Induction of Hydroxycinnamoyl-CoA: Hydroxyanthranilate *N*-Hydroxycinnamoyl-transferase (HHT) Activity in Oat Leaves by Victorin C

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Victorin C, a host-specific toxin produced by *Helminthosporium victoriae*, induced hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT, EC 2.3.1) activity in oat leaves (*Avena sativa* L., a cultivar carrying *Pc-2* gene). This enzyme activity catalyzes the final step of biosynthesis of oat phytoalexins, avenanthramides. The HHT activity was detected after 12 h of victorin C application and reached to a maximum by 18 h. The induction of HHT was dose-dependent. All of the putative precursors of avenanthramides acted as substrates for HHT. These findings indicate that the accumulation of avenanthramides by victorin C treatment is due to induction of HHT. The enzyme activity showed highest specificity to 5-hydroxyanthranilate for the anthranyl moiety, while feruloyl-CoA was most effective for cinnamoyl moiety. HHT induced by victorin C showed significantly lower affinity for anthranilic acid relative to the enzyme induced by oligo-*N*-acetylchitooligosaccharides, another elicitor, suggesting that isozymes of HHT occur in this plant.

Introduction

In oats, a series of substituted *N*-cinnamoyl anthranilates, which are referred to as avenanthramides (**1-5**), have been shown to be phytoalexins (Miyagawa *et al.*, 1995). These phytoalexins are induced not only by the inoculation of pathogens (Mayama *et al.*, 1982), but by the treatment of leaves with various elicitors, such as chitin and chitosan oligomers (Bordin *et al.*, 1991), victorin C (Mayama *et al.*, 1986), heavy metals (Fink *et al.*, 1990) and calcium ionophore A23187 (Ishihara *et al.*, 1996). Among these elicitors, we have recently reported that chitin oligomers, or oligo-*N*-acetylchitooligosaccharides, induce hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT) activity in oat leaves, which well involved in the biosynthesis of the phytoalexin in oats (Ishihara *et al.*, 1997).

Victorin C is a host-specific toxin produced by *Helminthosporium victoriae* (Meehan and Murphy, 1946), the casual agent of oat victoria blight disease, and exhibits a specific elicitor activity at a low concentration (Mayama *et al.*, 1986). Although it is unclear how victorin C induce the phytoalexins, it has the following characteristics as an elicitor, compared to chitooligosaccharides: 1) it is specifically elicitor-active against oat cultivars carrying *Pc-2* gene (Mayama *et al.*, 1986); 2) it increases the leakage of electrolytes from the oat cell (Sammddar and Scheffer, 1971; Ullrich and Novacky, 1991); 3) it specifically induces an avenanthramide congener, **4** (Miyagawa *et al.*, 1996a). For better understanding the effects of victorin C on oats, we examined the induction of HHT by this toxin, and characterized the enzyme in terms of the biochemical and kinetic parameters to compare with that induced by the treatment with chitooligosaccharides.

Materials and methods

Plant material

Oat seeds (*Avena sativa* L., a cultivar carrying *Pc-2* gene) were soaked in H_2O at room temperature for 12 h in darkness. The soaked seeds were

Abbreviations: HHT, Hydroxycinnamoyl-CoA: Hydroxyanthranilate *N*-hydroxycinnamoyltransferase.

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then sown in wet vermiculite, and maintained at 20 °C for seven days under continuous artificial light.

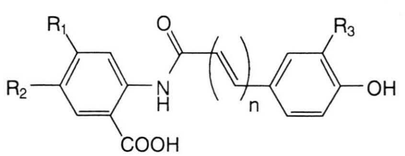
Chemicals

Avenanthramide A (**1**), B (**2**) and D (**3**) were prepared using the procedures described by Collins (1989). Avenanthramide G (**4**), L (**5**), C (*N*-(3′,4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid), *N*-(4′-hydroxycinnamoyl)tyramine, *N*-(4′-hydroxybenzoyl)-5-hydroxyanthranilic acid, *N*-cinnamoyl-5-hydroxyanthranilic acid and *N*-(4′-hydroxycinnamoyl)-3-hydroxyanthranilic acid were synthesized as described elsewhere (Miyagawa *et al.*, 1995; Miyagawa *et al.*, 1996a; Ishihara *et al.*, 1997).

Hydroxycinnamoyl-CoA thioesters were prepared by transesterification of hydroxycinnamoyl-*N*-hydroxysuccinimide esters (Stöckigt and Zenk, 1975).

Enzyme extraction and assay

The lower epidermis of 7-day-old primary oat leaves (a cultivar carrying *Pc-2* gene) was peeled off, and 5 cm segments were taken 1–6 cm from the leaf tip. The segments were floated in a 10 ml solution of victorin C, which was prepared as described (Mayama *et al.*, 1986), with the peeled surface in contact with the solution. After an 18 h incubation, the leaf segments were homogenized in 10 volumes of 0.1 M sodium phosphate buffer (pH 7.5) containing 14.4 mM mercaptoethanol. The homogenate was then centrifuged (12,000*g*, 10 min), and the supernatant was used as the enzyme solution. All operations were carried out at 4 °C. The protein content in the enzyme solution was determined according to the methods of Bradford (1976).



1: Avenanthramide A, R_1=H, R_2=OH, R_3=H, n=1
2: B, R_1=H, R_2=OH, R_3=MeO, n=1
3: D, R_1=H, R_2=H, R_3=H, n=1
4: G, R_1=OH, R_2=H, R_3=H, n=1
5: L, R_1=OH, R_2=H, R_3=H, n=2

Structures.

The reaction mixture consisted of 10 µl of enzyme solution, 10 µl of 10 mM 5-hydroxyanthranilic acid, 10 µl of 0.5 mM *p*-coumaroyl-CoA and 70 µl of 0.1 M sodium phosphate buffer at pH 7.0. After 20 min incubation, the reaction was stopped by adding of AcOH. The amount of product (avenanthramide A, **1**) was determined by HPLC analysis using an ODS column (Wakosil-II 5C18HG) as described previously (Ishihara *et al.*, 1997).

Fractionation of HHT by salting-out

To study the characteristics of HHT activity induced by victorin C, a crude extract of the leaf segments, prepared after 18 h of elicitor treatment, was partially purified by salting-out with $(NH_4)_2SO_4$. Sixty percent of the initial activity was recovered in the fraction which precipitated between 30 and 45% saturation. The fraction was desalted on a Sephadex G-25, equilibrated with 0.02 M sodium phosphate buffer (pH 7.0, 14.4 mM mercaptoethanol). By storing at −30 °C after addition of one volume of glycerol, the enzyme solution could be preserved for 1 month with activity loss of less than 10%.

Results

Induction of HHT activity by victorin C

Figure 1a shows the results of time course experiments for changes in HHT activity in the leaf segments treated with victorin C at 100 pg/ml concentration. While little HHT activity was detectable at 0 h, the activity was significantly increased after 12 h, and high activity was detected from 18 h until 36 h. The activity then declined to about 10% of the maximal value 48 h after treatment. Little HHT activity was observed in the control leaf segments. The change in HHT activity was compatible with the time course of the production of **1** in oat leave segments, as shown in Fig 1b. Compound **1** was first detected 12 h after application of victorin C and increased up to 48 h. Thereafter, the accumulation of **1** leveled off.

Figure 2 shows the effect of victorin C concentration on the induction of HHT activity and the production of **1** in oat leaf segments. The HHT activity in crude extracts from leaves treated with victorin C was measured 18 h after application.

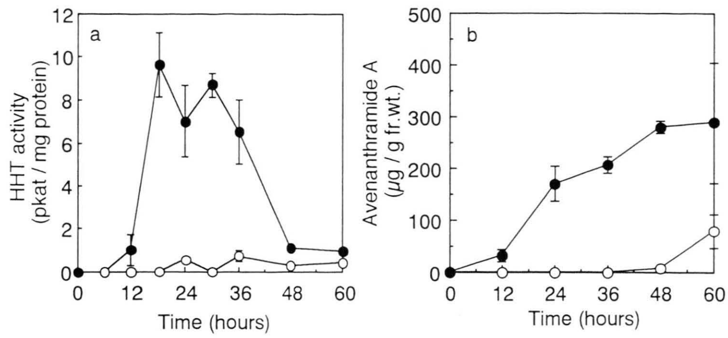


Fig. 1. Changes in HHT activity (a) and accumulation of avenanthramide A (b). At time 0, oat leaf segments were floated in 100 pg/ml victorin C solution (●) or distilled H_2O (○) and incubated at 20 °C under artificial light. HHT activity and amount of **1** are plotted as a function of time after the initiation of elicitor treatment. The results are expressed as the means of triplicate experiments with ± SD.

The activity was detected in leaves treated with victorin C at concentrations of not less than 0.01 ng/ml. The induction of HHT activity was dependent on concentration up to 1 ng/ml, but at concentrations higher than this, the induction was inhibited and the activity was reduced to 50% of the maximal value at 100 ng/ml. The elicitor-concentration dependence of the HHT activity induction was almost identical with that of production of **1** in leaf segments.

Properties of HHT induced by victorin C

To study the characteristics of HHT activity induced by victorin C, a crude extract of the leaf segments, prepared after 18 h of elicitor treatment, was partially purified by salting-out with $(NH_4)_2SO_4$.

The dependence of induced HHT activity on pH is shown in Fig. 3. The maximum activity was observed at pH 7.0 with half activity at pH 6.0 and 8.5 using 0.1 M sodium phosphate and GTA (0.05 M 3,3-dimethylglutaric acid, 0.05 M tris(hydroxymethyl)aminomethane, 0.05 M 2-amino-2-methyl-1,3-propanediol) buffer solution.

The substrate specificity of the induced HHT activity for various substituted anthranilic acids is shown in Table I, with *p*-coumaroyl-CoA being used as the acyl donor. Among the tested compounds, only the anthranilates which constitute naturally occurring avenanthramides acted as substrates, while no conversion was observed for 3-hydroxyanthranilic acid and tyramine. Even among the effective substrates, the specificity was rather high. The best substrate was 5-hydroxyanthranilic acid, based on both apparent K_m value and V_{max}/K_m value.

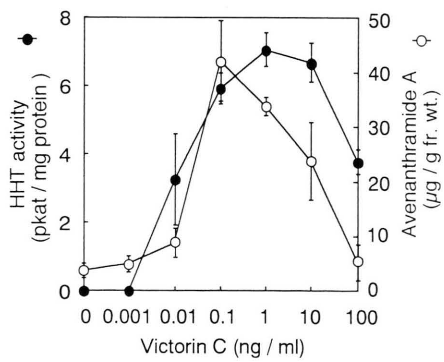


Fig. 2. Induction of HHT activity (●) and avenanthramide A (○) by various concentrations of victorin C. Oat leaf segments were floated in solutions containing various concentrations of victorin C. HHT activity in oat leaf tissue was measured 18 h after the initiation of elicitor treatment using 5-hydroxyanthranilic acid and *p*-coumaroyl-CoA as substrates. Amount of **1** produced in oat leaves was determined 24 h after the initiation of elicitor treatment. The results are expressed as the means of triplicate experiments with ± SD.

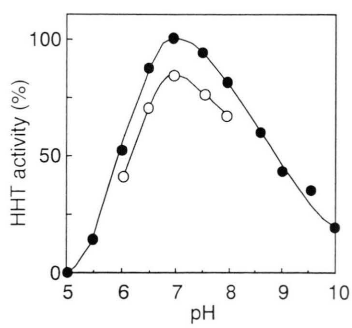


Fig. 3. Dependence of HHT activity on pH. HHT activity in oat leaf tissue was measured in 0.1 M sodium phosphate (○) and GTA (●) buffer using 5-hydroxyanthranilic acid and *p*-coumaroyl-CoA as substrates.

Table I. Substrate specificity of HHT for the anthranilic moiety. *p*-Coumaroyl-CoA (0.5 mM) was used as the common acyl donner.

Substrate	$10^5 \times K_m$ [M]	Relative V_{max} [%]	$10^{-5} \times V_{max}/K_m$
Anthranilate	340	100	0.29
5-Hydroxyanthranilate	12	59	4.9
4-Hydroxyanthranilate	120	41	0.34
3-Hydroxyanthranilate	n.d.[a]	n.d.	–
Tyramine	n.d.	n.d.	–

[a] n.d., not detectable.

Table III. Induction of avenanthramides by victorin C. Oat leaf segments were floated in 100 pg/ml victorin C solution. Amounts of avenanthramides were determined 24 h after the initiation of treatment by HPLC. Each value represents the mean ± SD. of three experiments.

Avenanthramides	µg/g fr. wt.	%
A (**1**)	223 ± 70.1	100
B (**2**)	n.d.[a]	–
D (**3**)	45.4 ± 7.11	20
G (**4**)	55.4 ± 14.5	25
L (**5**)	17.1 ± 9.45	7.7

[a] n.d., not detectable.

Table II shows the HHT activity in the presence of various cinnamoyl-CoA derivatives, when 5-hydroxylanthranilic acid was used as the acyl acceptor. All of the CoA derivatives tested acted more or less as substrates. Among these, the best substrate was feruloyl-CoA, on the basis of V_{max}/K_m value, followed by avenalumoyl-CoA and cinnamoyl-CoA. *p*-Coumaroyl- and caffeoyl-CoA were rather poor substrates.

In Table III, the amounts of avenanthramides produced in oat leaves after the elicitation by victorin C are shown. As to the case of the elicitation by penta-*N*-acetylchitopentasaccharide (Ishihara *et al.*, 1997), **1** was produced in the largest amount. In miner components, the production of **3** and **4** was evident, while **2** was hardly detectable.

Discussion

The treatment of oat leaves with victorin C significantly induced HHT activity. The maximum amount of extractable activity occurred 18 h after elicitor treatment, when the formation of avenanthramide appeared to occur most actively (Fig. 1). This finding, together with the elicitor-concentration dependence of the HHT activity induction (Fig. 2), suggests that the induction of HHT activity in oat leaves is responsible for the accumulation of **1**.

Table II. Substrate specificity of HHT for the cinnamoyl moiety. 5-Hydroxyanthranilic acid (1 mM) was used as the common acyl accepter.

Substrate	$10^5 \times K_m$ [M]	Relative V_{max} [%]	$10^{-5} \times V_{max}/K_m$
Feruloyl-CoA	4	100	7.1
Cinnamoyl-CoA	27	65	2.4
p-Coumaroyl-CoA	16	24	1.5
Caffeoyl-CoA	18	9	1.1
Avenalumoyl-CoA	4.4	16	3.6
4-Hydroxybennzoyl-CoA	5.2	1.6	0.31

The study of properties of HHT induced by victorin C indicated that the enzyme accepts all putative precursors of avenanthramides, while the substrate specificity of the enzyme activity does not always account for the amount of product. As to the anthranyl part of avenanthramides, 5-hydroxyanthranilic acid, the component of **1**, was the best substrate for the HHT among the substituted anthranilic acids tested, which was compatible with the finding that **1** was produced in the largest amount in victorin C-treated leaf segments. On the other hand, its counterpart, *p*-coumaroyl-CoA, was not necessarily the best substrate. A discrepancy between the substrate specificity and the amount of products was also observed for other cinnamoyl substrates; in spite of the highest specificity for feruloyl-CoA, the production of **2** was suppressed in the leaves, which was typical of the elicitation by victorin C (Miyagawa *et al.*, 1996b); no conjugate of anthranilates with cinnamic acid nor caffeic acid was detected in the elicited oat leaves in this study, although the corresponding CoA derivatives were active as HHT substrates. These results show that the composition of the avenanthramides in leaves may depend on other factors, such as the amounts of available substrates in leaves and the metabolic transformations after the production.

The reactivities of substrates for HHT observed in this study were generally the same as those for HHT activity induced by oligo-*N*-acetylchitooligosaccharide elicitor (Ishihara *et al.* 1997), except that anthranilic acid had a far lower affinity for the enzyme induced by victorin C relative to the enzyme induced by oligo-*N*-acetylchitooligosaccharide. This suggests that several isozymes of HHT occur in this plant and that the respective induction of isozymes depends on the nature of the elicitor. The purification and characterization of induced HHT in oat leaves will be an important step to understand this phenomenon in relation to microbe-plant interaction.

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