β-1,3-Glucooligosaccharide Induced Activation of Four Enzymes Responsible for *N-p*-coumaroyloctopamine Biosynthesis in Potato (*Solanum tuberosum* cv.) Tuber Tissue

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β-1,3-Glucooligosaccharide, *N-p*-Coumaroyloctopamine, *Solanum tuberosum*

Potato tuber disks, when treated with laminarin, a β-1,3-glucooligosaccharide from *Laminaria digitata*, accumulate a hydroxycinnamoyl amide compound, *N-p*-coumaroyloctopamine (*p*-CO). The biosynthesis of *p*-CO was investigated by feeding experiments, in order to show that the precursors of *N-p*-coumaroyl and octopamine moieties of *p*-CO are L-phenylalanine and L-tyrosine, respectively. The treatment of potato tuber tissue with laminarin resulted in elevated activities of four enzymes which are putatively involved in *p*-CO biosynthesis: phenylalanine ammonia lyase (PAL; EC 4.3.1.5), 4-hydroxycinnamic acid:CoA ligase (4CL; EC 6.2.1.12), hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT; EC 2.3.1.110) and tyrosine decarboxylase (TyrDC; EC 4.1.1.25). Among these, the response of TyrDC was specific to laminarin treatment, thus indicating that the regulation of TyrDC activity is critical for the accumulation of *p*-CO in potato tuber tissue.

Introduction

Plants exhibit a variety of inducible defense responses against infection by pathogens. From the chemical point of view, such responses consist of diverse changes in secondary metabolism, resulting in the syntheses of antifungal compounds (Kuć, 1995), as well as of polymeric compounds which serve as a physical barrier against intrusion by pathogens (Matern *et al.*, 1995).

In potato (*Solanum tuberosum*), the chemical defense responses are characterized by the induction of sesquiterpenoid phytoalexins such as rishitin (Katsui *et al.*, 1968; Tomiyama *et al.*, 1968), and hydroxycinnamoyl amide compounds, which contain tyramine and octopamine as the amine donors (Clarke, 1982). While the former phytoalexin accumulation has been observed only in tuber tissue (Rohwer *et al.*, 1987), the latter is related to phenylpropanoid metabolism following pathogen infection in a tissue-independent manner (Clarke, 1982; Keller *et al.*, 1996), and thus appears to be of more concern. Although these amide compounds show no practical antifungal activity themselves (unpublished data), it has been proposed that they are deposited into cell walls and utilized for physical reinforcement of the wall (Clarke, 1982; Facchini *et al.*, 1999; Keller *et al.*, 1996; Negrel *et al.*, 1996).

An accumulation of hydroxycinnamoyl amides in potato was first observed in the tuber tissue infected with the late blight fungus, *Phytophthora infestans* (Clarke, 1982). Accumulation also occurs in leaves infected with *P. infestans* (Keller *et al.*, 1996) and in suspension-cultured cells which have been treated with an elicitor preparation from the same fungus (Pi elicitor) (Schmidt *et al.*, 1998). Recently, we reported that the treatment of tuber tissue with laminarin, a β-1,3-glucooligosaccharide derived from *Laminaria digitata*, causes an accumulation in a manner similar to the Pi elicitor, and the saccharide-nature of the elicitor may be responsible for the induction of the amide compounds (Miyagawa *et al.*, 1998). The hydroxycinnamoyl amides in potatoes are thought to be synthesized from a hydroxycinnamoyl CoA ester and the corresponding amine precursors. The enzyme which catalyzes this cin-

Abbreviations: CoA, coenzyme A; *p*-CO, *N-p*-coumaroyloctopamine; CHX, cycloheximide; 4CL, 4-hydroxycinnamic acid:CoA ligase; PAL, phenylalanine ammonia lyase; THT, hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase; TyrDC, tyrosine decarboxylase.

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namoyl transfer reaction, hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT; EC 2.3.1.110), has been purified and its cDNA has already been cloned. (Hohlfeld *et al.*, 1996; Hohlfeld *et al.*, 1995; Negrel and Javelle, 1997; Schmidt *et al.*, 1999). Schmidt *et al.* (1998) have reported that the accumulation of amide compounds is preceded by rapid and transient increases of the activities of THT activity in suspension-cultured potato cells treated with the Pi elicitor. They also have demonstrated that the activities of other enzymes involved in amide synthesis, including phenylalanine ammonia-lyase (PAL) for the cinnamoyl moiety and tyrosine decarboxylase (TyrDC) for the amine moiety are changed in the same manner. Moreover, THT has been studied in potato in relation to wound response (Negrel *et al.*, 1993) and in other plants with regard to changes in activity caused by elicitor- and stress-stimulation (Villegas and Brodelius, 1990).

Available data indicate that there is some difference between tuber tissue and cultured-cells, regarding the accumulation of hydroxycinnamoyl amide compounds in potatoes following the elicitation. Tuber tissue produces a large amount of *N*-*p*-coumaroyloctopamine (*p*-CO) (Miyagawa *et al.*, 1998), while the cultured-cells mainly accumulate tyramine amides, such as *N*-*p*-coumaroyl- and *N*-feruloyltyramine (Keller *et al.*, 1996; Schmidt *et al.*, 1998). Although octopamine has been found in some plant species (Smith, 1977; Wheaton and Stewart, 1970), neither its role nor biosynthesis in plants has yet been elucidated and thus the production of *p*-CO in potato tissue raises an interesting subject. We have recently shown that the absolute configuration of the octopamine moiety of *p*-CO in potato is *S* (Matsuda *et al.* submitted), in contrast to that of octopamine found in animals (Goosey and Candy, 1980; Starratt and Bodnaryk, 1981).

In this study we wish to report the biosynthetic origins of *p*-CO and the effects of the β-1,3-glucooligosaccharide elicitor on the activities of four enzymes which are associated with the formation of hydroxycinnamoyl amides in potato tuber tissue. The results indicate that the activities of these enzymes in potato tuber tissues are regulated in a more complex manner than that occurs in suspension culture cells.

Materials and Methods

Plant material

All potato tubers (*Solanum tuberosum* cv. Eniwa) used in this study were stored at 4 °C for a minimum of 6 months following harvesting. The internal part of the tuber was cut into disks (8 mm diameter, 2 mm thick), and washed with water for 30 min. Washed disks were incubated at 18 °C in the dark under wet conditions for 24 h prior to treatment with an aqueous solution of laminarin.

Chemicals

Laminarin from *Laminaria digitata* was purchased from SIGMA Chemical Co. Laminariheptaose was purchased from Seikagaku Co., JAPAN. Cycloheximide was purchased from Nacalai Tesque Co., JAPAN. *p*-CO and *N*-feruloyloctopamine were synthesized as described by Negrel *et al.* (1993). *p*-Coumaroyltyramine and *N*-feruloyltyramine were prepared according to the methods of Villegas and Brodelius (1990). *p*-Coumaroyl CoA was prepared by transesterification of hydroxycinnamoyl-*N*-hydroxysuccinimide esters as described by Stöckigt and Zenk (1975). [2,3,4,5,6-^{5}H] L-phenylalanine and [2,6-^{2}H] L-tyrosine were purchased from ISOTEC, USA.

Quantification of phenolic amide compounds

10 µl of an aqueous laminarin solution (1.0 mg/ml) was applied to potato tuber disks, followed by incubation for 24 h. Three disks were combined, weighed, and extracted with 2.5 ml of 2% acetic acid (aq.) at 100 °C for 10 min. These extracts were centrifuged at 3000 rpm for 10 min, followed by HPLC analysis of the supernatant (column: Wakosil 5C18, 4.6 × 150 mm, solvent: 40% methanol in water containing 0.1% H_3PO_4, flow rate: 0.8 ml/min, detection: UV 310 nm).

Feeding experiments with [2,3,4,5,6-^{5}H] L-phenylalanine and [2,6-^{2}H] L-tyrosine

A 10 µl aliquot of 10 mM [2,3,4,5,6-^{5}H] L-phenylalanine or [2,6-^{2}H] L-tyrosine dissolved in 0.1 M Tris[tris(hydroxymethyl)aminomethane]HCl buffer (pH 7.5) was applied on a tuber disk following treatment with a 10 µl aliquot of laminarin solution (1.0 mg/ml). This treatment was repeated at six hour intervals after the initial appli-

cation. After 20 h *p*-CO was extracted as described above, and a 1.0 ml aliquot of the extract was evaporated *in vacuo*. The dried extracts were redissolved in 100 µl of methanol, and analyzed by LC-API-MS. Positive ion-spray ionization mass spectra measurements were carried out using a Parkinermer-Sciex API-165 instrument (ion-spray voltage: 5 kV, orifice voltage: 30V, nebulizer gas, curtain gas: nitrogen) combined with a Shimadzu LC-10A HPLC system. (column: Cosmosil 5C18, 4.6 × 150 mm, solvent: 40% methanol in water containing 0.1% acetic acid, flow rate: 0.8 ml/min).

Enzyme assays

A 10 µl aliquot of an aqueous laminarin solution (1.0 mg/ml) was applied to potato tuber disks which were then incubated for a specified time.

Phenylalanine ammonia lyase (PAL)

Three disks (about 0.5 g) were homogenized with sea sand in five volumes of 0.1 M sodium phosphate buffer (pH 7.5) containing 2-mercaptoethanol (14.4 mM). The homogenate was centrifuged at 12,000×*g* for 10 min, and the supernatant was desalted on a PD-10 column. The protein fraction was used as the crude enzyme extract. The reaction mixture consisted of 60 µl of crude enzyme extract and 600 µl of 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM L-phenylalanine as the substrate. The mixture was incubated at 30 °C for 60 min, and the reaction was quenched by the addition of 60 µl of 1N HCl. After the addition of methanol (720 µl), the amount of *trans*-cinnamic acid produced was determined by HPLC (column: Wakosil 5C18, 4.6 × 150 mm, solvent: 70% methanol in water containing 0.1% H_3PO_4, flow rate: 0.8 ml/min, detection: UV 280 nm).

4-Hydroxycinnamic acid:CoA ligase (4CL)

Three disks (about 0.5 g) were homogenized with sea sand in five volumes of 0.1 M sodium phosphate buffer (pH 7.5) containing 2-mercaptoethanol (14.4 mM) and glycerol (25% v/v). The homogenate was centrifuged at 12,000×*g* for 10 min and the supernatant was used as the crude enzyme extract. Aliquots (0.15 ml) of crude extract were incubated at 30 °C in a solution composed of 0.3 ml of 5.0 mM *p*-coumaric acid, 0.75 ml of 20 mM ATP containing 20 mM $MgCl_2$, and 0.2 M KH_2PO_4-NaOH buffer (pH 7.5). The reaction was initiated by adding of 0.3 ml of 1.0 mM CoA and the suspension was incubated for 10 min. 4CL activity was determined from the increase in absorbance at 333 nm using a molar extinction coefficient of 2.2 × 10^4 a described by Zenk *et al.* (1980).

Hydroxycinnamoyl-CoA:tyramine N-(hydroxycinnamoyl)transferase (THT)

Three disks (about 0.5 g) were frozen in liquid N_2 and homogenized with sea sand in five volumes of 0.1 M sodium phosphate buffer (pH 7.5) containing 2-mercaptoethanol (14.4 mM). The homogenate was centrifuged at 12,000×*g* for 10 min, and the supernatant was used as the crude enzyme extract. The reaction mixture contained 10 µl of 500 µM *p*-coumaroyl CoA, 10 µl of 10 mM octopamine hydrochloride, 70 µl of 0.1 M sodium phosphate buffer (pH 8.0) and 10 µl of crude enzyme extract. The mixture was incubated at 30 °C for 10 min, and the reaction was quenched by the addition of 20 µl of acetic acid. *p*-CO levels were quantified by HPLC (column: Wakosil 5C18, 4.6 × 150 mm, solvent: 40% methanol in water containing 0.1% H_3PO_4, flow rate: 0.8 ml/min, detection: UV 310 nm).

Tyrosine decarboxylase (TyrDC)

A crude enzyme extract was prepared by the same procedure as described for the PAL assay. The reaction mixture contained 90 µl of the crude enzyme extract, 30 µl of 10 mM L-tyrosine, 30 µl of 1.0 mM pyridoxal-5'-phosphate, and 150 µl of 0.1 M sodium phosphate buffer (pH 7.5). The mixture was incubated at 30 °C for 60 min and after the addition of 30 µl of 1 N HCl to quench the reaction, the induced tyramine was converted to dansyl tyramine by a modification of the method described by Smith and Davis (1985). An 100 µl of the reaction mixture was added to an aliquot (200 µl) of saturated Na_2CO_3 and 400 µl of 7.5 mg/ml dansyl chloride in acetone in sealed vials. And the vials were incubated at 60 °C for 60 min in the dark. Following the addition of 100 µl of a proline solution (100 µg/ml), the mixture was incubated for an additional 30 min under identical conditions. The mixture was extracted with 800 µl of ethyl acetate, followed by removal of the organic

phase under a steady stream of N_2. The residue was resuspended in 500 µl methanol, filtered through a PTFE filter (0.45 µM, ADVANTEC), and analyzed by HPLC (column: Wakosil 5C18, 4.6 × 150 mm, gradient system (methanol : water (0.1% phosphate)): from 60 : 40 to 95 : 5 in 27 min, flow rate: 1.0 ml/min, temperature: 35 °C, detection fluorescence: excitation 365 nm, emission 510 nm). Dansyl tyramine was eluted at 19.2 min under these conditions.

Protein quantification

The protein content in the crude extract was determined according to the methods of Bradford (1976) using bovine serum albumin as a standard.

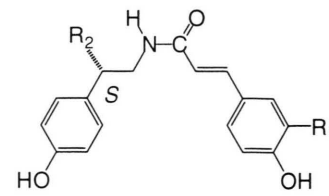
Quantification of cell deposited p-CO

Cell wall fractions of potato tuber disks were prepared as described Keller *et al.* (1996). The cell wall fraction (a white powder) was suspended in 2.0 ml of 0.3 N sodium methoxide in 80% methanol (aq.), and stirred continuously under N_2 for 2 h at 80 °C followed by 1 h at room temperature. The hydrolysate was acidified by the addition of 50 µl 85% H_3PO_4 (aq.) and centrifuged for 10 min at 3000 rpm. Following removal of the supernatant *in vacuo*, the residue was resuspended in 1.0 ml of water and extracted twice with 2.0 ml of diethyl ether. The extract was dried and redissolved in 20% methanol (aq.), 20 µl of which was used for HPLC analysis (column: Wakosil 5C18, 4.6 × 150 mm, gradient system (methanol : water (0.1% phosphate)): from 20 : 80 to 80 : 20 in 50 min, flow rate: 0.8 ml/min, temperature: 35 °C, detection: UV 310 nm). *p*-CO, which was eluted at 22.6 min under these conditions, was identified by co-chromatography with an authentic sample and detected via the pseudomolecular ion (*m/z*: $[M+H]^+$ 300) by LC-API-MS.

Results

Composition of phenolic amides induced by laminarin

To date, four kinds of compounds (*N*-*p*-coumaroyloctopamine (*p*-CO), *N*-feruloyloctopamine, *N*-*p*-coumaroyltyramine, *N*-feruloyltyramine; Fig. 1) have been described as inducible phenolic amides in potato tuber tissue (Clarke, 1982). The changes



R_1 = H, R_2 = OH; *N*-*p*-coumaroyloctopamine (*p*-CO)
R_1 = H, R_2 = H; *N*-*p*-coumaroyltyramine
R_1 = OCH_3, R_2 = OH; *N*-feruloyloctopamine
R_1 = OCH_3, R_2 = H; *N*-feruloyltyramine

Fig. 1. Structures of hydroxycinnamoyl-amides found in potato tuber tissues.

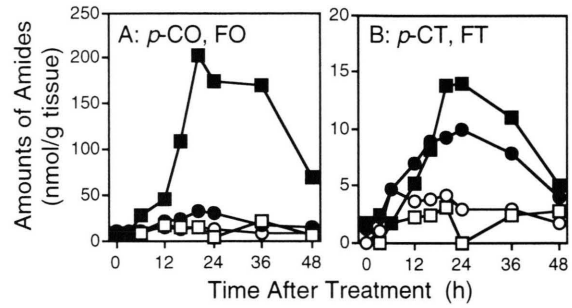


Fig. 2. Time course for the production of four hydroxycinnamic amide compounds in potato. The disks were prepared 24 h prior to the treatment. At time 0, potato tuber disks were treated with a 10 µl aliquot of 1.0 mg/ml laminarin solution (full symbols) or distilled H_2O (open symbols) and incubated at 18 °C under dark condition. (A) Amounts of *N*-*p*-coumaroyloctopamine (*p*-CO, rectangles) and *N*-feruloyloctopamine (FO, circles). (B) Amounts of *N*-*p*-coumaroyltyramine (*p*-CT, rectangle) and *N*-feruloyltyramine (FT, circles). The results are the mean of duplicate experiments.

in the amount of these compounds were analyzed, in order to characterize the features of the elicitor response. As shown in Fig. 2, the amounts of all four hydroxycinnamoyl amides were increased in laminarin treated tuber disks, reaching maximum levels after 24 h. No induction of these amides was observed in control disks. Among these compounds, the increase in the level of *p*-CO was greatest, with levels reaching 200 nmol/g tissue.

Incorporation [2,3,4,5,6-^{5}H] L-phenylalanine (phe-d_5) and [2,6-^{2}H] L-tyrosine (tyr-d_2) into p-CO

In order to clarify the biosynthetic origins of *p*-CO which was most abundantly accumulated in the potato tuber tissue treated with elicitor as de-

scribed above, the incorporation of L-phenylalanine and L-tyrosine was examined using stable-isotope labeled compounds.

Following treatment of the potato tuber disk with the labeled compounds and a laminarin solution (1.0 mg/ml) for 20 h, the incorporation rates were analyzed by LC-API-MS. Table I shows the relative intensities of the observed pseudomolecular *p*-CO ion (*m/z* 300 [M+H$^+$]) and its isotope ions. While the exogenous application of phe-d_5 significantly increased the intensity of the *p*-CO-d_4 ion (*m/z* 304), a corresponding increase in *p*-CO-d_8 ion (*m/z* 308) was not observed. Since one deuterium is thought to be replaced by a hydroxyl group, these results indicate that phenylalanine was incorporated into only one of the *p*-coumaroyl- and octopamine moieties that constitute *p*-CO. Similarly, the application of tyr-d_2 resulted in an increase in the ion intensity from *p*-CO-d_2 (*m/z* 302), but no increase in that from *p*-CO-d_4 (*m/z* 304). On the other hand, when phe-d_5 and tyr-d_2 were applied simultaneously, an increase in the *p*-CO-d_6 (*m/z* 306) ion intensity was clearly observed, in addition to those from *p*-CO-d_4 and *p*-CO-d_2. These results strongly suggest that L-phenylalanine and L-tyrosine are, respectively, incorporated into the different moieties of *p*-CO in the elicited potato tuber tissue.

Activation of four enzymes responsible for p-CO biosynthesis by treatment with laminarin

The results of feeding experiments showed that *p*-CO accumulated in potato tuber tissue is synthesized by the pathway shown in Fig. 3. In this pathway, phenylpropanoid-metabolism-related enzymes such as PAL and 4CL are involved in the synthesis of the *p*-coumaroyl moiety, TyrDC for that of octpamine-moiety, and THT for the formation of amide bond. Thus, the effects of laminarin treatment on the activities of these biosynthetic related enzymes were examined. The time-dependent changes in each enzyme activity are shown in Fig. 4. All enzyme activities in the control tissue increased to some extent in the absence of elicitor treatment. In particular, THT and 4CL activities were increased to 102.8 and 46.2 p katal / mg protein, respectively. Laminarin treatment caused an additional transient activation of these enzymes. While the maximal levels of THT and 4CL activities reached about twice those of control, the activity of PAL was 3 times the control and that of TyrDC was 5 fold greater than control levels. Following maximal induction, all enzyme activities declined to control levels within 48 h after treatment.

CHX (10 μg/ml) inhibited the activation of PAL, THT and TyrDC in the laminarin-treated tuber disks, with the activities decreasing to that of control levels (Table II). Concomitantly, the accumulation of *p*-CO in the tuber tissue was inhibited by CHX in a dose dependent manner, and the level of *p*-CO decreased to 1/12 that of the positive control at a concentration of 25 μg/ml, as shown in Fig. 5. These results indicate that laminarin activates de novo protein biosynthesis, leading to the accumulation of *p*-CO.

Table I. Intensity of the molecular ion peak in the mass spectrum of *N*-*p*-coumaroyloctopamine produced by potato tuber tissues fortified with [2,3,4,5,6-^{2}H] L-phenylalanine (phe-d_5) and [2,6-^{2}H] L-tyrosine (tyr-d_2).

m/z	control	phe-d_5	tyr-d_2	phe-d_5 + tyr-d_2
300	100	100	100	100
301	23.5	23.5	30	24.7
302	6.0	6.0	25.6	18.3
303	3.4	3.4	6.9	6.2
304	2.4	34.2	2.4	30.6
305	2.4	8.2	2.4	8.0
306	2.2	2.6	1.2	9.1
307	3.5	6.8	3.3	10.4
308	3.4	3.8	3.2	5.3
				(%)

Intensity of [M+H$^+$] ion (*m/z*: 300) was arbitrary set to 100%.

Effects of the elicitor concentration on enzyme activity

To investigate the effects of the elicitor concentration on the enzyme activities, an aliquot of various concentration of laminarin was treated on potato tuber disks for 16 h and the activities of PAL, 4CL, THT and TyrDC were determined. The activities of these enzymes were increased in a dose dependent manner up to 1.0 mg/ml (Fig. 6). Beyond this laminarin concentration, the activity of each enzyme reached a plateau and no increase was observed up to 10.0 mg/ml. These profiles were in good agreement with that observed for the relationship between the accumulated amount of *p*-CO in potato tuber disks and laminarin concen-

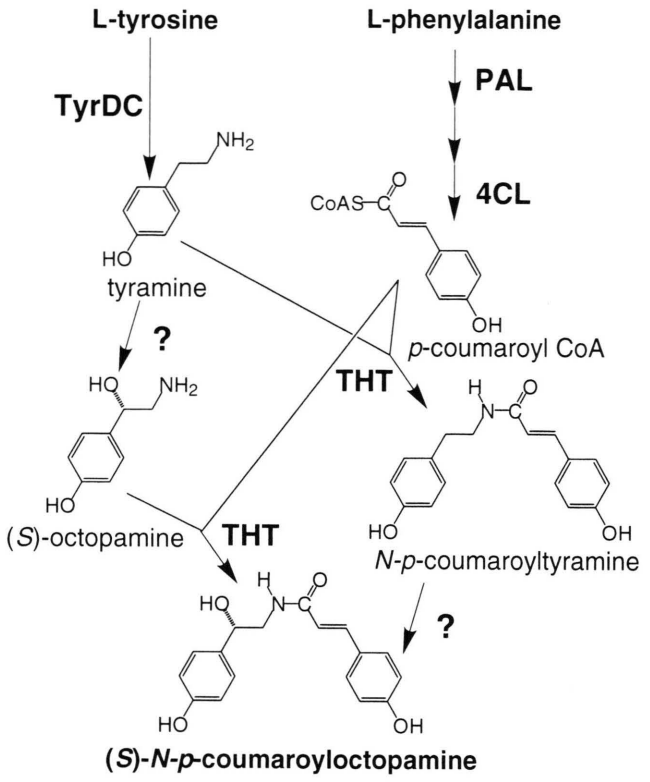


Fig. 3. Proposed pathways for the biosynthesis of *p*-CO in potato tuber tissue. Two possible pathways are shown, in which *p*-CO was produced by the direct condensation between octopamine and the *p*-coumaroyl CoA thioester (left pathway), and the oxidation of *N*-*p*-coumaroyltyramine (right pathway). PAL, phenylalanine ammonia lyase: 4CL, 4-hydroxycinnamic acid: CoA ligase: THT, hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase: TyrDC, tyrosine decarboxylase. The steps marked with a question mark have not been established.

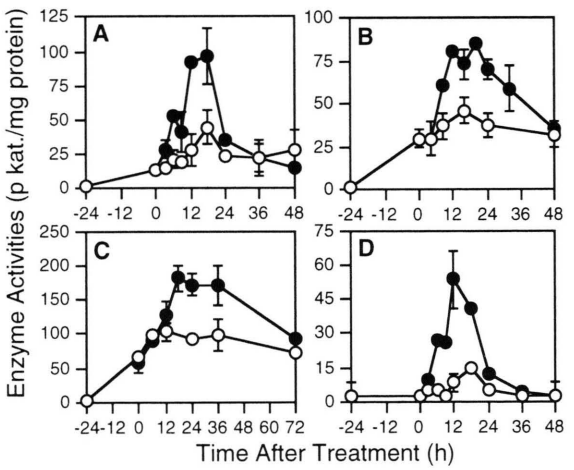


Fig. 4. Activation of PAL (A), 4CL (B), THT (C) and TyrDC (D) in potato tuber disks treated with laminarin. The disks were prepared 24 h prior to the treatment with an aqueous solution of laminarin (1.0 mg/ml). Closed circles represent activities of enzymes in disks treated with laminarin and open circles those of the control disks. The results are expressed as the mean of triplicate experiments ±SD.

Table II. Effects of cycloheximide (CHX, 10 μg/ml) on the laminarin induced activation of enzymes responsible for *p*-CO biosynthesis.

Enzyme	Enzyme activities (pkatal / mg protein)		
	Control	Laminarin 1.0 mg/ml	Laminarin + CHX
PAL	34.1 ± 1.8	120 ± 3.3	22.8 ± 0.5
THT	135 ± 3.8	235 ± 31.5	62.7 ± 7.7
TyrDC	13.8 ± 1.7	91.8 ± 13.4	16.7 ± 1.5

All enzyme activities were determined at 16 h after laminarin treatment. The results are expressed as the average of triplicates ± SE. PAL, phenylalanine ammonia lyase: THT, hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase: TyrDC, tyrosine decarboxylase.

tration (Miyagawa *et al.*, 1998), indicating that the activation of these enzymes are responsible for the accumulation of *p*-CO.

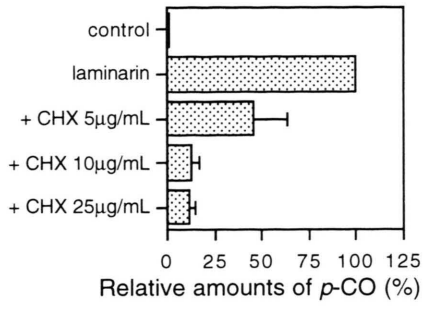


Fig. 5. Effects of the cycloheximide (CHX) on the laminarin-induced accumulation of *p*-CO in potato tuber disks. The Tuber disks were treated with laminarin (1.0 mg/ml) in the presence of the specified concentration of CHX, and levels of induced *p*-CO were quantified 24 h after treatment. The results are expressed as the means of triplicates ±SD.

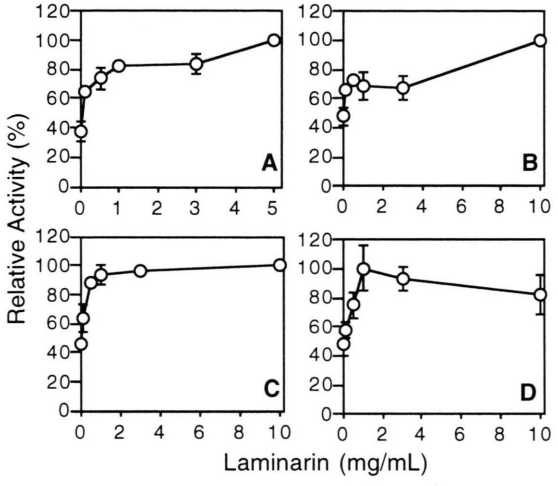


Fig. 6. Effects of the laminarin concentration on the activities of PAL (A), 4CL (B), THT (C) and TyrDC (D). The disks were prepared 24 h prior to treatment with an aqueous solution of laminarin (1.0 mg/ml). The levels of the enzymes were determined 16 h after laminarin treatment. The results are expressed as the means of triplicates ±SD.

Effects of laminariheptaose

In order to verify that the ability of laminarin to activate these enzymes is due to the β-1,3-glucooligosaccharide linkages, the effect of a chemically-pure glucooligosaccharide on enzyme activity was examined. Laminariheptaose (a β-1,3-glucoheptasaccharide) was chosen as a model oligosacharide, because of its demonstrated ability to induce *p*-CO in potato tuber tissue (Miyagawa *et al.*, 1998). The activities of PAL, 4CL, THT and TyrDC 16 h after treatment with laminariheptaose (3.0 mg/ml) are shown in Table III. All the examined enzymes were activated to levels higher than the control. However in all cases, enzyme levels induced by laminariheptaose were less than those induced by laminarin. These results are consistent with the induction of *p*-CO in tuber disks by laminarihaptaose and laminarin, and the effects of these two saccharides on potato tuber tissue are likely to be essentially the same.

Table III. Effects of laminariheptaose on the activity of enzymes responsible for *p*-CO biosynthesis.

Enzyme	Enzyme activities (pkatal / mg protein)		
	Control	Laminarin 1.0 mg/ml	Laminari-heptaose 3.0 mg/ml
PAL	30.9 ± 5.7	74.0 ± 3.9	58.5 ± 1.2
4CL	45.5 ± 4.0	70.2 ± 3.5	54.3 ± 4.4
THT	135 ± 8.8	235 ± 31.5	208 ± 11.3
TyrDC	13.8 ± 1.7	62.7 ± 13.4	33.2 ± 1.3

All enzyme activities were determined at 16 h after laminarin treatment. The results are expressed as the average of triplicates ± SE. PAL, phenylalanine ammonia lyase: 4CL, 4-hydroxycinnamic acid: CoA ligase: THT, hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase: TyrDC, tyrosine decarboxylase.

Deposition of p-CO in cell wall

It has been proposed that phenolic amide compounds are produced for deposition in the cell wall, in order to form physical barriers to the attack by pathogens (Clarke, 1982; Facchini *et al.*, 1999; Keller *et al.*, 1996; Negrel *et al.*, 1996). Thus, the amount of unextractable *p*-CO in cell walls was examined, in order to evaluate the exact activity of amide compounds biosynthesis. After the potato tuber tissue was thoroughly extracted by various solvents, the obtained cell wall fraction was hydrolyzed under alkaline conditions. The large amount of *p*-CO was recovered from the cell walls of laminarin treated tissue which reached levels as high as about 560 nmol/g tissue after 48 h (Table IV). In contrast, only small amounts of *p*-CO were recovered from the control cell wall fraction by the same procedure. This result indicates that neither soluble nor cell wall bound *p*-CO is hardly formed in the control tissues, so that almost

Table IV. Effects of the laminarin on the amounts of *p*-CO deposited in the cell walls of potato tuber disks.

Time after laminarin treatment (h)	*p*-CO (nmol / g fresh tissue)	
	Control	Laminarin treated
0	9.2 ± 2.4	5.4 ± 1.8
48	40.9 ± 10.8	562 ± 157

The results are expressed as the means of triplicates ±SD.

no biosynthesis of *p*-CO occurs in the absence of laminarin treatment, even though a significant activation of some biosynthesis-related enzymes had occurred.

Discussion

A variety of hydroxycinnamoyl amide compounds in plants have been documented (Chaves and Roque, 1997; Chen *et al.*, 1998; Martin-Tanguy *et al.*, 1978; Mühlenbeck *et al.*, 1996; Yamamoto *et al.*, 1991; Yoshihara *et al.*, 1981). Amoung them, *N*-*p*-coumaroyloctopamine (*p*-CO), *N*-feruloyloctopamine, *N*-*p*-coumaroyltyramine, and *N*-feruloyltyramine (Fig. 1) have been found in potato tuber tissue (Clarke, 1982). Treatment with an aqueous solution of laminarin resulted in increased levels of all four amide compounds tested (Fig. 2). While the inductions of *N*-feruloyloctopamine, *N*-*p*-coumaroyltyramine, and *N*-feruloyltyramine did not exceed the level of 30 nmol/g fresh tissue, the changes in the level of *p*-CO were more drastic and reached 203.4 nmol/g of fresh tissue 24 h after application. This accumulation profile in tuber tissue was observed in several other potato cultivars tested (data not shown). The accumulation of similar phenolic amide compounds has been observed in some plants such as tobacco, tomato, and onion under stressed conditions (McLusky *et al.*, 1999; Mühlenbeck *et al.*, 1996; Negrel and Martin, 1984; Pearce *et al.*, 1998), and in suspension-cultured potato cells treated with Pi elicitors (Schmidt *et al.*, 1998). However, increases of octopamine amides of this magnitude have not been described to date and therefore might be characteristic of potato tuber tissue treated with β-1,3-glucooligosaccharides. It has been proposed that hydroxycinnamoyl amide compounds are deposited into the cell walls of the tuber tissues and utilized for physical reinforcement against pathogen infections (Clarke, 1982; Facchini *et al.*, 1999; Keller *et al.*, 1996; Negrel *et al.*, 1996). In agreement with this theory, a large amounts of *p*-CO was recovered from the cell wall of laminarin treated potato tuber tissue (Table IV).

Feeding experiments indicated that L-phenylalanine and L-tyrosine are precursors of *p*-CO biosynthesis (Table I). The results also showed that these precursors were separately incorporated into two components of *p*-CO, namely the *p*-coumaroyl and octopamine moieties. Since a *p*-coumaroyl moiety is often found as a component of secondary metabolites of the plant phenylpropanoid pathway originating from L-phenylalanine, the octopamine moiety of *p*-CO is likely to be of L-tyrosine origin. It has been suggested that tyramine amides of hydroxycinnamic acids are synthesized from the same precursors (Villegas and Brodelius, 1990). On the basis of this information, we suggest possible pathways leading to *p*-CO biosynthesis (Fig. 3). However, the issue whether *p*-CO is formed by direct condensation between octopamine and the *p*-coumaroyl CoA thioester (left pathway in Fig. 3), or whether it is formed by the oxidation of *N*-*p*-coumaroyltyramine (right pathway) remains unknown. As has been shown in our previous report, the octopamine moiety of *p*-CO in potato tuber has the *S* configuration (Matsuda *et al.* submitted), being opposite to that of neuroactive octopamine present in animals (Goosey and Candy, 1980; Midgley *et al.*, 1989; Starratt and Bodnaryk, 1981). Work to further clarify the biosynthetic pathway of *p*-CO in potato tuber tissue is now in progress.

The treatment of potato tuber disks with an aqueous solution of laminarin caused an increase in enzyme activities related to the biosynthesis of hydroxycinnamoyl amide compounds, namely PAL, 4CL, THT and TyrDC (Fig. 4). The results herein are consistent with findings of suspension-cultured potato cells (Schmidt *et al.*, 1998), in which the activation of these enzymes, except for 4CL, was demonstrated to occur as the result of treatment with an elicitor preparation from *P. infestans*. Treatment with laminariheptaose also caused a significant activation of these enzymes in tuber tissue (Table III), indicating that the β-1,3-glucosaccharide structure is an important factor

for the elicitor activity. The observed activation by the oligosaccharide was thought to be the result of *de novo* protein biosynthesis, since it was strongly inhibited by CHX (Fig. 5, Table II). Among the enzyme activities studied, those of PAL, 4CL, and THT were found to be elevated prior to elicitor treatment (Fig. 3A, B, C) which was probably due to the wounding of the tuber tissue, as the result of the preparation of sample disks (Negrel *et al.*, 1993). However, the activation of these enzymes in control tissues resulted in negligible production of either the soluble or cell wall bound forms of hydroxycinnamoyl amides (Fig. 2, Table IV), so that they appeared to be "idling". By contrast, a significant level of activation of TyrDC in tuber tissue occurred only after treatment with laminarin (Fig. 3D). Recent work has demonstrated that mRNA of THT is specifically accumulated in potato suspension culture cell treated with Pi elicitor. However, the time courses of the enzyme activity in potato tuber tissue (Fig. 4) implied that TyrDC is more closely associated with the induced biosynthesis of hydroxycinnamoyl amides than other enzymes such as PAL and THT. The importance of TyrDC has also been shown in the hydroxycinnamoyltyramine biosynthesis of suspension cultured tobacco cells after treatment with a chitosan elicitor (Villegas and Brodelius, 1990).

It has been shown that plant defense responses are triggered both by tissue wounding and by chemical signal substances, or elicitors, of pathogen origin (Bögre *et al.*, 1997; Díaz and Merino, 1998; Negrel *et al.*, 1993). The elucidation of the signaling systems relevant to these stimuli in plants is a matter of great concern. Accordingly, the specific activation of TyrDC by laminarin in the wounded potato tissue observed in this study is of particular importance, since it suggests the presence of a signaling pathway stimulated by saccharide elicitors in potato which is independent of that for wounding. Future work will focus on elucidating the chemical basis of elicitor-active saccharides as well as its perception mechanisms in plants.

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