Localization, Accumulation, and Antifungal Activity of Chitinases in Rye (Secale cereale) Seed

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Received June 28, 2001; Accepted August 29, 2001

In order to understand a physiological role of chitinases in rye, the localization and accumulation of rye seed chitinase-a and -c (RSC-a and -c) in the seeds were studied by immunochemochemical methods. An antiserum specific to the chitin-binding domain (CB-domain), which is an N-terminal part of RSC-a, and an antiserum specific to the catalytic region of RSC-a and RSC-c were used. An immunoblot analysis detected both RSC-a and RSC-c in the endosperm of the rye seed. Immunohistochemical staining indicated that RSC-a was localized in only thealeurone cells, whereas RSC-c existed at least in thestarch endosperm and was also likely to exist in the aleurone cells. It was found by ELISA and an immunoblot analysis that RSC-a and -c were accumulated in the seed during the later stage of development. Both chitinases and the Cat-domain exhibited antifungal activity toward Trichoderma species, while theCB-domain did not. Observation of the inhibition of hyphal growth of the T. species suggests that the two chitinases acted in different ways.

Key words: chitinase; chitin-binding domain; rye seed; aleurone; antifungal activity

There are several different types of chitinase in higher plants and they are thought to play a role in plant defense against fungal attack in different ways.10 Rye seed chitinase-a (RSC-a) is a class I chitinase consisting of anN-terminal chitin-binding (CB) domain and a catalytic (Cat) domain connected by a flexible linker.12 The CB-domain is homologous to hevein, wheat germ agglutinin (WGA), and pokeweeds lectin (PL) that are referred to aschitin-binding proteins. Rye seed chitinase-c (RSC-c) is a class II chitinase with 92% sequence similarity to the Cat-domain of RSC-a, but lacking theN-terminal CB-domain.12,13 RSC-a has 3 times higher chitinase activity than that of RSC-c when using colloidal chitin as an insoluble substrate.14 We are interested in knowing whether this difference in enzymatic activity is related to the physiological role of chitinases in vivo.

To learn about this role, information about the localization and accumulation of chitinases is very important. The localization and accumulation of chitinases in developing barley seed has been shown by a northern blot analysis, immunoblot analysis, activity staining, and in-situ RNA hybridization.9-10 In these experiments, however, no attention has been paid to distinguishing between class I and class II chitinases.

Yamagami et al. have previously isolated theCB-domain and the Cat-domain after limited thermolysin hydrolysis of RSC-a.10 The chitinase activity of the Cat-domain, using colloidal chitin, was decreased to the level of RSC-c. We produced a polyclonal antibody that could discriminate between class I and class II chitinases by using the CB-domain as an antiserum. We describe here that the CB-domain antiserum could discriminate between RSC-a and RSC-c. The localization and accumulation during seed development of RSC-a and RSC-c were determined by experiments using the CB-domain antiserum and RSC-c antiserum.

The antifungal activity of chitinases derived from various plants has been investigated,15 but a correlation between the structure and enzymatic activity of chitinase and the antifungal activity of the protein has been unclear. A difference in the antifungal activi-
ity between RSC-a and RSC-c has been observed by some assays. In this paper, the relationship among the localization, accumulation, and antifungal activity of RSC-a and RSC-c is discussed.

Materials and Methods

Materials. Rye seeds were purchased from Takii Seed Co. (Kyoto, Japan). Developing spikes were harvested after flowering at the indicated intervals days up to 51 DAF. The seed tissues were dissected, immediately frozen in liquid nitrogen, and stored at −70°C. RSC-a and RSC-c were prepared from rye seeds, and the CB-domain (Glul-Pro48) and Cat-domain (Val49-Ala302) were isolated from a thermolysin digest of RSC-a as described by Yamagami et al.11) The reduced and carboxymethylated CB-domain was prepared as described by Yamagami et al.11) PL-B and PL-C were prepared from pokeweed roots as described by Kino et al.,12) and PL-D1 and PL-D2 were prepared as described by Yamaguchi et al.13) Trichoderma sp., isolated from the sediment of sugarcane bagasse, was used for assessing the antifungal activity. All other reagents were of analytical grade.

Preparation of the antisera. A KLH-CB-domain conjugate for immunization was prepared from 1 mg each of KLH and the CB-domain dissolved in 2 ml of 0.1 M sodium bicarbonate. A 10 mg amount of DMS was added to the mixture as a solid ten times at 5 min intervals; the pH of the solution was maintained at 9.0 by titrating with 0.1 M NaOH. The solution was incubated at room temperature for 2 h after the last addition. After this incubation, the reaction mixture was stored at −70°C as the KLH-CB-domain conjugate. The KLH-CB-domain conjugate and RSC-c were separately emulsified with an equal volume of Freund's complete adjuvant and intracutaneously injected into rabbits twice times at two-week intervals at a dose of 0.5 mg/rabbit. These antigens with incomplete adjuvant were next injected twice at two-week intervals three weeks after the first injection. Antisera were prepared from blood of the rabbits 2 weeks after the last injection, these antisera being designated as the CB-domain antiserum and RSC-c antiserum, respectively.

Competitive indirect ELISA. All washing steps were carried out with PBS containing 0.05% Triton-X 100 (PBST). First, 96-well plates were coated overnight at 4°C with 50 μl of RSC-a or RSC-c (0.1 μg/well) in a sodium phosphate buffer (pH 7.2) as an immobilized antigen. After one wash, the wells were blocked for 2 h with 100 μl of PBS containing 1% BSA. After another wash, 30 μl of an antiserum (1:5,000) in PBS was added to the well just after 20 μl of a serial dilution of the competitive antigen in PBS had been added, and the mixture was incubated for 2 h. After washing again, 50 μl of horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000) in PBST was added to the wells, and the mixture was incubated for 2 h. After washing again, color development was initiated by the addition of o-PDA/hydrogen peroxide, allowed to develop up to 10 min and terminated by the addition of 100 μl of 2 M sulfuric acid. Antibody binding was determined from the absorbance at 490 nm with an Inter Med microplate reader. Antibody binding is represented by B/B₀[(%)] = ((y − yₐₗ)/(yₚₐ₉ − yₐₗ)) × 100, with absorbance y and minimum and maximum absorbance yₚₐ₉ and yₐₗ, respectively. RSC-a, CB-domain, Cat-domain, RSC-c, PL-B, PL-C, PL-D1, or PL-D2 was used as a competitive antigen, and the amount was plotted as pmol/well, using molecular weights of 31,691, 4,639, 27,053, 26,089, 34,493, 13,747, 9,317, and 9,103, respectively.

Localization of RSC-a and RSC-c

(1) Detection of RSC-a and RSC-c in the organs of rye seed. The endosperm, embryo, or testa containing the pericarp of mature seeds was homogenized with 5-fold (w/v) distilled water in a mortar and pestle, and then left to stand for 4 h at 4°C. After centrifugation, each supernatant was used for evaluating the chitinase activity and for an immunoblot analysis.

Immunoblot analysis. Each sample was subjected to electrophoresis on 15% polyacrylamide gel containing sodium dodecyl sulfate by the method of Laemmli.15) After electrophoresis, the proteins were transferred on to a nitrocellulose membrane (BA85, Schleicher & Schuell) for 2 h at 4°C, using 176 mA. The membrane was blocked overnight with PBS containing 2% BSA at 4°C and then treated with the CB-domain antiserum (1:5,000) or RSC-c antiserum (1:5,000) in PBS for 2 h at room temperature. Following a wash with PBST, the membrane was treated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000) in PBST for 2 h at room temperature. The reaction of peroxidase was developed with 4-chloro-l-naphthol/hydrogen peroxide.

Assay for the chitinase activity. The chitinase activity was assayed colorimetrically by using glycolchitin as a substrate as described by Yamada et al.16) Ten μl of the sample solution was added to 500 μl of a 0.2% (w/v) glycolchitin solution in a 0.1 M sodium acetate buffer at pH 5.0. After incubating at 37°C for 15 min, the reducing power of the reaction mixture was measured with the ferriferrocyanide reagent by the method of Imoto and Yagishita.17)

(2) Immunohistochemical analysis. Dry seeds were fixed overnight at 4°C in a solution of 4% paraformaldehyde in a 100 mM sodium phosphate
buffer (pH 7.0). The fixed tissues were dehydrated through 10, 30, 50, 70, and 90% ethanol, and three changes of 100% ethanol (30 min per step). The tissues were infiltrated for 1 h with 1:1 LR White acrylic resin, 100% ethanol, and then with pure LR White for 24 h. Final embedding was done in pre-dried OO gelatin capsules, before polymerization in nitrogen gas in an oven at 60°C for 5 days. Sections (5 μm thick) were cut with a glass knife in a microtome and mounted on glass slides. The sections for immunodetection were blocked with PBS containing 1% BSA for 1 h. Then, 30 μl of the normal serum (1:500), CB-domain antiserum (1:500), or RSC-c antiserum (1:500) in PBS containing 1% BSA was placed on the samples which were incubated overnight at room temperature. Any unbound antibodies were removed by washing with PBS. The tissue sections were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000) in PBST for 4 h at room temperature. Any unbound second antibodies were removed by washing with PBST. The reaction of peroxidase was developed with 3,3′-diaminobenzidine tetrahydrochloride/hydrogen peroxide.

**Accumulation of RSC-a and RSC-c during seed development.** The fresh weights of ten seeds at each development stage were measured. Extracts prepared as described in "Detection of RSC-a and RSC-c in the organs of rye seed" were used for assessing the protein content, chitinase activity, and immunoreactive RSC-a or RSC-c. The protein content in the extract was measured by the method of Lowry et al., using bovine serum albumin as a standard. Chitinase activity was measured by the above-mentioned method, and RSC-a or RSC-c was detected by an immunoblot analysis using RSC-c antiserum (1:5,000). Quantification of RSC-a and RSC-c was done by competitive indirect ELISA, using the CB-domain antiserum and RSC-c antiserum, respectively.

**Assay for the antifungal activity.** Three bioassays for detecting the inhibition of fungal growth were carried out using *Trichoderma* sp. as the test fungus. The hyphal-extension inhibition assay was run on an agar disk (6 mm in diameter) with the fungus placed in the center of a Petri dish containing potato dextrose agar (PDA) in 1.5% (w/v) agar. The plate was incubated at room temperature for 12 h. Wells were subsequently punched into the agar at a distance of 15 mm from the center of the Petri dish. The samples to be tested were placed in the wells in 10 μl of distilled water. Radial hyphal extension inhibition was observed after incubating at room temperature for 24 h.

The linear hyphal-extension inhibition assay was run on several agar disks with the fungus placed in a straight line on a PDA plate (see Fig. 6(E)). The plate was incubated at room temperature for 12 h. Wells were subsequently punched into the plate in parallel with the line at a distance of 15 mm. The samples to be tested were placed in the wells in 10 μl of distilled water. Inhibition of the linear hyphal extension was observed after incubating at room temperature for 24 to 30 h.

A hyphal passing-through slit assay was devised as the third assay. Wells were punched into the PDA plate with a comb at a distance of 15 mm from the agar disks with the fungus that had been placed in straight line (see Fig. 7(D)). The plate was incubated at room temperature for 12 hours. The samples to be tested were then placed in the wells in 5 μl of distilled water. The hyphae passing through the slits between the wells were observed after incubating at room temperature for 24 to 48 h.

**Results**

**Characterization of the CB-domain antiserum**

To investigate the specificity of the CB-domain antiserum, competitive indirect ELISA was performed by using RSC-a as the solid-phase antigen. RSC-a, the CB-domain (Glu1-Pro48), Cat-domain (Val49-Ala302), and RSC-c having 92% sequence similarity to the catalytic domain of RSC-a were used as competitors. The liquid-phase CB-domain inhibited dose-dependently the binding of the CB-domain antiserum to solid-phase RSC-a. Neither liquid-phase RSC-c nor the Cat-domain affected the binding (Fig. 1). These results suggest that the CB-domain antiserum bound specifically to the CB-domain. This assay could quantitatively detect the liquid-phase CB-domain (0.05 μg-0.5 μg/well) and RSC-a (0.2 μg-2 μg/well).

Pokeweed lectin-B, which consists of seven domains with about 50-60% homology to the CB-domain, did not affect the binding. Pokeweed lectin-C, -D1 and -D2, which respectively have three, two, and two domains with about 50-60% homology to the CB-domain, also did not affect the binding (data not shown).

The CB-domain has five disulfide bonds. The 2CM-CB-domains and 10CM-CB-domains, in which one disulfide (Cys15-Cys42) and all five disulfides were respectively reduced and carboxymethylated, were used as competitors. The 2CM-CB-domain and 10CM-CB-domain inhibited the binding at 55% and 25%, respectively, to the native CB-domain at an amount of 400 pmol. These results suggest that the CB-domain antiserum bound only to the CB-domain of RSC-a, specifically and conformation-dependent.

**Specificity of the RSC-c antiserum**

Competitive indirect ELISA showed that RSC-c, RSC-a, and the Cat-domain inhibited dose-depen-
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The curves show the competition for the CB-domain antiserum between immobilized RSC-a (3.2 pmol/well) and the competing free antigens: ○, CB-domain; ●, RSC-a; △, RSC-c; ▲, Cat-domain; ▲, PL-B; ○, 2CM-CB-domain; ●, 10CM-CB-domain. Each result is the mean ± SE of three independent determinations.

Localization of RSC-a and RSC-c in rye seed

Extracts containing pericarp (TP) were used as an assay of the chitinase activity. Chitinase activity was detected only in the endosperm (data not shown). Part of these extracted proteins was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel and then blotted to a nitrocellulose membrane (Fig. 3(A)). The CB-domain antiserum was used for detecting RSC-a (Fig. 3(B)), and the RSC-c antiserum for detecting RSC-c (Fig. 3(C)). RSC-a and RSC-c were both detected in the endosperm, but were not present in the embryo and testa containing pericarp. These results agree with localization of chitinase activity and indicate that RSC-a and RSC-c were both localized in the endosperm.

Localization of RSC-a and RSC-c in the endosperm

To investigate the localization of RSC-a and RSC-c in the endosperm, immunolocalization was performed by using an embedded section of the endosperm. Only the aleurone cells (AL) were stained when using CB-domain antiserum (Fig. 4(B)), while both the aleurone cells and starchy endosperm (SE) were stained when using the RSC-a antiserum (Fig. 4(C)). These results show clearly that RSC-a was localized only in the aleurone cells, whereas RSC-c existed at least in the starchy endosperm and was likely to have existed in the aleurone cells as well.

Accumulation of RSC-a and RSC-c during seed development

The fresh weight of seeds of rye increased gradually from 6 DAF, reached a maximum by 23 DAF, maintained a high level until about 44 DAF, and then rapidly decreased due to desiccation (Fig. 5(A)). The total protein contents increased from 6 DAF, reached a maximum by 44 DAF, and then maintained the level. Chitinase activity showed a marked increase from 23 DAF, reached a maximum by 40 DAF, and then maintained the level (Fig. 5(B)).

RSC-a and RSC-c were detected by 23 DAF by an immunoblot analysis (Fig. 5(B), inset) and competitive indirect ELISA (Fig. 5(B), plots). These contents reached a maximum by 40 DAF and were maintained at this level until maturation had been completed. These results agree with the expression of chitinase activity and suggest that RSC-a and RSC-c were synthesized in the later stage of seed development.

Antifungal activity of rye seed chitinases and its domains

The antifungal activity was determined by using the conventional hyphal-extension inhibition assay on agar plates with Trichoderma sp. as the test fungus. RSC-a, RSC-c, and the Cat-domain inhibited hyphal extension at 90 pmol/well, but the CB-domain did not (Fig. 6(A)). Addition of the CB-domain to the Cat-domain did not strengthen the inhibitory ability of the latter (Fig. 6(A), wells 4 and 6). The CB-domain did not inhibit hyphal extension at 862 pmol/well (data not shown). It should be mentioned that RSC-a, RSC-c, and the Cat-domain exhibited similar inhibition zones, i.e. of "V" form.
Proteins extracted from matured rye seeds, their endosperm, embryo, and testa containing pericarp were resolved by SDS-PAGE (A), transferred on to a nitrocellulose membrane, and probed with the CB-domain antiserum (B) or RSC-c antiserum (C). Lane c, purified RSC-c; lane a, purified RSC-a; lane Wh, whole seed; lane En, endosperm; lane Em, embryo; lane TP, testa containing pericarp. a and c with an arrow indicate the respective positions of the RSC-a and RSC-c bands.

On the other hand, in the linear hyphal-extension inhibition assay, dose-dependent inhibition of linear hyphal extension by both RSC-a and RSC-c was observed after 24 and 30 hours of incubation (Figs. 6(B) and 6(D), and 6(C) and 6(E)). After 24 hours of incubation, RSC-a and RSC-c inhibited hyphal extension to a similar extent in wells 1, 2 and 3 (Figs. 6(B) and 6(C)), although RSC-c was slightly more effective than RSC-a as can be seen in well 4. After 30 hours of incubation, hyphal extension between the wells was inhibited in the case of RSC-a, but not in the case of RSC-c (Figs. 6(D) and 6(E)). Similar results were obtained with only the Cat-domain or with the Cat-domain plus CB-domain to those of RSC-c (data not shown). It should be mentioned with respect to the hyphal growth that the inhibition zone formed by RSC-a took the form of a “V”, while that by RSC-c took the form of a “U” by 24 h. By 30 h, however, the inhibition zone formed by RSC-a retained the form of a “V”, whereas that by RSC-c took the form
of a "horseshoe" or "teardrop" (Figs. 6(D) and 6(E), wells 1 and 2). It is thought that the speed of hyphal extension in slits between the wells contributed to the form of the inhibition zone. To make these observations clearer, the hyphae passing-through slit assay was devised (Fig. 7). The rate of hyphae passing through the slits from the wells containing RSC-a was lower than that through the slits from the wells containing RSC-c. In addition, the hyphae affected by RSC-a were abnormally extended (gathered at high density), whereas those by RSC-c were normally extended (widely diffused at low density). These observations suggest that the actions of RSC-a and RSC-c toward the hyphae were different from each other.

Discussion

The localization of some chitinases in barley seed has been shown. Protein C (class II chitinase) has
be the synthesis of only the RNA fraction of the starchy endosperm. It has been suggested that class I and C (class II) have been detected in both aleurone cells and starchy endosperm.\(^7\) mRNA encoding 26-kDa chitinase (class II) has been detected only in the aleurone cells.\(^8\) Robert et al. have identified an enhancer/silencer sequence directing the aleurone-specific expression of a barley chitinase gene encoding 26-kDa chitinase.\(^8\) According to these findings for barley seed, it is thought that several isoforms of class I or class II chitinase may exist and they are each localized specifically in the aleurone cells and/or starchy endosperm. Several of these experiments did not use a probe that could discriminate between class I and class II chitinase, it is ambiguous whether fractionation of the aleurone cells and starchy endosperm was successful. We followed two strategies to exclude this uncertainty by making a polyclonal antibody that could discriminate between class I and class II chitinases, using the CB-domain as the antigen. We also employed an immunohistochemical procedure, using the embedded sections, to avoid fractionation of the cell components.

The CB-domain antiserum bound equally to RSC-a and the CB-domain, but did not bind to the Cat-domain and RSC-c. These results indicate that the CB-domain antiserum bound to the N-terminal CB-domain of RSC-a and certainly discriminated between RSC-a and RSC-c. The CB-domain of RSC-a is homologous with hevein, wheat germ agglutinin (WGA), and pokeweed lectin (PL) that are referred to as chitin-binding proteins.\(^9\) Pokeweed lectin-B, -C, -D1, and -D2, in which each domain has about 50–60% homology to the CB-domain, did not bind to the CB-domain antiserum. The positions of eight cysteine residues are conserved in chitin-binding proteins. Since Yamagami et al. have revealed that the CB-domain of RSC-a had an extra disulfide bond between Cys15 and Cys42 in chitin-binding proteins,\(^1,10\) it was suggested that the extra disulfide bond influenced the antigenicity. The affinity of the 2CM-CB-domains, in which the disulfide bond between Cys15 and Cys42 was cleaved, to the CB-domain antiserum was less than that of the native CB-domain. The 10CM-CB-domain, in which all disulfide bonds were cleaved, had little affinity to the CB-domain antiserum. These results suggest that a three-dimensional structure was needed to bind the CB-domain antiserum to the chitin-binding domain.

The RSC-c antibody bound to RSC-c, RSC-a, and the Cat-domain, but did not bind to the CB-domain. The affinity of the RSC-c antiserum to RSC-a was slightly weaker than to the Cat-domain and even weaker than to RSC-c. These results suggest that the RSC-c antiserum could bind to the Cat-domain of RSC-a.

The immunoblot analysis, using the CB-domain antiserum and RSC-c antiserum, indicated that RSC-a and RSC-c were present only in the endosperm, and not in the embryo, testa, or pericarp. This result agrees with the localization of some barley seed chitinases, except for CH3 that has been identified as a class I chitinase localized primarily in the embryo.\(^11\) Our experiments, using the immunohistochemical technique, show that at least RSC-a as a class I chitinase was localized only in the aleurone cells. It seems advantageous in defense against fungi that RSC-a containing the CB-domain, which has high affinity to an insoluble substrate, is located in aleurone cells, the outer layer of the endosperm.

To investigate the accumulation of RSC-a and RSC-c in developing seeds, the immunoblot analysis was performed by using the RSC-c antiserum. The experiments indicate that RSC-a and RSC-c were synthesized in the later stage of embryogenesis. These results agree with the accumulation of mRNA of the
barley seed chitinases. The appearance of RSC-a and RSC-c in the later stage of seed development suggests a role for these enzymes in defense against fungi in the quiescent and germinating seed.

The existence in rye seeds of some isoforms of class I and class II chitinases has been confirmed by the purification process of rye seed chitinases. Our antisera possibly bound to these isoforms. Since RSC-a and RSC-c account for 70% of all the isoforms in rye seeds, the existence of isoforms seems to have little influence on the results of localization and accumulation.

Various in vitro studies have demonstrated a growth inhibitory effect of chitinases against fungi. However, it is poorly understood whether a difference in the structure or enzymatic activity of chitinase is related to the potency of the antifungal activity of the protein. Chitinase C (class II) derived from barley seed inhibited the growth of the Trichoderma viride fungus as well as chitinase T (class I) did. Tob chitinase (class I) and TobA H chitinase (Tob chitinase without the chitin-binding domain) have been found capable of inhibiting the growth of T. viride, although Tob was about three times more effective than TobA H. Tobacco class I chitinase has inhibited the growth of Fusarium solani, but class II chitinase did not. The mutant protein of the 26-kDa chitinase (class II) of barley that does not possess chitinolytic activity had 15% of the antifungal activity of the wild type of chitinase. The mutant protein of class I chitinase (derived from chestnut seed) that had no chitinolytic activity had as much antifungal activity as the wild type of chitinase. These results may suggest that the CB-domain of class I chitinase alone possesses antifungal activity and/or that part of the Cat-domain of class II chitinase does.

In our hyphal-extension inhibition assay, RSC-c and the Cat-domain inhibited hyphal extension as well as RSC-a did, but the CB-domain did not. In the linear hyphal-extension inhibition assay, however the forms of the inhibition zone produced by RSC-a and RSC-c were different from each other. In the hyphae passing-through slit assay, RSC-a more persistently inhibited hyphal growth than RSC-c did. These observations suggest that the action site of RSC-a was different from that of RSC-c on the fungal cell wall. Considering that the inhibition by only the Cat-domain or Cat-domain combined with CB-domain was similar to that by RSC-c in these assays, the difference in inhibition of hyphal growth between RSC-a and RSC-c is possibly attributable to the presence of the CB-domain in RSC-a.

In a part of the fungal mature cell wall distant from the hyphal tip, the polysaccharides are cross-linked to form mixed chitin-glucan fibers and may be overlaid by other polysaccharides and protein layers. Thus, at the hyphal tip, the exposed nascent chitin chains are accessible to hydrolysis by chitinase, whereas the chitin layer in the mature cell wall is inaccessible to degradation by the enzyme. RSC-a might better hydrolyze the mature cell wall or the site near mature cell wall than RSC-c can. The CB-domain may have affinity to this putative action site of RSC-a on the fungal cell wall. Consequently, the cell walls distant from the hyphal tip would be degraded by RSC-a and hyphal growth would be persistently inhibited. In the linear hyphal-extension assay, the courses for branching and extension of the hyphae would be influenced a little by RSC-c, resulting in the U-form inhibition zone. If RSC-a acts on the root of hyphal branches, it would limit the courses of branching and extension of hyphae to the horizontal axis, thus resulting in the formation of a V-form inhibition zone. For the same reason, the delayed vertical hyphal extension by RSC-a would cause abnormal growth and finally persistent inhibition of the hyphae.

Application of the hyphae passing-through slit assay, in which the wells, slits, and area over the wells are respectively regarded as an aleurone layer, wound, and starchy endosperm of seed, allows effective inhibition by RSC-a and/or RSC-c against invasion by fungus from the wound to be experimentally reproduced.

In summary, the location of RSC-a in the outer layer in the later stage of seed development seems to bring about persistent inhibition of the hyphal growth of invading fungi in the quiescent seeds.

Acknowledgments

We thank S. Toyama and J. Moromizato (Ryukyu University, Japan) and T. Ohnnuma (Kyushu University, Japan) for their practical advice and discussions.

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