Immunolocalization and functional role of *Sclerotium rolfsii* lectin in development of fungus by interaction with its endogenous receptor

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Many fungi are known to secrete lectins, but their functional roles are not clearly understood. *Sclerotium rolfsii*, a soilborne plant pathogenic fungus capable of forming fruiting bodies called sclerotial bodies, secrete a cell wall–associated Thomsen-Friedenreich antigen–specific lectin. To understand the functional role of this lectin, we examined its occurrence and expression during development of the fungus. Furthermore, putative endogenous receptors of the lectin were examined to substantiate the functional role of the lectin. Immunolocalization studies using FITC-labeled lectin antibodies revealed discrete distribution of lectin sites at the branching points of the developing mycelia and uniformly occurring lectin sites on the mature sclerotial bodies. During development of the fungus the lectin is expressed in small amounts on the vegetative mycelia and reaching very high levels in mature sclerotial bodies with a sudden spurt in secretion at the maturation stage. Capping of the lectin sites on the sclerotial bodies by lectin antibodies or haptons inhibit strongly the germination of these bodies, indicating functional significance of the lectin. At the maturation stage the lectin interacts with the cell wall–associated putative endogenous receptor leading to the aggregation of mycelium to form sclerotial bodies. The lectin–receptor complex probably acts as signaling molecule in the germination process of sclerotial bodies. Using biotinylated lectin, the receptors were identified by determining the specific lectin binding to lipid components, extracted from sclerotial bodies, and separated on thin-layer chromatograms. Preliminary characterization studies indicated that the receptors are glycosphingolipids and resemble inositolphosphoceramides. These findings together demonstrate the importance of lectin–receptor interactions to explain hitherto speculated functional role of the lectins and also the glycosphingolipids of fungi.

**Key words:** fungal lectins/glycosphingolipids/glycosylinositolphosphoceramides/lectin receptor/*Sclerotium rolfsii* lectin

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Introduction

Lectins are ubiquitous and are being intensively studied in plants, animals, and bacteria because they specifically recognize and bind to carbohydrates present on cell surfaces. Although occurrence of lectins in fungi has been known for a while (Gold and Balding, 1975), they did not receive much attention compared to bacterial lectins. It has been shown that lectins are common in fungi, and in the recent past several lectins were purified and characterized (Guillot and Konska, 1997; Kawagishi, 1995; Kellens et al., 1989; Pemberton, 1994; Wang et al., 1998a). Majorit y of the fungal lectins isolated were from the fruiting bodies and rarely from the vegetative mycelia (Gioi llant et al., 1993; Kellens et al., 1992; Wang et al., 1998b; Oda et al., 2003).

Contrary to established roles of bacterial lectins in host–parasite interactions, functional roles assigned for fungal lectins are speculative. Although many believe that fungal lectins do mediate host–parasite interactions (Rudiger, 1998) similar to bacterial adhesins, several other roles are also put forth. Often physiological roles for fungal lectins were attributed based on the location and selection of the source for isolation (Barak and Chet, 1990; Elad et al., 1983; Inbar and Chet, 1994; Kellens and Peumans, 1990). Some of the roles assigned to fungal lectins are storage proteins (Kellens and Peumans, 1990), fungal–fungus interactions (mycoparasitism), and host–parasite interactions (Fukazawa and Kagaya, 1997; Hostetter, 1994; Rudiger, 1998). Another argued function gaining greater attention is their involvement in morphogenesis and development of the fungi (Cooper et al., 1997; Yatohgo et al., 1988). However, none of these assigned roles are established.

Similar uncertainty also exists for the functional roles of membrane-bound fungal sphingolipids referred to as phytosphingosines. Inositolphosphorylceramides (IPCs), a family of membrane lipids with characteristic glycosyl moieties, occur exclusively in fungi (Dickson, 1998). These molecules, once believed to be simply structural components of membranes, are now implicated for their diversified roles as second messengers that activate intracellular signal transduction pathways leading to regulation of cell cycle and growth (Dickson, 1998). Our findings with *Sclerotium rolfsii* lectin (SRL) and its putative endogenous receptor suggest that the cell wall–associated lectins and the sphingolipids in fungi with obscure functions collude to carryout important biological function in the development of the fungus.

Earlier we reported the purification and the fine sugar specificity of a Thomsen-Friedenreich antigen–specific lectin from *S. rolfsii* (Swamy et al., 2001; Wu et al., 2001); its
preliminary X-ray crystallographic data has been reported recently (Leonidas et al., 2003). Interesting sugar specificity exhibited by SRL toward Galβ1→3GalNAc-ser/thr, an oncofetal antigen, prompted us to study its functional role. The present article describes the localization and expression of the lectin and identification of its putative endogeneous glycosphingolipid receptor, to explain the functional role of the lectin and the receptor in the development and morphogenesis of S. rolfsii.

Results

Localization of SRL

Immunolocalization of SRL in vegetative mycelia and sclerotial bodies using fluorescein isothiocyanate (FITC) anti-SRL demonstrated that the lectin is detected in small amounts on the mycelia and large amounts in sclerotial bodies. Binding of the FITC anti-SRL to vegetative mycelia and sclerotial bodies of S. rolfsii as seen under fluorescence microscope are presented in Figure 1. The interaction of FITC anti-SRL with the lectin occurring on the mycelia was observed as discrete intense fluorescence spots at the branching points of the mycelium (Figure 1a). However, uniform weak fluorescence was observed all along the hyphal surface (Figure 1a, inset). These observations indicate that although the lectin is distributed all along the hyphae but occur more densely at the branching points. Immature sclerotial bodies in the final stage of formation still associated with highly branched mycelia around showed dense mass of congre gated lectin sites arising due to aggregation of mycelium (Figure 1b) in addition to weaker fluorescence on nonaggregated regions. In the completely matured sclerotial bodies, intense uniform fluorescent label (Figure 1c) was seen over the entire surface of the mature sclerotial bodies, revealing uniform distribution of lectin at very high levels compared to vegetative mycelia.

SRL expression during development

To ascertain the expression of lectin quantitatively during the course of growth and development of S. rolfsii, lectin content in developing mycelia and sclerotial bodies was determined by hemagglutination assay. Results of the expression of lectin in the mycelium and sclerotial bodies on different days of growth are shown in Figure 2a and b, respectively. The dry weight of mycelial mass increases progressively after fourth day with concomitant increase in protein and total sugar contents until the ninth day. However, the mycelial lectin content increases slowly, but not as a function of mycelial growth; instead a sudden spurt in lectin content was observed between 13th and 15th days (Figure 2a). Subsequently mycelial mass decreases rapidly, as did the protein and sugar content, resulting in the aggregation of mycelium leading to the onset of sclerotial bodies. As the formation of sclerotial bodies continued, the mycelial mass decreased rapidly (Figure 2a). In contrast, lectin, protein, and total sugar contents increased linearly in the sclerotial bodies with the increase in their dry weight (Figure 2b).

At any given stage of growth, the specific activity of lectin in the sclerotial bodies is ~1000-fold higher compared to mycelium. These results reflect the previous observations of immunolocalization, and it becomes apparent that much of the lectin was secreted at once during the aggregation of mycelia to form sclerotial bodies. Probably the lectin secreted will help the mycelial filaments cross-link and form sclerotial bodies because of its specific sugar-binding property.

Inhibition of germination by neutralizing antibody

To further investigate the involvement of lectin in growth of the fungus, we examined the germination of sclerotial bodies after capping the lectin sites by anti-SRL. Interestingly, sclerotial bodies treated with anti-SRL did not germinate (Figure 3b) even after 7 days, but the sclerotial bodies treated with normal rabbit serum germinated normally and lavish growth was observed (Figure 3a). Similarly, the mycelia treated with anti-SRL also failed to grow. Not only anti-SRL but also SRL-binding glycoproteins fetuin and mucin inhibited the germination similarly (data not shown). Observations of inhibition of germination by extraneous lectin-binding molecules suggests that...
the lectin expressed in response to nutrient stress interacts with the endogenous receptor, apart from facilitating the mycelial aggregation, to form sclerotial bodies, which also play key role in germination process.

**Endogenous receptor of SRL**

Specific binding of biotinylated SRL to the lipid components of sclerotial bodies separated on thin-layer chromatography (TLC) plates, as demonstrated by avidin peroxidase reaction, lead to the identification of endogenous glycolipid receptors. After TLC, the chromatogram was treated with periodate-treated bovine serum albumin (p-BSA) to abolish nonspecific binding and allowed to interact with biotinylated SRL. Developed blue color revealed the presence of two SRL binding bands and were assigned as receptor bands RI and RII (Figure 4, 1). To confirm the specificity of the SRL binding, another parallel chromatogram was treated with biotinylated peanut agglutinin (PNA) (Figure 4, 2). Both the SRL binding lipid bands (RI and RII) coincided with the PNA binding bands, and because SRL and PNA have common sugar specificity, this confirmed the specificity of lectin binding.

Considering the sugar specificity of SRL and PNA, it may be concluded that the receptors RI and RII are glycolipids containing Galβ1→3GalNAc-glycoconjugate moiety. To get an insight into the chemical nature of the lectin binding receptors, parallel chromatograms were sprayed with specific detection reagents: orcinol to detect glycolipids (Figure 4, 3), ninhydrin to detect free amino groups (Figure 4, 4), and ammonium molybdate to detect phospholipids (Figure 4, 5). Staining for glycolipid with orcinol showed an intense
band for RI but a faint band for RII at the concentration of lipid applied on TLC. However when fivefold excess lipid was applied for TLC, RII also showed as prominent orcinol positive band (Figure 4, 6) coinciding with SRL binding bands (Figure 4, 7), indicating the glycolipid nature of RI and RII. However, the resolution of separation of other lipid components at this lipid concentration applied was not satisfactory.

Identification of free amino groups in the receptors on TLC by spraying with ninhydrin indicated positive reaction only for RI but not for RII (Figure 4, 4), even with excess amounts of lipid. However positive indication for the presence of phosphate moiety in both RI and RII was demonstrated by detection with ammonium molybdate and the color developed with tetramethyl benzidine/H₂O₂ substrate. Analytical identification of lectin-binding lipid components, chromatograms after development sprayed with orcinol reagent to detect glycolipids (3) ninhydrin to detect free amino groups (4) and ammonium molybdate reagent to detect phospholipid (5) components. Fivefold excess lipid (10 μl) loaded to TLC plates to confirm the glycolipid staining by orcinol for RI and RII (6), without alteration in the mobility of SRL binding bands (7).

Discrepancies could not be ruled out. Indeed both the receptors contain Galβ1→3GalNAc, an essential criterion for binding by SRL and PNA.

Discussion
For establishing the physiological role of lectins, it would be significant to have detailed information on their distribution and cellular localization. The distinction between intracellular and cell surface location of agglutinin would be of paramount importance for understanding its function. The results of immunolocalization and the expression studies revealed that SRL is formed initially on the young hyphae in small amounts, and very high levels accumulated rapidly at the time of sclerotial body formation.

Similar observations were made in *Rhizoctonia solani*, where in the lectin occurring in small amounts on young hyphae increased dramatically at the time of maturation and accumulated in mature sclerotina (Kellens and Peumans, 1990). Lectin accumulated in the sclerotina, represented as high as 40% of the total sclerotial protein, hence they concluded that the lectin serve as reserve storage protein as in plant seeds. In contrast Cooper *et al.* (1997) suggested that the small, saline-soluble galactose-binding lectins (fungal galectins) secreted by many fungi are developmentally regulated with high expression in fruiting bodies. A fucose-specific lectin secreted by *Rhizopus stolonifer* only under spore-forming conditions reported recently (Oda *et al.*, 2003) supports this latter view.

Our results of localization and expression of SRL revealed that the SRL is developmental-stage specific lectin secreted in response to stress, playing key role in the formation of sclerotial bodies rather than simply serving as reserve storage protein. Germination of sclerotial bodies is another event in the development of the fungus, which also involves the role of the lectin as shown by lectin capping studies. Capping of the lectin sites by anti-SRL strongly inhibits germination of the sclerotial bodies. Similar inhibition was also found by treating these bodies with mucin or fetuin, with which SRL strongly binds (Swamy *et al.*, 2001). These observations suggest that the lectin in a bound form with any extraneous lectin-binding molecules, such as antibodies, fetuin, or mucin will result in inhibition of sclerotial body germination. For the onset of sclerotial body germination, lectin–receptor complex could be mediating as critical signaling molecule, probably this signaling event is interrupted when the lectin is complexed with anti-SRL or a hapten.

Recently it was shown that the disruption of the glucosyl ceramide synthesis using inhibitors of UDP-Glc, ceramide glucosyltransferase, leads to inhibition of spore germination, cell cycle, and hyphal growth in *Aspergillus nidulans* and *Aspergillus fumigatus* (Levery *et al.*, 2002). Membrane-bound glycosyl ceramides are reported to be widely distributed in many fungi, and during their syntheses, sugar moieties are added directly to ceramides, which are referred to as glycosylinositol phosphoceramides (GIPCs). This class of glycosyl ceramides occurring in fungi (Lester and Dickson, 1993; Dickson, 1998) are mostly glucosylceramides. However there are also reports of galactosylceramides occurring in some fungi (Levery *et al.*, 2000; Toledo
Streptavidin horseradish peroxidase, tetramethyl benzidine/Duk was carried out according to the procedure described by Glass (1981). Biotinylation of SRL and PNA FITC-labeled SRL antibodies were prepared as described on Sephadex G-50 column. Specificity of anti-SRL was fate precipitation (Livingston, 1974) and chromatography immunizing rabbits (New Zealand breed) with purified 2001). Antibodies for SRL (anti-SRL) were obtained by for studying the expression of lectin at different stages of terose agar slants containing 5% dextrose. Cultures grown on different days, washed thoroughly with distilled water, and powdered after freeze drying. Dried mycelium (10 mg/ml) was homogenized with PBS (50 mM, pH 7.2) using pestle and mortar, briefly sonicated, and kept for extraction on a rotary shaker (150 rpm) for 1 h. The homogenate was subjected to centrifugation at 10,000 × g for 30 min, at 4°C (Kubota refrigerated centrifuge, RA 300F angle rotor). Clear supernatant after ultrafiltration on 0.2 μ membrane was used for the determination of lectin activity.

In sclerotial bodies. Sclerotial bodies formed during the course of development were harvested from each flask (in triplicate) after picking up manually from the culture broths on different days, washed thoroughly with distilled water, and powdered after freeze drying. Dry powder of the scleroti- bodied was suspended in phosphate buffered saline (PBS) containing p-BSA (3%) and incubated for 30 min at 37°C to block nonspecific binding by lectin antibodies, followed by extensive washing with PBS. Subsequently the filaments were incubated with FITC anti-SRL in PBS (50 μg/ml) for 30 min with gentle shaking. Excess unbound antibodies were removed by washing with PBS on centrifugation. Essentially the same procedure was adopted for lectin localization on sclerotic bodies. Interaction of FITC anti-SRL on vegetative mycelia and sclerotial bodies was observed and photographed under fluorescence microscope (Carl Zeiss Jenalumar, model Fluoval 2) using excitation filter G-247 in the path of excita- tion light and barrier filter B 450 in the path of emitted light.

### Materials and methods

Stock cultures of *S. rolfsii* were maintained on potato dextrose agar slants containing 5% dextrose. Cultures grown on Byrde’s liquid synthetic media (Byrde et al., 1956) were used for studying the expression of lectin at different stages of development and growth of the fungus. SRL was purified from sclerotial bodies as described earlier (Swamy et al., 2001). Antibodies for SRL (anti-SRL) were obtained by immunizing rabbits (New Zealand breed) with purified SRL, further fractionating serum by 50% ammonium sulfate precipitation (Livingston, 1974) and chromatography on Sephadex G-50 column. Specificity of anti-SRL was confirmed by Ouchterlony immunoprecipitation method. FITC-labeled SRL antibodies were prepared as described by Goldman (1968). p-BSA for blocking nonspecific sites for immunolocalization studies was prepared essentially according to Glass (1981). Biotinylation of SRL and PNA was carried out according to the procedure described by Duk et al. (1994) using N-hydroxysuccinimido biotin. Streptavidin horseradish peroxidase, tetramethyl benzidine/H₂O₂ (TMB/H₂O₂), and silica-coated high-performance TLC glass plates were purchased from Sigma Chemical (St. Louis, MO). All other chemicals used were of analytical reagent grade, and the reagents were prepared in twice glass-distilled water.

### Immunolocalization of lectin

Localization of lectin in vegetative mycelia and immature and mature sclerotial bodies of *S. rolfsii* during development was carried out by immunolabeling the lectin sites with FITC anti-SRL. Vegetative mycelial mass from the *S. rolfsii* culture broth of 10 days was washed repeatedly by centrifugation at 8000 × g. Washed mycelial filaments were suspended in phosphate buffered saline (PBS) containing p-BSA (3%) and incubated for 30 min at 37°C to block nonspecific binding by lectin antibodies, followed by extensive washing with PBS. Subsequently the filaments were incubated with FITC anti-SRL in PBS (50 μg/ml) for 30 min with gentle shaking. Excess unbound antibodies were removed by washing with PBS on centrifugation. Essentially the same procedure was adopted for lectin localization on sclerotic bodies. Interaction of FITC anti-SRL on vegetative mycelia and sclerotial bodies was observed and photographed under fluorescence microscope (Carl Zeiss Jenalumar, model Fluoval 2) using excitation filter G-247 in the path of excitation light and barrier filter B 450 in the path of emitted light.

### Determination of SRL activity during growth and development of *S. rolfsii*

In mycelia. For the determination of lectin activity in developing mycelia, *S. rolfsii* cultures were grown in Byrde’s mineral medium (100 ml) in different flasks by innoculating one sclerotial body for each flask. On different days from the date of inoculation, the mycelial mass formed was collected from three flasks separately (triplicate) after filtration on a sintered funnel. Individual mycelial lots of each day were separately washed with distilled water and freeze-dried. Dried mycelium (10 mg/ml) was homogenized with PBS (50 mM, pH 7.2) using pestle and mortar, briefly sonicated, and kept for extraction on a rotary shaker (150 rpm) for 1 h. The homogenate was subjected to centrifugation at 10,000 × g for 30 min, at 4°C (Kubota refrigerated centrifuge, RA 300F angle rotor). Clear supernatant after ultrafiltration on 0.2 μ membrane was used for the determination of lectin activity.

In sclerotial bodies. Sclerotial bodies formed during the course of development were harvested from each flask (in triplicate) by picking up manually from the culture broth on different days, washed thoroughly with distilled water, and powdered after freeze drying. Dry powder of the sclerotic bodies was suspended in PBS (5 mg/ml), sonicated to obtain homogeneous suspension and extracted for 1 h on a rotary shaker. Clear supernatant obtained on centrifugation at 8000 × g for 30 min was used for the determination of lectin activity. Lectin activity in the extracts of mycelia and sclerotial bodies harvested on different days was determined by hemagglutination assay. Total carbohydrate, protein content, and dry weight were also determined. The results of the lectin activity, total sugar, protein content, and dry weight of mycelium/sclerotial bodies were plotted against the age of culture.

### Hemagglutination assay

The hemagglutinating activity of the lectin was routinely assayed by serial twofold dilution technique of Liener and
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Analytical characterization of lectin binding receptors

Analytical characterization of lectin-binding receptors resolved on TLC plates was carried out using specific spraying reagents. The chromatograms were dried and sprayed separately for detection of glycolipids by orcinol, free amino groups by ninhydrin, and phospholipids by ammonium molybdate reagents. Reagents and the methods used were essentially as described by Kates (1972). After the development of the color the plates were photographed.

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Abbreviations

BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; IPCs, inositolphosphorylceramides; GIPCs, glycosylinositol phosphorylceramides; PBS, phosphate buffered saline; PNA, peanut agglutinin; SRL, Sclerotium rolfsii lectin; TLC, thin-layer chromatography.

References


References

Hill (1953) in microtiter assay plates using trypsinized human (O group) erythrocytes. The highest dilution of the extract causing visible hemagglutination was regarded as the titer and the protein content in the highest dilution causing visible agglutination as 1 unit of hemagglutination activity (minimum concentration of protein required for agglutination). Total activity in the extracts was determined from the total protein content.

Estimation of protein and sugar

Protein concentration was estimated by the method of Lowry et al. (1951) using BSA as standard. The total sugar content was estimated by the phenol-sulfuric acid method of Dubois et al. (1956) using glucose as standard sugar.

Inhibition of sclerotial body germination by anti-SRL

Sclerotial bodies from a fresh culture were incubated for 10 min at 37°C with anti-SRL in a screw-capped vial. Treated sclerotial bodies were allowed to germinate in a petri dish containing Byrde’s media with 1.5% agar at 28°C. As control, a sclerotial body that was treated with normal rabbit serum was inserted in another well of the plate. Germination of the sclerotial body in the plate was photographed after fourth day of inoculation.

Identification of putative endogenous lectin receptor

Extraction and thin layer chromatography of membrane lipids. Total lipids were extracted from the powdered mature sclerotial bodies using 20 volumes (w/v) of chloroform: methanol (2:1). The extract was dried by rotary evaporation and partitioned between chloroform and water. The resulting organic layer was washed twice with water and finally dried by flushing nitrogen and stored at –20°C for further fractionation by TLC. Dried lipid extracted from sclerotial bodies in chloroform (2 μl) was applied by streaking onto high-performance TLC plates (1 × 10 cm), and developed with chloroform:methanol:water (13:5:0.9), and dried at 60°C for 1 h.

Biotinylated lectin labeling assay

Lectin-binding lipid components on TLC plates were identified by enzyme-linked lectinosorbent detection using biotinylated SRL. In principle, the method we adopted is similar to solid-phase methods, used to demonstrate binding of viruses and bacteria and to characterize receptor carbohydrates based on their specific binding to glycolipids separated on thin-layer chromatograms (Karlsson, 1989). In this detection assay, the TLC plates with fractionated lipid components were immersed in PBS containing polyvinyl pyrrolidone (1%) for 5 min. Subsequently the plates were blocked with p-BSA (3%) in PBS for 1 h to prevent nonspecific binding and washed gently three times with PBS and incubated overnight at 4°C in screw-capped tubes containing biotinylated SRL or biotinylated PNA in PBS (20 μg/ml). The plates were gently washed with PBS to remove unbound lectin and treated with streptavidin horseradish peroxidase. The plates were finally washed with distilled water and incubated in tetramethyl benzidine/H₂O₂ for 5 min; the blue-colored bands developed were photographed immediately.


