Many fungi are known to secrete lectins, but their functional roles are not clearly understood. *Sclerotium rolfsii*, a soil-borne 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 inhibits 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; Pembertone, 1994; Wang et al., 1998a). Majority of the fungal lectins isolated were from the fruiting bodies and rarely from the vegetative mycelia (Giolliant 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–fungal 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 phyosphingosines. Inositolphosphorylceramides (IPC), 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 carry out 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 congregated 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.

Endogeneous 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

Fig. 2. Expression of SRL at different stages of development. Lectin content in (a) vegetative mycelium and (b) sclerotial bodies, expressed as total hemagglutination activity (diamonds) on different days of growth. Growth was expressed in terms of dry weight (open circles), carbohydrate (closed circles) and protein content (squares). Mean values of triplicate experimental sets are presented.

Fig. 3. Effect of capping the lectin by anti-SRL on germination of sclerotial body. (a) Sclerotial body treated with normal rabbit serum showing normal growth. (b) Binding of anti-SRL strongly inhibits the germination after 3 days of incubation.
associated with the receptor bands leading to such
could probably be a modified form of RI. Heterogeneity
(ethanolamine or serine). These differences suggest that RII
higher content of carbohydrate and with free amino groups
staining revealed that RI is different than RII by having
the receptors (RI and RII) identified are ceramide-type
5). From these analytical findings, we concluded that both
strated by detection with ammonium molybdate (Figure 4,
ence of phosphate moiety in both RI and RII was demon-
amounts of lipid. However positive indication for the pres-
TLC by spraying with ninhydrin indicated positive reaction
orcinol reagent to detect glycolipids (3) ninhydrin to detect free amino
components. Fivefold excess lipid (10
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).

Fig. 4. Identification by lectin labeling and analytical characterization of
lectin-binding lipid components extracted from the sclerotial bodies on
thin-layer chromatograms. Lectin binding receptors from the lipids (2 µl)
separated were identified by treating the chromatograms with
biotinylated SRL (1), biotinylated PNA (2) incubated with avidin–
horseradish peroxidase 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
bands (Figure 4, 7), indicating the glycolipid nature of RI
positive band (Figure 4, 6) coinciding with SRL binding bands
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 pres-
ence of phosphate moiety in both RI and RII was demon-
strated by detection with ammonium molybdate (Figure 4,
5). From these analytical findings, we concluded that both
the receptors (RI and RII) identified are ceramide-type
glycosphingophospholipids. Also, orcinol and ninhydrin
staining revealed that RI is different than RII by having
higher content of carbohydrate and with free amino groups
(ethanolamine or serine). These differences suggest that RII
could probably be a modified form of RI. Heterogeneity
associated with the receptor bands leading to such
discrepancies could not be ruled out. Indeed both the recep-
tors 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 distribu-
tion and cellular localization. The distinction between intra-
cellular 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 deval-
mental 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 stor-
age 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
ermination 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 sig-
aling 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
glucoisyltransferase, leads to inhibition of spore germina-
tion, cell cycle, and hyphal growth in Aspergillus nidulans
and Aspergillus fumigatus (Levery et al., 2002). Membrane-
bound glycosyl ceramides are reported to be widely distri-
buted in many fungi, and during their synthetises, sugar
moieties are added directly to ceramides, which are referred
to as glycosylinositol phosphorylceramides (GIPCs). This
class of glycosyl ceramides occurring in fungi (Lester and
Dickson, 1993; Dickson, 1998) are mostly glycosylcer-
imides. However there are also reports of galactosylcer-
imides occurring in some fungi (Levery et al., 2000; Toledo
et al., 1999, 2000). GIPCs are believed to act as signaling molecules and are implicated for their diversified roles in viability (Zhong et al., 2000) and cell growth (Chung et al., 2001). We believe that the inhibition of spore germination by disrupting the ceramide synthesis using inhibitors of glycosyl transferases in A. nidulans and A. fumigatus (Leverey et al., 2002) is analogous to our observed inhibition of sclerotal body germination by capping the lectin sites on sclerotal bodies.

Identification of lectin binding receptors in the lipid extracts of sclerotal bodies by specific lectin labeling method substantiates the significance of the lectin–receptor interaction in the development of the fungus. Considering the sugar specificity of SRL, which binds specifically to Galβ1→3GalNAc residues of glycoproteins (Swamy et al., 2001), it becomes evident that lectin-binding receptor could essentially contain this moiety. Preliminary characterization studies of the lectin-binding receptors after TLC using specific detection reagents indicate that the receptor RI and RII resemble phytoceramides reported in fungi (Dickson, 1998), in particular IPCs. The lectin receptor identified in S. rolfsii appears to be galactosyl ceramide. Based on our findings, we propose that the lectin expressed abundantly on the mycelia at the time of sclerotal body formation facilitates the aggregation of the mycelium by interacting with endogenous glycosyl ceramide receptor(s) having specific carbohydrate moiety. Furthermore, it appears that the lectin–receptor complex formed at the time of sclerotal body formation will remain intact during the dormant stage, and the complex would undergo changes to facilitate germination. In the near future these findings would shed light on the importance of fungal lectins as well the IPC receptors.

Materials and methods

Stock cultures of S. rolfsii were maintained on potato dextrose agar slants containing 5% dextrose. Cultures grown on Byrne'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 sclerotal 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 sclerotal bodies. Interaction of FITC anti-SRL on vegetative mycelia and sclerotal bodies was observed and photographed under fluorescence microscope (Carl Zeiss Jenalumar, model Fluoval2) 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 Byrne's mineral medium (100 ml) in different flasks by inoculating one sclerotal 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 sclerotal bodies. Sclerotal bodies formed during the course of development were harvested from each flask (in triplicate) by picking up manually from the culture broths on different days, washed thorougly with distilled water, and powdered after freeze drying. Dry powder of the sclerotal 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 after centrifugation at 8000 × g for 30 min was used for the determination of lectin activity. Lectin activity in the extracts of mycelia and sclerotal 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/sclerotal 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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and finally dried by flushing nitrogen and stored at 4°C. The resulting organic layer was washed twice with water and dried by rotary evaporation of the solvent. Total lipids were extracted from the powdered sclerotial bodies using 20 volumes (w/v) of chloroform/methanol (2:1). The extract was dried by rotary evaporation of the solvent 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.

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 lectin sorbent detection using biotinylated SRL. In principle, the method we adopted is similar to solid-phase methods, used to demonstrate binding of viruses and bacteria to and 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 polyethylene glycol (5 mg/ml) and incubated overnight at 4°C in a screw-capped vial. 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 a screw-capped vial. The plates were blocked with PBS to remove unbound lectin and treated with streptavidin horse-radish 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.

Analytical characterization of lectin binding receptors

Analytical characterization of lectin-binding receptors resolved on TLC plates was carried out using specific spray 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; IPFs, inositolphosphorylceramides; GIPCs, glycosylinositol phosphorylceramides; PBS, phosphate buffered saline; PNA, peanut agglutinin; SRL, Sclerotium rolfsii lectin; TLC, thin-layer chromatography.

References


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