 (7).

trans-N-*Feruloyltyramine* (**9**). UV λ_{max} nm: 290, 318. GC-MS (TMSi) *m/z* (rel. int.): 457 $[M]^+$ (11), 442 $[M - Me]^+$ (10), 338 (2), 264 (100), 249 (37), 234 (9), 219 (7), 192 (70), 177 (15), 145 (6), 117 (4).

Examination of the metabolism of the compounds. Cell cultures were harvested 16 hr after elicitation and the cells were removed by filtration. 5 ml of the medium was extracted for HPLC analysis. H_2O_2 (1.2 M, 300 μl) or H_2O, respectively, was added to 30 ml of medium; alternatively H_2O_2 was applied to the boiled medium. 10, 20, 30, 60 and 240 min after the additions, 5 ml of medium was extracted and analysed by HPLC. Every 10 min, fresh H_2O_2 was added to the medium. In another approach H_2O_2 and horseradish peroxidase (5 U ml^{-1} medium) was added to the boiled medium. Peroxidase activity was measured according to ref. [25]. For catalase experiments, catalase (100 000 U) was added to cell cultures 4 hr after application of yeast elicitor. Control cultures were supplemented with boiled (20 min) catalase. After 4, 8, 24 and 28 hr, 3 ml of culture medium was extracted for HPLC analyses and fresh catalase was added at each indicated time interval.

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