エンバクのファイトアレキシン・アベナルミンの誘導活性をもつエリシターについて

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Potential Elicitors for Avenalumin Accumulation in Oat Leaves

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Abstract

Chitin, chitosan, oligo galacturonic acid, glucan and some of constituent monosaccharides were tested of their elicitor activities for avenalumin accumulation in oat leaves and a bioassay method for its elicitation was evaluated. Among the compounds tested, a water-soluble chitosan (DP: 37, acetyl. rate 39%) was the most potent elicitor, followed by chitin, glycol chitin, oligo galacturonic acid and glucan in the order. No accumulation of avenalumin I was elicited by aminosugar monosaccharides. Effects of the molecular size and acetylation rate of chitosan on elicitor activity were determined using chitosans with DPs 37, 10, 8 and 5, and acetylation rates 39, 30, 15 and 6%, respectively. All the chitosans were effective elicitors; however, the high molecular chitosan (DP: 37) seemed to be more active elicitor than the others especially at low concentrations. A significant finding was that the chitosans elicited a differential production of avenalumin I in oat cultivars of different genetic backgrounds. Certain cultivars such as Shokan-1 and Pc-51 were always sensitive to the chitosans and accumulated larger quantities of avenalumin, whereas a cultivar like Pc-45 accumulated only about 1/4 the amount in the sensitive cultivars. It was shown that infiltration of elicitor solutions into intercellular spaces of the primary leaves by hypodermic injection was found much more efficient for the assay of differential elicitor activity than the assay with epidermis-detached leaf segments.

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Key words: phytoalexin, elicitor, avenalumin, chitosan, oat.

INTRODUCTION

In host-parasite interactions, the parasitic specificity of a pathogen and specific expression of host resistance have been observed at the levels of species-species and cultivar-race combinations. It has been theoretically assumed that the products of avirulence genes of a pathogen interact with the products of genes for resistance in the host resulting in the activation of inducible defence mechanisms[9,11,13,20]. Numerous fungal and bacterial metabolites such as polysaccharides, glycoproteins, peptides, fatty acids and hydrolytic enzymes have been implicated in the elicitation of resistance reactions[4,5,15]. Most of these elicitors have been reported to be non-specific in race-cultivar interaction, although a few race-specific elicitors have been known[2,6,14].

Previous studies in crown rust of oats suggested that the cultivar-specific resistance as differentiated by production of avenalumin I is probably initiated and regulated by mutual recognition between the rust fungus and oat plant cells[16,17]. Specific elicitation by victorin of avenalumin I in the lines containing Pc-2 gene also suggested the significance of a signal recognition for the expression of specific resistance in crown rust of oats[18]. It is thus important to specify some

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signal compounds responsible for the differential induction of avenalumin accumulation in the race-cultivar system.

In this study, various polysaccharides, some of which have been known as elicitors for other phytoalexins, were examined for their elicitor activities in avenalumin I production and some bioassay methods were evaluated not only for elicitation of avenalumin accumulation but also for the study of induced resistance or susceptibility in the crown rust system.

MATERIALS AND METHODS

Plants. The oat lines used in the present study were Shokan-1, Iowa X469, Iowa X-424, Pc-38, Pc-35, Pc-39, Pc-40, Pc-45, Pc-47, Pc-48 and Pc-51 which carry respective independent gene for resistance to various races of *Puccinia coronata* f. sp. *avenae*. Iowa X469 and Iowa X424 are near-isogenic lines and Pc-35, 39, 40, 45, 47 and 48 are also near-isogenic each other. The seeds were husked, soaked in water for 2 hr and incubated at 20°C for 24 hr on a wet filter paper in petri dish. The germinated seeds were then planted on vermiculite in a planter and grown in a growth chamber at 20°C for 16 hr light period. The 8-day-old seedlings were used for the experiments.

Elicitor activity. Various sugar compounds such as chitin, glycol chitin, chitosans, oligo galacturonic acid, glucan, aminosugar monosaccharides were tested for their elicitor activities for avenalumin production. Oligo galacturonic acids (DP 6, 8, 10 and 12) were prepared from *Ficus* pectin (Fushimi Pharmaceutical Co., Marugame, Japan) and various chitosan preparations derived from crab shell were obtained from Kurita Kogyo Co., Tokyo, Japan (Table 1), all being highly soluble in water. Chitin was suspended in water and blended by sonication. All other chemicals were purchased from Nacalai Tesque Company, except glucan from Sigma Chemical Company. These compounds were dissolved in water containing 0.01% Penicillin G and Cephalosporin. Elicitor activity for avenalumin I production was determined by two bioassay methods. (1) The lower epidermis layers were peeled away from primary leaves and the exposed mesophyll cells of 5 cm leaf segments were floated on elicitor solutions by facing the exposed side down. (2) The elicitor solutions were infiltrated by injection into intercellular spaces of primary leaves without stem-cutting according to the method of Hagborg[10]. The treated leaves were kept at 20°C in a continuous light condition for 24 hr after treatment. The amount of avenalumin I accumulated was estimated by a high-performance liquid chromatography with a Hitachi-HPLC 638-30 using an authentic avenalumin I as standard as described elsewhere[17].

Electrolyte leakage and viability of oat cells. The effects of elicitor compounds on oat cell viability were determined by electrolyte leakage. Electrolyte leakage was estimated by measuring conductivity of the elicitor solution after 24 hr of incubation with a conductivity meter, TOA CM-205. Viability of oat cells was also examined under a fluorescence microscope of the inner monolayer cells of coleoptile which had been treated and stained with fluorescein diacetate (FDA)[18].

Table 1. Molecular characteristics of soluble chitosans used in this experiment

Properties	Chitosan preparations			
	I	II	III	IV
Molecular weight (10^3)	6.5	1.7	0.9	1.4
Degree of polymerization	37	10	5	8
Degree of acetylation (%)	39	30	6	15

RESULTS

Potential elicitors of avenalumin I production

A range of putative elicitors were screened on the basis of their elicitor activity of avenalumin I in lower epidermis-stripped oat leaves (Table 2). With this bioassay, the most potent elicitor was found to be chitosan preparation in the four cultivars tested. The amount of avenalumin accumulated in Pc-38 was significantly less than those in other cultivars. Chitin and soluble glycol chitin had also apparent elicitor activity, especially in Iowa X469 and Shokan-1. None of the aminosugar monosaccharides was active in the induction of avenalumin accumulation as observed with their polymers. The β-glucans from barley and laminarin, an algal polysaccharide, were found to be poor elicitors for avenalumin accumulation. The oligo galacturonic acid, DP 6, a common component of pectin, showed some elicitor activity comparable to chitin, but inferior to glycol chitin.

Effects of different molecular weight and N-acetylation of chitosan on elicitor activity

Since chitosan I showed the highest elicitor activity among the compounds tested in the bioassay with epidermis peeled leaves, chitosans with different molecular weight and degree of acetylation were examined further for their elicitor activities (Table 3). Chitosans I, II, III and IV with 39, 30, 6 and 15% of N-acetylation, respectively, were equally active as high concentrations and no clear difference in avenalumin induction was found with elicitors of different degrees of polymerization. However, there seemed to be a tendency that elicitor activity of chitosan I and II is higher in Shokan-1 than in Pc-38 especially at low concentrations. Dose dependency of avenalumin accumulation was observed with chitosans III and IV which have lower degrees of polymerization and acetylation. Leakage of electrolytes occurred generally at higher concentrations of chitosans (Table 3).

Cultivar difference on avenalumin I production by chitosans

The elicitor activity of chitosans I and III was further studied with various oat cultivars containing different Pc genes for resistance. As shown in the Tables 4 and 5, chitosans induced differential accumulation of avenalumin I in cultivars of different genetic backgrounds. The cultivars Shokan-1 and Pc-51 were more sensitive to both chitosans I and III than the other Pc lines. The cultivar Pc-45 accumulated the least amount of avenalumin I in response to chitosan I, about 1/5–1/4 of those in Pc-51 and Shokan-1, respectively. Dose-dependent ac-

Table 2. Elicitor activities of various sugar compounds for the accumulation of avenalumin I in primary leaves of oats

Treatment	Conc. (mg/ml)	Avenalumin I (μg/g fresh wt)			
		Iowa X469	Iowa X424	Shokan-1	Pc- 38
Chitosan I[a)]	0.1	317	305	375	43
Chitin	1.0	39	9	26	tr[c)]
Glycol chitin	1.0	133	18	86	tr
Glucosamine	1.0	8	14	5	4
N-acetyl glucosamine	1.0	0	0	10	2
N-acetyl galactosamine	1.0	0	0	6	2
Glucan	0.5	0	0	11	1
Laminarin	1.0	0	7	15	1
Oligo galacturonic acid[b)]	0.5	58	tr	22	12

a) DP: 37, Acetyl. 39%.
b) MW: 1056, DP:6.
c) tr: trace.

Table 3. Elicitor activities of chitosans with various degree of polymerization (DP) and N-acetylation for avenalumin I induction and release in the epidermis-detached primary leaves of oat

Chitosans	Conc. (mg/ml)	Pc-38		Shokan-1	
		Avl. I [a]	Leakage [b]	Avl. I	Leakage
Water		tr	13.0	tr	13.9
Chitosan I (DP: 37 Acetyl. 39%)	0.05	48	15.7	377	19.3
	0.1	76	18.7	101	18.3
	0.5	102	22.8	160	19.9
	1.0	75	18.1	168	18.5
Chitosan II (DP: 10 Acetyl. 30%)	0.05	tr	12.6	141	12.8
	0.1	tr	15.3	115	15.4
	0.5	128	17.1	100	18.4
	1.0	132	17.7	134	18.7
Chitosan III (DP: 5 Acetyl. 6%)	0.05	30	12.3	30	12.4
	0.1	36	14.8	23	13.6
	0.5	76	32.2	128	29.3
	1.0	118	57.1	417	47.1
Chitosan IV (DP:8 Acetyl. 15%)	0.05	0	15.3	0	15.7
	0.1	75	19.3	134	20.8
	0.5	289	19.6	270	20.8
	1.0	73	29.0	191	19.7

a) μg/g fresh wt.
b) μS/cm.

Table 4. Elicitor activities of chitosans as assessed by avenalumin I induction in oat cultivars of different genetic backgrounds

Cultivar	Avenalumin I (μg/g fresh wt)	
	Chitosan I [a]	Chitosan III [a]
Pc-51	628	399
Shokan-1	367	306
Pc-40	249	233
Pc-48	230	235
Pc-35	225	249
Pc-47	148	205
Pc-39	127	244
Pc-45	113	191

a) 1 mg/ml.

cumulation of avenalumin I was further determined with Pc-51, Shokan-1 and Pc-39 (Table 5). At 0.1 and 0.5 mg/ml of chitosans I and III, the differential accumulation of avenalumin I was observed among these cultivars. The cultivars Pc-51 and Shokan-1 were always sensitive to the elicitors. At the concentration of 1 mg/ml, however, the differential production became not so obvious because Pc-39 also responded to these elicitors and accumulated avenalumin I at a significant quantity. Avenalumin was excreted into the elicitor solution especially in 0.1 mg/ml chitosan I solution, while the secretion was prevented at higher concentrations (Table 5). Cytoplasmic strands with an intense yellow fluorescence were observed in the treated mesophyll cells and some cells in tissues treated at higher concentrations were unstainable with FDA. Oligo galacturonic acids of DP 6 to 12 did not elicit any differential accumulation of avenalumin I in these cultivars (data not shown).

Table 5. Chitosan-induced avenalumin I production in the mesophyll tissues and its release into elicitor solutions[a)]

Conc. of chitosans (mg/ml)	Cultivar	Avenalumin I (μg/g fresh wt)					
		Chitosan I			Chitosan III		
		Tissue	Sol.	Total	Tissue	Sol.	Total
0.1	Pc-51	495	4,568	5,063	99	329	428
	Shokan-1	360	1,396	1,756	73	140	213
	Pc-39	139	487	626	119	0	119
0.5	Pc-51	551	131	682	298	53	351
	Shokan-1	124	164	288	529	275	804
	Pc-39	98	0	98	113	72	184
1.0	Pc-51	628	528	1,156	399	0	399
	Shokan-1	367	225	592	306	0	306
	Pc-39	127	127	254	244	0	244

a) Avenalumin I accumulated in the leaf tissues and released into elicitor solutions was estimated 24 hr after treatment.

Table 6. Chitosan-induced avenalumin I accumulation in epidermis-peeled mesophyll tissue and in leaves infiltrated with elicitor solution by a hypodermic syringe

Treatment	Conc. (mg/ml)	Avenalumin I (μg/g fresh weight)			
		Pc-38		Shokan-1	
		Peeled	Injected	Peeled	Injected
Water		42	tr	7	tr
Chitosan I	0.1	61	tr	311	489
	1.0	451	37	593	729
Chitosan III	0.1	8	tr	35	tr
	1.0	540	tr	501	30

As the bioassay with the epidermis-stripped leaf segments involves a physical injury which may induce non-specific elicitation of avenalumin, the hypodermic-injection method was used to infiltrate elicitor solutions into intercellular spaces of primary leaves to compare the efficiency of these two bioassay methods for the evaluation of elicitor activity (Table 6). The differential induction of avenalumin I in Shokan-1 by chitosan I was much clearly observed in the elicitor-infiltrated leaves than those of which the epidermis had been detached and incubated on elicitor solution. In addition, chitosan I (DP 37 with 39% of acetylation), but not chitosan III, was found to be a specific elicitor for avenalumin I in Shokan-1. In the epidermis-stripped leaves, however, the specific induction of avenalumin was not so obvious. The differential induction by chitosan I was evident at 0.1 mg/ml, but became ambiguous at 1.0 mg/ml.

DISCUSSION

The experiments described in this paper were carried out as a primary step toward understanding the nature of signal compounds that may elicit avenalumin production in oat leaves of different genetic backgrounds. It was found that chitin and its soluble derivatives such as glycol chitin and chitosans were potent elicitor for avenalumin production. Especially, the soluble chitosans used in this experiment were by far more active than the other compounds including glucan. The previous study with a chitosan, however, showed a little elicitor activity due to the low solubility of the chitosan preparation used[18].

One of the most important findings in this study would be the differential elicitor activity of chitosans in various oat cultivars. Some cultivars such as Shokan-1 and Pc-51 were always sensitive to the chitosan elicitors and accumulated avenalumin I in larger quantity, while a cultivar like Pc-45 was induced to accumulate avenalumin at the least amounts. Although this differential induction cannot be directly related to the specific parasitism of crown rust races, it is interesting to know that such compounds as chitosans induced differential accumulation of avenalumin I in some of oat cultivars. The mechanisms of this differential recognition of chitosans in those cultivars are not known. However, it should be noted that the genetic backgrounds of Shokan-1 and Pc-51 were different from other Pc-lines which are near isogenic each other. The recognition of chitin or chitosans could be related to the induction of general resistance of oat leaves. The results also indicate the importance of precise chemical characterization of cell wall of crown rust fungus, although its major components are known to be chitin and glucans[1,19]. Recently, it has been shown that one of pathogenicity-related proteins is a chitinase, which is induced in plants in response to pathogens and other stresses[21]. There is a report of the presence of chitinases in the intercellular space of oat infected by rusts, suggesting that release of chitin or chitosan oligomers from fungal walls could be the primary event leading to the cascade of resistance expression[7,8]. The release of elicitors of phytoalexin accumulation by the action of host hydrolytic enzymes had been demonstrated in hyphal walls of *Phytophthora megasperma*[22]. As suggested by the present results, the induction of chitosanase should be examined in oat leaves in response to crown rust fungi.

The mode of action of chitosan in the elicitation of avenalumin I is not known, although changes in membrane properties leading to altered ion-transport has been suggested[3,11,12]. The present data suggest the alteration of host cell membrane property because chitosan at low concentration induced the excretion of avenalumin I into elicitor solutions, and the secretion was inhibited at higher concentrations where leakage of electrolytes was usually induced (Table 5). At low concentrations, chitosans sensitize cells to synthesize phytoalexins and to actively transport avenalumin through membrane.

It was found in the present study that the hypodermic injection of elicitors to intercellular spaces was very efficient in determining specific induction of phytoalexins in crown rust system. It allows further inoculation of rust fungi which can establish the infection only through stomata, hence makes the analysis of functional role of phytoalexins possible. Further study with the chitosans and fungal components of rust races is underway by using the hypodermic injection method not only to examine elicitor or suppressor activities of compounds of fungal origin but also to evaluate their functions in the induced resistance or susceptibility against crown rust infection.

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和 文 摘 要

A. P. A. Bordin・眞山滋志・谷 利一：エンバクのファイトアレキシン・アベナルミンの誘導活性をもつエリシターについて

キチン，キトサン，オリゴガラクツロン酸，グルカンなど各種糖物質のアベナルミンのエリシター活性について調べるとともに，同活性の生物検定法について検討した。水溶性キトサンが最も顕著なエリシター活性を示した。つぎに，キチン，グリコールキチン，オリゴガラクツロン酸の順に活性が認められたが，グルカンの活性は低かった。アミノ糖の単糖では活性は認められなかった。分子量（37，10，8 および 5 糖）およびアセチル化率（39，30，15，6%）の異なる水溶性キトサンの活性を比較したところ，いずれもエリシター活性を示したが，一般に高分子（37 糖）のキトサンは低濃度（0.05～0.1 mg/ml）でも活性を示し，顕著な濃度依存性は認められなかった。しかし，低分子（5～8 糖）キトサンのエリシター活性には濃度依存性

(0.05～1.0 mg/ml) が認められた。アセチル化率のエリシター活性に及ぼす影響は明確にはできなかった。キトサン処理葉肉細胞は高濃度では電解質の漏出が顕著で，部分的に死細胞が観察された。本研究結果における最も興味ある現象はキトサンのエリシター活性にはエンバク品種による差異が認められたことである。その品種間差異は特に低濃度 (0.05～0.1 mg/ml) で顕著で，キトサンのアベナルミン誘導活性は勝冠 1 号および Pc-51 では Pc-45 等他の Pc 系統に比べて有意に高かった。エリシターの生物検定法として，注射器によるエリシター物質の初生葉への表皮下注射法を検討したところ，表皮下注射法は表皮剝離法のような剝皮傷害による非特異的な誘導も少なく，キトサンのエリシター活性の品種間差もより顕著に認められた。表皮下注射法はエリシター物質の検討のみならず，とくにさび菌のように気孔感染菌の場合には各種成分のさび菌に対する誘導抵抗性や誘導罹病性活性の判定に有効と考えられる。