Original Article

Comparative Studies of Elicitors That Induce Phytoalexin in Oats

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The activity of the four elicitors, a *N*-acetylchitopentaose [(GLNAc)₅], a chitopentaose [(GLN)₅], Ag⁺ ion and victorin, which induce phytoalexins in oats were compared using the following assays: i) electrolyte leakage, ii) effects of Ca²⁺ on the induction of phytoalexin and iii) difference in induced-metabolite profiles. The results suggest that at least two distinct mechanisms are involved in phytoalexin induction in oats. Among the tested elicitors, (GLNAc)₅ appears to induce phytoalexin directly by binding to its putative receptor, while induction by other elicitors may be a secondary effect, presumably due to the damage of the plasma membrane caused by the elicitors.

INTRODUCTION

After recognition of a parasite, plants are able to induce a variety of defense responses, thus protecting themselves. Defense responses can be induced, in certain cases, by chemicals (elicitors), such as saccharides, peptides, lipids and inorganic metals. Certain types of physical treatment also can elicit a response. Elicitors are of interest as probes to clarify and understand signal transduction mechanisms for recognition of the pathogen, in order to initiate a defense reaction in plants. In addition, understanding of such responses can lead to the development of new types of metabolic modulators in plants.

In case of oats, it has been demonstrated that phytoalexin(s) play an important role in the rejection of crown rust fungus, *Puccinia coronata* f. sp. *avenae*.^{1,2)} Chemical studies on these phytoalexins first proposed that they are three kinds of substituted 4*H*-3,1-benzoxazin-4-ones named avenalumin I–III.³⁾ However, the succeeding study has demonstrated that they should be revised as *N*-acyl anthranilate derivatives, commonly referred to as avenanthramides. These derivatives contain 5-hydroxyanthranilic acid, and *N*-acyl groups consisting of coumaroyl (avenanthramide A, **1**), feruloyl (avenanthramide B, **2**) and 5-(4-hydroxyphenyl)-

2*E*, 4*E*-pentadienoyl (avenanthramide L, **3**) moieties.⁴⁾ Although avenanthramides have been isolated from the oat seeds,^{5–7)} none appear to be present in young healthy leaves prior to inoculation with incompatible race of the rust fungus.

- 1: R₁=H, R₂=OH, R₃=H, n=1
 2: R₁=MeO, R₂=OH, R₃=H, n=1
 3: R₁=H, R₂=OH, R₃=H, n=2
 4: R₁=R₂=H, R₃=OH, n=1
 5: R₁=R₂=R₃=H, n=1

Polysaccharides such as chitin and chitosan,⁸⁾ and certain heavy metal ions⁹⁾ have also been shown to act as elicitors of phytoalexin induction in oats. In addition, victorin, a host-specific toxin produced by the pathogenic fungus *Helminthosporium victoriae* is known to be an elicitor that acts specifically on oat cultivars carrying *Pc-2* gene.¹⁰⁾ Although these elicitors all induce phytoalexin, it is unlikely that the mechanisms leading to these responses are the same, in view of the physico-chemical diversity of the elicitors. In this study, the elicitor activities of saccharides (oligomers of D-glucosamine, or chitoooligosaccharides, and oligomers of *N*-acetyl-D-glucosamine, or *N*-acetylchitoooligosaccharides), a heavy metal ion (Ag⁺) and victorin were

Abbreviations: (GLN)₅, chitopentaose (D-glucosamine pentamer); (GLNAc)₅, *N*-acetylchitopentaose (*N*-acetyl-D-glucosamine pentamer).

compared in order to understand better the mechanism(s) of phytoalexin induction in oats.

MATERIALS AND METHODS

1. Plant Cultivars

The oat cultivars Shokan-1 and Pc-2 were grown on vermiculite in a growth chamber at 25°C under continuous illumination. Seven- to 8 day old seedlings were used in the studies.

2. Elicitors

Chitopentaose [(GLN)₅] and *N*-acetylchitopentaose [(GLNAc)₅] were purchased from Seikagaku Co. (Tokyo, Japan). Victorin (D) was prepared as described previously.¹⁰⁾

3. Standard Avenanthramides

Avenanthramides A (1), B (2) and D (5) were prepared using the procedures described by Collins.⁵⁾ Avenanthramides L (3) and G (4) were synthesized as described elsewhere.^{4, 11)}

4. Elicitor Activity

The lower epidermis of oat leaves was peeled off and 2 cm segments were prepared. The segments were allowed to float on an aqueous solution of elicitor for 24 hr under fluorescent light, with the peeled surfaces exposed to the solution. Avenanthramides which were secreted into the solution were then quantified by HPLC using a Wakosil II 5C18 column (4.6 × 150 mm) with methanol-water containing 0.1% H₃PO₄ (55 : 45) as mobile phase. Peaks were detected using a UV detector at 340 nm. For the case of treatment with Ag⁺, the leaf segments were allowed to float on the test solution without peeling off

the epidermis, in order to alleviate the toxic effect of the elicitor.

5. Electrolyte Leakage

Electrolyte leakage from the leaf segments was estimated by measuring the conductivity of the elicitor solution after 24 hr incubation with a HORIBA C-172 conductivity meter. The data were converted and expressed as the conductivity (μS/cm) of the 1 ml of solution on which 50 mg of the leaf segments had been allowed to float.

RESULTS

1. Time Course of Avenanthramide Production

In initial experiments, changes in the amount of avenanthramides produced by the oat leaf segments were examined after treatment with (GLNAc)₅ at the concen-

Fig. 1 Time course for the production of avenanthramide A (1) by oat leaf segments (cv. Shokan-1) treated with (GLNAc)₅ at a concentration of 1 mg/ml. Bars indicate the S.D. of 3 replications.

Fig. 2 Effects of the elicitor concentration on the production of avenanthramide A (1) by oat leaf segments. The cultivar Shokan-1 was used for the treatments with (GLNAc)₅, (GLN)₅ and Ag⁺, whereas Pc-2 was used for victorin. Concentrations are expressed in log scale (a) and in normal scale (b). Symbols represent means of duplicate experiments.

tration of 1 mg/ml. This was done in order to establish the analytical conditions. Figure 1 shows that **1**, the major induced component, was detected after 6 hr, and reached a maximum after 18 hr. Compound **1** was largely secreted into the test solution with only small amounts being detected in the leaf tissue. After 24 hr, the level of **1** gradually decreased. Based on these data, and for experimental convenience, elicitor activity was evaluated by quantifying **1** in the test solution after a 24 hr incubation period.

2. Effects of the Elicitor Concentration

Effects of the concentration of the four elicitors for the induction of **1** are shown in Fig. 2a.

The elicitor (GLNAc)₅ induced measurable amounts of **1** at a concentration of 0.05 mg/ml. The levels of induced **1** increased sharply up to a concentration of 1 mg/ml. Beyond this concentration, the increase became somewhat moderate (Fig. 2b), and no inhibition of induction was observed even at a concentration of 5 mg/ml.

In contrast to this saturation pattern of dose-dependence, induction by (GLN)₅, victorin and Ag⁺ was observed only within a limited range of elicitor concentrations, with an optimum clearly observed. At higher concentrations, induction was inhibited because of the toxic effects of the elicitors. Among these three elicitors, victorin was the most active, inducing significant amounts of **1** at concentrations as low as 1 pg/ml, and was effective over a relatively wide range of concentration up to 100 ng/ml. Compared with victorin, higher concentration of Ag⁺ and (GLN)₅ were required for phytoalexin production. It is also noteworthy that induction by (GLN)₅ occurred only within a narrow concentration range.

3. Electrolyte Leakage Caused by Elicitors

Chitosan and victorin are known to modify the cell membrane permeability of oats and cause an electrolyte leakage,^{10,12)} and this is generally thought to be related to their phytotoxicity. An increase in conductivity of the test solution was observed when the oat leaf segments were treated with (GLN)₅ or victorin at near optimum levels for the induction of **1** (Fig. 3). By contrast, (GLNAc)₅ at a concentration of 1 mg/ml caused negligible electrolyte leakage after treatment, suggesting that the cell membrane was largely undamaged by this elicitor.

4. Effects of Ca²⁺ Ion on the Induction of **1**

As described above, elicitor activities of victorin, (GLN)₅, and Ag⁺ were accompanied with some toxicity against oat leaves, while (GLNAc)₅ induced phytoalexin with no significant phytotoxicity. The toxicities of victorin and chitosan have been reported to be suppres-

Fig. 3 Electrolyte leakage from oat leaf segments treated with elicitors.

The cultivar Shokan-1 was used for the treatments with (GLNAc)₅ and (GLN)₅, whereas Pc-2 was used for victorin. Bars represent the S.D. of 3 experiments.

Fig. 4 Effect of Ca²⁺ on the production of avenanthramide A (**1**) by oat leaf segments treated with elicitors.

The elicitors are a) *N*-acetylchitopentaose ((GLNAc)₅, 1 mg/ml), b) victorin (1 ng/ml), c) chitopentaose ((GLN)₅, 0.1 mg/ml) and d) Ag⁺ (AgNO₃, 0.01 mg/ml). The cultivar Shokan-1 was used for the treatments with (GLNAc)₅, (GLN)₅ and Ag⁺, whereas Pc-2 was used for victorin.

sed by the addition of CaCl₂.^{12,13)} This suppression is thought to be due to the stabilization of membrane structure as a result of fortified electrostatic interactions between cationic Ca²⁺ and anionic constituents of the membrane. Taking this into account, the effects of Ca²⁺ on induction by the elicitors were examined in order to evaluate the possible relationship between elicitor activity and membrane dysfunction as caused by the elicitor.

As shown in Fig. 4, elicitor activities of victorin, (GLN)₅, and Ag⁺ were inhibited by CaCl₂ in a dose-

Table 1 Composition of induced metabolites in oat leaves treated with various elicitors.^{a)}

Metabolites	Shokan 1				Pc-2		
	(GLNAc) ₅ (1 mg/ml)	(GLN) ₅ (0.1 mg/ml)	AgNO ₃ (50 µg/ml)	P.c. ^{b)}	(GLNAc) ₅ (1 mg/ml)	(GLN) ₅ (0.1 mg/ml)	Victorin (1 ng/ml)
1	100	100	100	100	100	100	100
2	26.0	3.4	1.5	44.7	12.2	4.3	ND
3	20.4	1.4	6.7	28.7	17.0	1.6	3.0
4	2.8	13.0	15.1	0.3	8.9	10.1	84.6
5	1.9	10.7	3.9	1.8	2.7	3.6	38.4

^{a)} Values represent the relative peak area of the metabolites on HPLC, as detected at 340 nm.

^{b)} Inoculated with the uredospores of *Puccinia coronata* f. sp. *avenae* race 226.

dependent manner. Induction was almost completely inhibited by 100 µg/ml (*ca.* 1 mM) of CaCl₂. These experiments also revealed that electrolyte leakage from the victorin-treated oat leaves was significantly suppressed by the addition of CaCl₂ to the medium (data not shown).

In contrast, practically no inhibition by CaCl₂ was observed in the case of phytoalexin induction by (GLNAc)₅, suggesting again that this elicitor does not cause membrane damage of oat cells.

5. Composition of the Induced Metabolites

Elicitors used in this study induced five types of avenanthramides **1**–**5**. These compounds were identified by cochromatography with the synthetic standards on HPLC. Among them, compounds **1**–**3** have been shown to accumulate in oat leaves which have been inoculated with an incompatible rust fungus,⁴⁾ whereas the induction of **4** has been observed by treatment of oat leaves carrying *Pc-2* gene with victorin.¹¹⁾ Compound **5** was first found as the induced metabolite of oats in this study, although it has been previously described as a component of oat seeds.⁵⁾

All the elicitors used induced **1** as the major component, but the composition of the other metabolites was dependent on the elicitor (Table 1). In addition to **1**, (GLNAc)₅ induced **2** and **3** as minor components in cultivars of Shokan 1 and Pc-2. In the elicitation by (GLN)₅, however, the induction of these two compounds was suppressed, and the induction of **4** was relatively evident. Similar composition profiles were found for the case of treatment of Shokan 1 with Ag⁺ and for that of Pc-2 with victorin. In the latter case, it was noticeable that victorin completely inhibited the production of **2**. In Table 1, the composition of the metabolites induced in oat leaves (Shokan 1) after inoculation with the incompatible race of rust fungus, *P. coronata* f. sp. *avenae*, is also shown. In the pathogen-inoculated leaves, **2** and **3** were produced as the minor components along with the major component of **1**. This pattern is similar to the induction caused by (GLNAc)₅.

DISCUSSION

Four types of elicitors which consisted of saccharides, a heavy metal and a fungal secondary metabolite were compared in terms of phytoalexin-inducing activities against oats. With the exception of (GLNAc)₅, the elicitor activities of the tested compounds were accompanied by phytotoxic effects to the extent that they induced no phytoalexin at the concentrations higher than a certain critical point. It seems probable that these toxic compounds induce phytoalexin at sublethal concentrations, where some "dying" oat cells might produce phytoalexin itself or, alternatively, a signal that induces other healthy cells to produce it. However, phytotoxic compounds do not always induce phytoalexin in oats. Fungal phytotoxins against gramineous plants such as ophiobolin¹⁴⁾ and cochlioquinone¹⁵⁾ as well as herbicides such as DCMU, glyphosate and diquat, showed no elicitor activity (data not shown). Thus, elicitor activity of the phytotoxic compounds appeared to be rather specific, and the results obtained in this study suggest that their activity may be indirect and may well be related to membrane dysfunction caused by them.

(GLNAc)₅ is different and unique in that it induces phytoalexin production with no apparent toxic effect on oat cells. Available data suggest that (GLNAc)₅ does not affect membrane integrity, but appears capable of stimulating a signal transduction system for the induction of defense reactions in oats, where some putative receptor for chitin fragments may be involved. With respect to the biological activities of *N*-acetylchitoooligosaccharides, these have been observed to affect not only the induction of the phytoalexin,¹⁶⁾ but the activity of an enzyme involved in the phytoalexin biosynthesis in cultured rice cells,¹⁷⁾ and the induction of lignification in wheat.¹⁸⁾ *N*-Acetylchitoooligosaccharides have been also reported to be active in dicotyls as well, effecting lignification in carrot,¹⁹⁾ and induction of chitinase in melon.²⁰⁾

The above described findings suggest that there are at least two distinct mechanisms for phytoalexin induction, and this difference in action is reflected by the composi-

tion of the induced metabolites (Table 1). (GLNAc)₅ induced three types of metabolites 1–3, whereas other toxic elicitors characteristically induced 4 and 5. Although the mechanism responsible for this difference is unknown at present, it may be assumed that the signal transduction systems activated by these two kinds of elicitors, (GLNAc)₅ and others, respectively induce the different sets of enzymes responsible for the biosynthesis of avenanthramides. In this context, it is noteworthy that the pattern induced by (GLNAc)₅ resembled the pattern induced by the inoculation of incompatible rust fungus. This suggests that the *N*-acetylchito-oligosaccharides may play a significant role in inducing phytoalexin in the rust fungus-infected oat leaves. Since endo-type chitinase activity has been detected in the intercellular space in oat leaves,²¹⁾ polymeric chitin, which is a major constituent of the cell walls of rust fungus, could undergo fragmentation by this chitinase during the primary state of the host-parasite interaction, and yield *N*-acetylglucosamine oligomers, which, in turn, could elicit the metabolic changes in host plant as was observed in this study. Furthermore, in cultured rice cells, treatment with chitin fragments was observed to induce and enhance the chitinase activity,¹⁷⁾ along with the induction of phytoalexins,¹⁶⁾ which appears to be an efficient mechanism for the rejection of the invading pathogens. The presence of chitin-fragment binding sites has been reported for the membrane fraction of cultured rice plant cells.²²⁾

In conclusion, *N*-acetylchitoooligosaccharides represent interesting probes for elucidating defense reactions in gramineous plants including oats. They could serve for elucidating the yet unknown mechanism of metabolic regulation in plants, leading to development of new types of plant growth modulators. Further studies of the activity of *N*-acetylchitoooligosaccharides in oats are now in progress.

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要 約

エンバクにファイトアレキシンを誘導するエリシターの比較研究

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エンバクに対してファイトアレキシン誘導活性を示す4種のエリシター(*N*-アセチルキトペンタオース, キトペンタオース, 銀イオンおよびピクトリン)について, それらの1)電解質漏出作用, 2)エリシター活性に及ぼすカルシウムイオン添加の影響, 3)エリシターが誘導する代謝物の組成を比較した。いずれの場合においても*N*-アセチルキトペンタオースとそれ以外のエリシターは異なった作用を示し, この結果からエンバクにおけるファイトアレキシン誘導機構は少なくとも2種類存在することが示唆された。*N*-アセチルキトペンタオース以外のエリシターでは, それらがもたらす細胞膜傷害作用が引き金となって2次的に誘導が起こると考えられるのに対して, *N*-アセチルキトペンタオースによる誘導は, 何らかのレセプターを介した, より直接的な機構によるものと推察される。