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Surface Galactolipids of Wheat Protoplasts as Receptors for Soybean Agglutinin and Their Possible Relevance to Host-Parasite Interaction

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ABSTRACT

Soybean agglutinin, a lectin specific for *N*-acetyl-D-galactosamine and D-galactose, was previously shown to agglutinate wheat leaf protoplasts (Larkin 1978 Plant Physiol 61: 626–629). We investigated the receptors for soybean agglutinin on the plasma membrane of these protoplasts. After treatment of the protoplasts with galactose oxidase, they were no longer agglutinated by the lectin, whereas upon reduction of the galactose oxidase-treated protoplasts with sodium borohydride the susceptibility to agglutination was restored. Analysis of the glycolipids of protoplasts surface labeled by the galactose oxidase-borotritide method, revealed that the radioactivity was mainly present in monogalactosyldiglyceride and digalactosyldiglyceride. The same galactolipids were identified as the only receptors for soybean agglutinin by direct binding of the ^{125}I-labeled lectin to a thin layer chromatogram of the glycolipids of wheat leaf protoplasts.

The role of surface glycoconjugates in many cell recognition phenomena is well established. Glycoproteins and glycolipids are important functional molecules in a wide range of animal plasma membranes (7, 14, 24) but little is known about their location and function in higher plants (18, 22, 23). The participation of glycoconjugates in the control of host-parasite relationships has also been investigated (10). Preliminary studies have shown that treatment of wheat leaves with SBA,[2] a lectin specific for D-galactose, causes a marked delay in the resistance reaction against the wheat stem rust (*Puccinia graminis*) (Kogel, Schrenk, Sharon and Reisener, paper in preparation). Protoplasts are agglutinated by SBA, demonstrating that this lectin binds to the plasma membrane (13). It was therefore of interest to identify the receptors for SBA on the surface of wheat protoplasts.

MATERIALS AND METHODS

Materials. Wheat cultivar 'Little Club' was used. The grains were soaked for 1 d on wet filter paper, and then planted in soil and grown in a greenhouse. The plants were kept in the dark 1 d before harvesting.

Onozuka R-10 cellulase from *Trichoderma viride* (1.5 units/mg) and fluorescein diacetate were from Serva, NaB[^{3}H]$_4$ (14.9 mCi/mmol) was from the Radiochemical Center (Amersham). Galactose oxidase (46 units/mg), MGDG, DGDG, and hemoglobin (bovine 2 × crystallized) were purchased from Sigma. D-Galactose was from Pfanstiehl. Ficoll-Paque was from Pharmacia, and activated silicic acid (Unisil) from Clarkson Chemical Co., Williamsport, PA. SBA was a gift from Dr. Halina Lis (Department of Biophysics, The Weizmann Institute of Science); it was iodinated by the Bolton and Hunter method (3) and repurified by gel filtration using Sephadex G-50 (Pharmacia, Sweden) chromatography as described. Specific activity of the [^{125}I]SBA was 2 μCi/μg SBA. HPTLC plates precoated with Silica Gel 60 were from E. Merck, West Germany. TLC plates (13179 silica gel) were from Kodak, Rochester, NY. Poly(isobutylmethacrylate) was from Polysciences, Warrington, PA. All other reagents were analytically pure.

Preparation and Purification of Protoplasts. After removal of the lower epidermis, the primary leaves of 9-d-old plants were layered in Petri dishes to which was added isolation medium (6 ml) together with 1% Onozuka R-10 cellulase. Isolation medium was prepared according to Larkin (12): to 1 L of 0.5 M mannitol was added 150 mg $CaCl_2 \cdot 2H_2O$; 250 mg $MgSO_4 \cdot 7H_2O$; 100 mg KNO_3; 27.2 mg KH_2PO_4; 2.5 mg $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.16 mg KI; 0.025 mg $CuSO_4 \cdot 5H_2O$; the pH was adjusted to 6.0 with HCl.

After 3 h at room temperature, the undigested material was removed with tweezers. Two volumes of a crude protoplast suspension were layered on the top of one volume of Ficoll-Paque (12) in a 10-ml centrifuge tube. After spinning down at 100*g* for 5 min, the unbroken protoplasts were removed from the interface. Finally the protoplasts were washed three times with isolation medium by centrifugation at 100*g* for 5 min and gentle resuspension. The integrity of the protoplasts and absence of contaminating chloroplasts were verified by microscopic examination. Protoplasts were counted using a Thomas Chamber.

Viability Staining of Protoplasts. Staining of protoplasts was carried out with fluorescein diacetate according to the method of Widholm (27). After each labeling step, representative samples (about 200 cells) were counted.

Radioactive Labeling of the Surface of Protoplasts. *Pretreatment.* Protoplasts (10^5) were suspended in 0.5 ml of isolation medium containing 30 mM Hepes buffer (pH 7.2). One mM $NaBH_4$ (100 μl) in 1 mM NaOH was added. After 15 min, the $NaBH_4$/NaOH solution was removed by washing the protoplasts four times in isolation medium.

Galactose Oxidase Treatment (7, 15). Pretreated protoplasts (in 0.5 ml of isolation medium containing 30 mM Hepes buffer [pH 7.2]) were treated with 1 mg of galactose oxidase. Incubation was at 35°C for 1 h with gentle shaking. The protoplasts were

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[2] Abbreviations: SBA, soybean agglutinin; HPTLC, high-performance thin layer chromatography; MGDG, monogalactosyldiglyceride; DGDG, digalactosyldiglyceride; PBS, phosphate buffered saline; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine.

washed first with 0.1 M D-galactose (in isolation medium containing only 0.4 M mannitol) and then washed three times with isolation medium without added galactose.

Radioactive Labeling. The enzymically oxidized protoplasts were reduced for 30 min at room temperature with about 2.0 mCi $NaB[^3H]_4$ in 0.5 ml isolation medium containing 30 mM Hepes buffer (pH 7.2), and then washed four times with isolation medium.

Agglutination Test. Protoplasts (3×10^4) were suspended in 200 µl isolation medium containing 10 mM Hepes buffer (pH 7.2) and 50 µg/ml SBA and agglutination was followed under a microscope. Untreated protoplasts were completely agglutinated upon incubation for 10 min at room temperature. The agglutination was completely inhibited by inclusion of 0.1 M D-galactose in the medium.

Extraction of Glycolipids. Glycolipids were extracted according to Öquist and Liljenberg (21) with some modifications. Protoplasts (2×10^6) of eight wheat leaves were shaken extensively in a round-bottomed flask (100 ml) with a mixture containing 15 ml water and 60 ml chloroform:methanol (1:2, v/v) for 5 min. The mixture was filtered (Whatman filter paper 1) and then transferred to a separatory funnel. Water and chloroform were added to a final ratio of 1:1:0.9 (v/v) for chloroform: methanol:water. The mixture was allowed to stand until a biphasic system was obtained. The two phases were collected separately; the material from the organic phase was treated as described below.

Treatment of the Aqueous Phase. The material from the aqueous phase was precipitated with TCA (10% w/v) and the pellet was analyzed by PAGE. A resorcinol test for polar glycosphingolipids (26) was performed on an aliquot from the aqueous solution.

Separation of Glycolipids. The organic phase (total lipids) was evaporated, redissolved in 2 to 3 ml of chloroform, and applied to a column (10 × 1.5 cm) of silicic acid (2 g). Lipids with increasing polarity were eluted according to Öquist and Liljenberg (21) with minor modifications, as follows: (a) chloroform (about 150 ml) to remove Chl, and carotinoids; (b) acetone (20 ml) for elution of sulfolipids and glycolipids; (c) chloroform:methanol (1:47, v/v, 50 ml) for elution of phospholipids. The fractions were evaporated to dryness and analyzed by TLC.

Thin Layer Chromatography. The fractions were dissolved in chloroform and separated by TLC (1), using chloroform:methanol:water (60:30:5, v/v) as solvent. For HPTLC separation, the solvent used was chloroform:methanol:acetic acid:water (100:20:12:8, v/v). The plates were air-dried and the lipids were visualized by iodine vapors or other specific reagents (1). Alternatively, the plates were stained with anisaldehyde, to visualize the glycolipids (9). MGDG and DGDG were used as standards.

Detection of ^3H-labeled Glycolipids. The extraction and chromatography of glycolipids from labeled protoplasts was done as described above. To minimize losses of radioactivity during the purification steps, unlabeled protoplasts (1×10^6) were added to the labeled sample. After separation, the air-dried silica gel plates were sliced and the slices (0.3 cm) were placed in vials. To each vial was added 100 µl H_2O and 3.5 ml of a scintillation mixture consisting of 25% Lumax and 75% xylene. After shaking overnight, radioactivity was measured in a Packard counter. In a control experiment, equivalent areas were scratched off with a spatula and the silica gel was transferred to the vials and treated as above. The two techniques gave essentially the same results, so that the cutting procedure was routinely used.

Specific Binding of $[^{125}I]$SBA to Glycolipids. Binding of $[^{125}I]$ SBA to protoplast glycolipids separated by TLC was analyzed by an adaptation of the autoradiographic assay described by Mag-

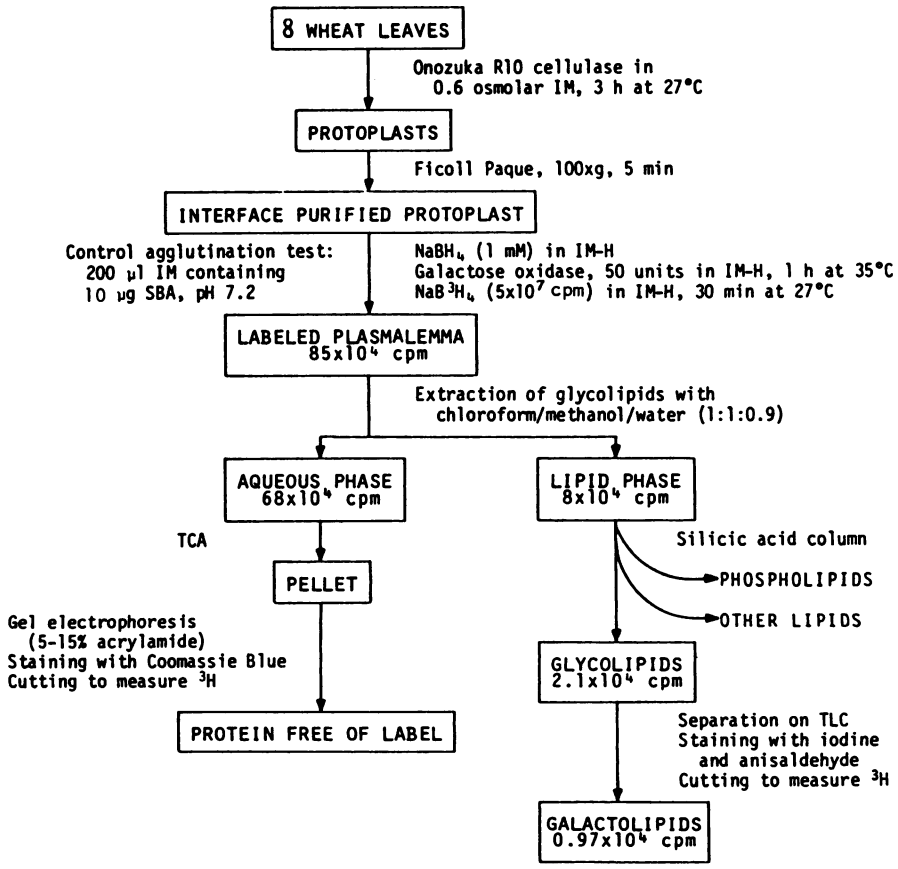

FIG. 1. Preparation of wheat leaf protoplasts, labeling of plasma membranes by galactose oxidase-$NaB[^3H]_4$ treatment, and identification of labeled compounds. IM, Isolation medium (see "Materials and Methods"); IM-H, isolation medium (0.5 ml) adjusted to pH 7.2 by addition of 20 mM Hepes buffer.

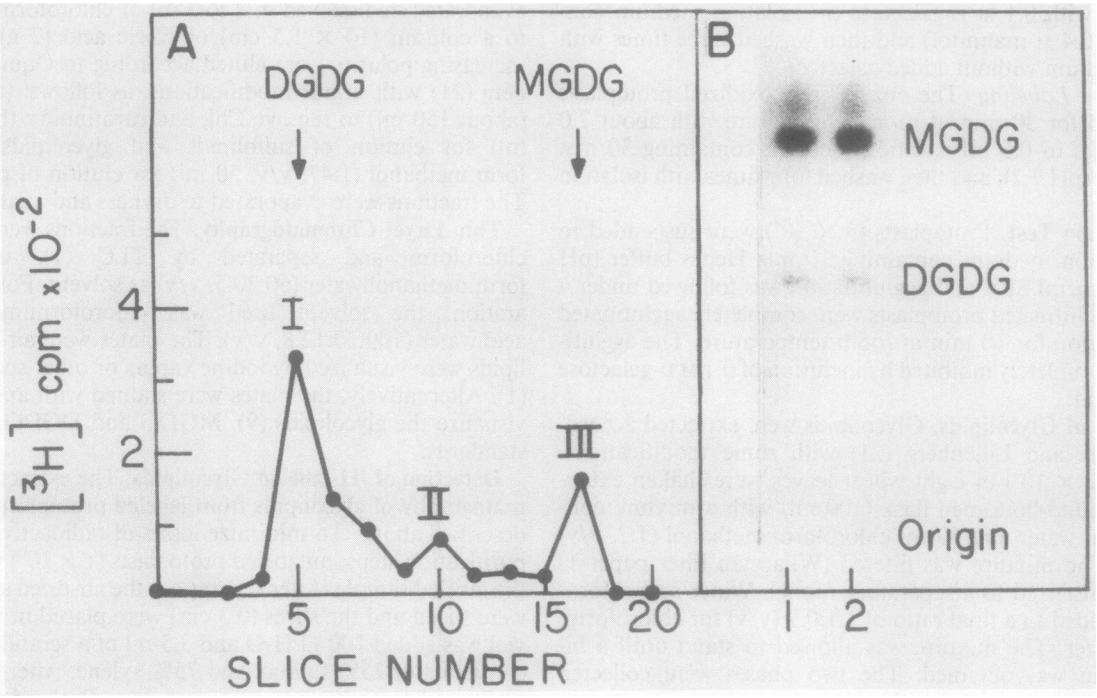

FIG. 2. TLC of glycolipids labeled by galactose oxidase and sodium borotritide method. A, Profile of radioactivity in the glycolipid fractions of ^{3}H-labeled protoplasts. Separation was done on TLC plates using chloroform:methanol:water (60:30:5, v/v). Each lane of the TLC was cut into slices of 3 mm width and the radioactivity measured. The small peak (II) was not identified. The two large peaks were identified as DGDG (I) and MGDG (III), by comparison of their migration rates with those of authentic standards. B, Visualization of glycolipids by iodine vapors. Lane 1, standards; lane 2, protoplast glycolipids.

nani *et al.* (16, 17). The procedure was used as follows: the glycolipids, after partial purification on the silicic acid column, were separated on HPTLC plates (3 × 7 cm) using chloroform:methanol:acetic acid:water (100:20:12:8, v/v) as solvents. The chromatograms were dried and later dipped for 1 min in a solution of poly(isobutylmethacrylate) (0.2% w/v in hexane). After air-drying for 2 min, the chromatograms were sprayed with PBS, immersed in a Petri dish containing 0.25% hemoglobin and 0.1% azide in PBS, and allowed to stand in this solution for 2 h at 4°C. The excess solution was drained, the plates were overlayed with [^{125}I]SBA in PBS (50 μl/cm^2, 2.0 × 10^6 cpm/ml), and incubated in a moist chamber for 16 h at 4°C. The unbound lectin was later removed by six successive dippings for 1 min each time in cold PBS. The plates were air-dried and exposed to x-ray film (Agfa-Gevaert Curix) at −70°C for 30 h.

Gel Electrophoresis. Gels were prepared according to Laemmli (11), using a polyacrylamide gradient from 5 to 15%. To the pelleted protoplasts was added immediately 0.5 ml PBS containing 0.1% SDS and 1% Triton X-100. After 2 h, sample buffer was added to give a final ratio of 3:1. Samples containing 30, 80, and 110 μg protein were placed on the gel and run for 5 h at 20 mamp. Alternatively, ^{3}H-labeled protoplasts (1 × 10^5 cpm) were applied and run under the same conditions. The gels were frozen, cut into 0.2 cm pieces, and the pieces transferred into vials. Solubilization was done by adding 200 μl Soluene 100 (Packard), followed by standing overnight. Finally, 4 ml of the scintillation mixture (Lumax S25, Lumac, Holland) was added and the radioactivity was counted.

For detection of galactoproteins, the unstained gels were incubated in the presence of [^{125}I]SBA (2 × 10^6 cpm/ml) according to Burridge (4). After washing, the gels were dried and exposed to Agfa-Geveart Curix x-ray film.

RESULTS

Since protoplasts are fragile, it was necessary to stabilize them. The isolation medium of Larkin (12) used in this study gave satisfactory results. About 90% of the protoplasts remained alive, as shown by the fluorescein diacetate test and microscopic examination. The stability of the protoplasts appears to depend upon the pretreatment of the leaves. Apparently, it was advantageous to keep the leaves in the dark 1 d prior to cutting. It should be noted that broken protoplasts were removed by repeating the purification steps described for the isolation of protoplasts following each step of the ^{3}H-labeling procedure. The absence of chloroplasts was examined microscopically and the viability of protoplasts was controlled after each purification step.

The preparation of the protoplasts, their labeling with NaB[^{3}H]$_4$, their isolation and the identification of labeled compounds, are summarized in Figure 1. Pretreatment of protoplasts with unlabeled $NaBH_4$ prior to treatment with galactose oxidase, resulted in the reduction of cell constituents which otherwise would interfere with the ^{3}H-labeling procedure. The distribution of radioactivity in the various fractions isolated from the [^{3}H]-labeled protoplasts after the galactose oxidase/NaB[^{3}H]$_4$ treatment is given in Figure 1.

In a control experiment, protoplasts were not treated by galactose oxidase but only with NaB[^{3}H]$_4$. Although radioactivity was incorporated into the protoplasts, after separation 83% was found in the aqueous phase and 8% in the lipid phase. Purification on silicic acid column left 1.3% of radioactivity in the acetone fraction but no counts were found upon analysis of the galactolipids after TLC separation and cutting (see "Materials and Methods"). We therefore concluded that this incorporation of radioactivity was nonspecific.

Treatment of protoplasts (5 × 10^4 in 0.5 ml isolation medium) with galactose oxidase (10 units) at 35°C for 1 h, inhibited their agglutination by SBA (50 μg/ml). Addition of $NaBH_4$ (100 μl of 1 mM) restored the agglutination almost completely (85% of protoplast agglutinated).

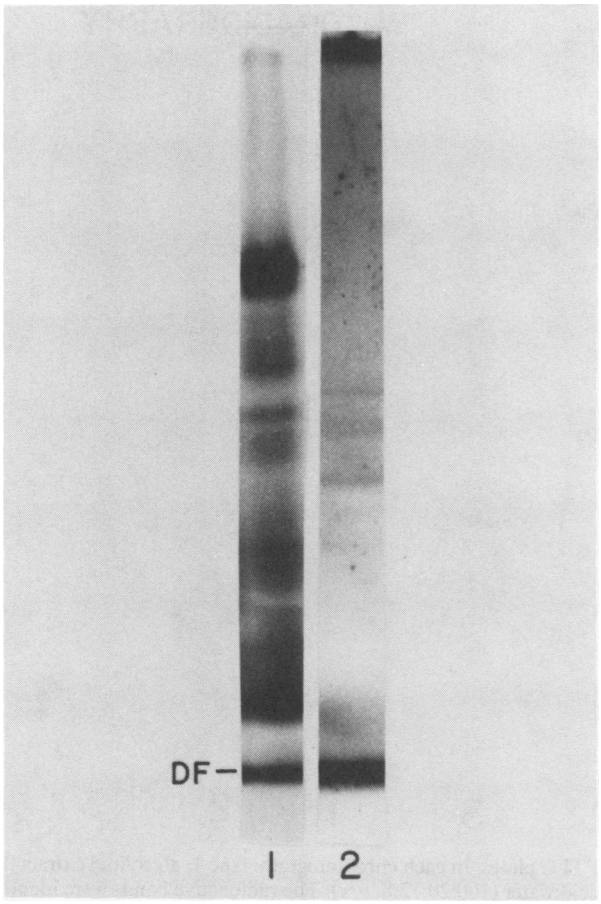

FIG. 3. SDS-PAGE of total protein extract of wheat leaf protoplasts. 1, Staining with Coomassie blue; 2, autoradiogram after staining with [^{125}I]SBA; DF, dye front.

The glycolipid phase of labeled protoplasts was analyzed by TLC and the results are presented in Figure 2. Two main radioactive peaks (Fig. 2A) were found; they were iodine positive (Fig. 2B) and had a mobility identical with MGDG and DGDG.

In the aqueous phase, no resorcinol-positive material (26) was found, which confirmed the absence of polar glycosphingolipids. Results of PAGE performed on the proteins of the TLC pellet of this phase are shown in Figure 3. Staining with Coomassie blue gave a distribution of protein bands over the whole range of mol wt and staining with [^{125}I]SBA gave some radioactive bands, which indicate the presence of galactoproteins in the total fraction of protoplasts. However, gel electrophoresis of the total protein fraction of galactose oxidase/NaB[^{3}H]$_4$-treated protoplasts did not reveal any radioactive bands, neither after slicing of the gels nor after fluorography, suggesting that galactoproteins are not present on the surface of the protoplasts.

Binding of protoplast glycolipids to [^{125}I]SBA was performed after their separation on HPTLC plates (see "Materials and Methods") (Fig. 4). The DGDG resolved into two bands after separation by HPTLC (Fig. 4), although separation by conventional TLC gave only one clear band (Fig. 2). In contrast, MGDG was revealed as one distinct band in both types of TLC (Figs. 2 and 4). This may be due to the greater heterogeneity in the fatty acid composition of DGDG than that of MGDG, as reported in the literature (20). Staining of glycolipid samples with anisaldehyde revealed a number of bands, whereas by iodine vapors mainly MGDG and DGDG were detected. Autoradiography (Fig. 4) performed after overlaying with [^{125}I]SBA clearly shows binding to MGDG and DGDG, thus confirming that these glycolipids are the only glycolipids on the surface of the protoplasts which contain the galactose moieties accessible to the lectin.

DISCUSSION

Several attempts have been made to determine the localization and the molecular function of genes for resistance and virulence in race-specific host-parasite interactions (10). Extensive genetic analyses of 'gene-for-gene' resistance in a large number of hosts have demonstrated that single genes can be responsible for the resistant phenotype. The genetic data are consistent with the hypothesis that incompatibility between host and parasite is the result of a protein-protein or protein-glycoconjugate interaction.

Recently there has been an intensified search for the products of some resistance genes (6). It was suggested that the products of these genes are membrane receptors (5).

Analyses of plasmalemma are not very numerous because this subcellular fraction is difficult to isolate from plant tissues (18). During grinding and homogenizaton of plant tissues, the plasmalemma is ruptured either into large fragments that co-sediment in the gradients with nuclei and mitochondria or into small vesicles that co-sediment with microsomes and vesicles of other organelles. As a result, isolated plasmalemma is always contaminated by other cell compounds. Our approach of investigating plasma membrane receptors by labeling the surface of protoplasts has the advantage of avoiding the problematic step of membrane isolation. However, it was of great importance to obtain intact protoplasts which were free from cellular debris. In our studies, particular attention was paid to prevent contamination by chloroplasts, since these organelles contain large amounts of MGDG and DGDG (8, 18). This was achieved by using a special isolation medium containing Hepes buffer and by purifying the protoplasts over Ficoll-Paque after every step of the labeling procedure. Our experiments have shown that treatment of the protoplast with galactose oxidase at 35°C for 1 h causes loss of their ability to be agglutinated by SBA, as was found earlier for human erythrocytes (15). These results indicated that the enzyme oxidized the Gal/GalNAc moieties of surface glycoconjugates of wheat protoplasts, because the modified sugar residues no longer exhibited affinity for SBA.

Galactose oxidase has a mol wt of around 76,000 (2) and it can be assumed that it does not easily penetrate the surface membrane of protoplasts. Therefore, only surface Gal/GalNAc moieties are oxidized by the enzyme (7, 25). On labeling of protoplasts with galactose oxidase/NaB[^{3}H]$_4$, the lipid phase contained two radioactive galactolipids, MGDG and DGDG, while no radioactivity was found in the control experiment.

Since either glycolipids or glycoproteins can serve as lectin receptors, analyses for both of these glycoconjugates were performed at different stages of the extraction. Galactoprotein autoradiography of the total extract of protoplasts revealed some positive bands upon staining of gel electrophoretograms with [^{125}I]SBA. However, no ^{3}H-labeled glycoproteins were found in the aqueous phase from the galactose oxidase/NaB[^{3}H]$_4$-treated protoplasts, which suggests the absence of galactoproteins from the surface of the protoplasts. Since staining with [^{125}I]SBA of gel electrophoretograms is more sensitive than fluorography of tritium-labeled compounds, a detailed study on galactoproteins in wheat protoplasts is being carried out.

Magnani *et al.* (16, 17) have reported an *in situ* binding assay in which the glycolipids are separated on TLC prior to incubation with a radiolabeled ligand. This method was used for identification of lectin-binding glycolipids in mammalian cells (19). This direct technique was adapted by us to demonstrate the specific binding of SBA to galactolipids in the extract of the wheat leaf protoplasts.

Our experiments show that only MGDG and DGDG, out of

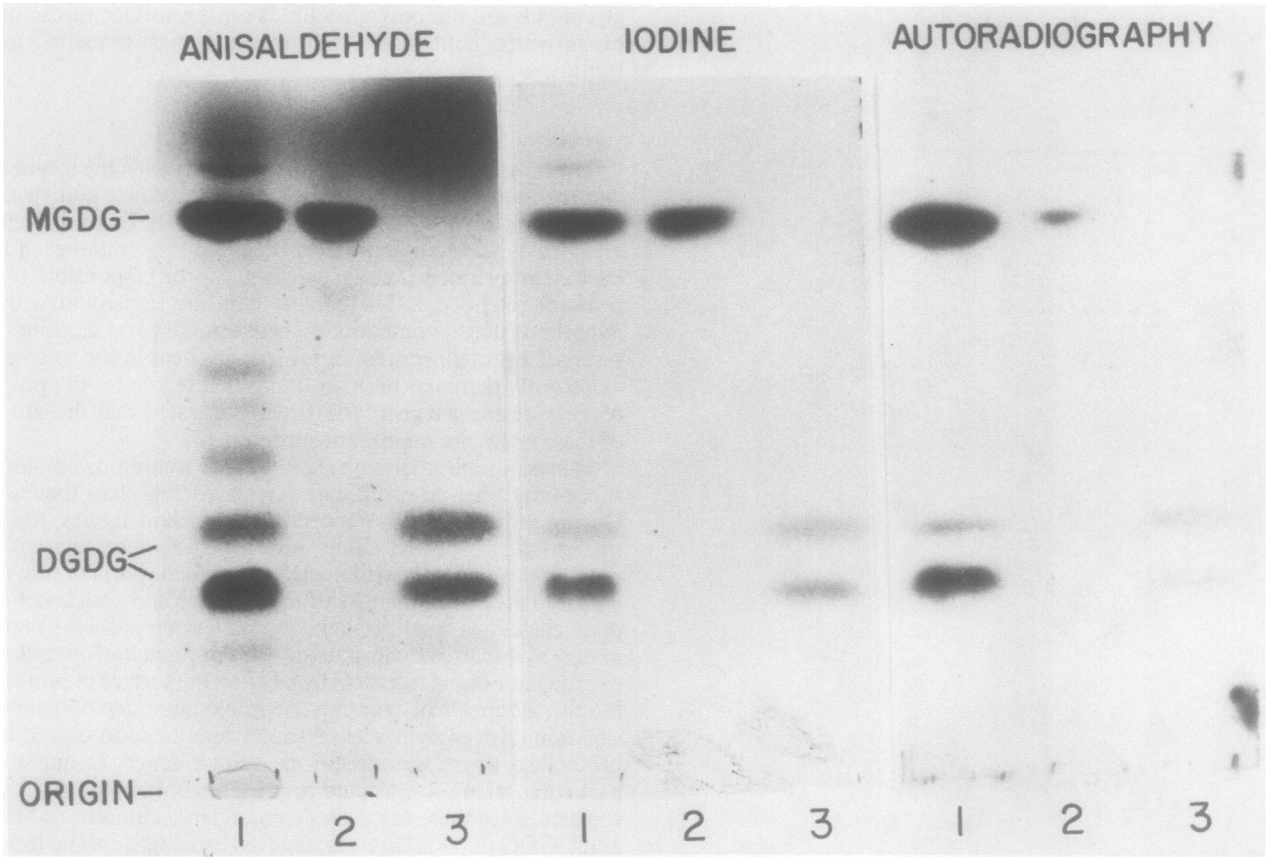

FIG. 4. Binding of [^{125}I]SBA to total lipid extract from wheat protoplasts on HPTLC plates. In each chromatogram: lane 1, glycolipid extract; lane 2, MDGD; lane 3, DGDG. The solvent system was chloroform:methanol:acetic acid:water (100:20:12:8, v/v). The radioactive bands were identified by comparison with parallel chromatograms stained by anisaldehyde and iodine.

all the glycolipids found in the extract of wheat leaf protoplasts, have the ability of binding to SBA. These findings are in full agreement with those obtained in the galactose oxidase/ NaB[^{3}H]$_4$ experiment, where the same galactolipids were ^{3}H-labeled, and thus confirm their presence on the membrane of wheat protoplasts. As wheat protoplasts bind SBA (13, see also "Materials and Methods") it can be concluded that the galactolipids found on the wheat plasma membrane are receptors for this lectin. Since preliminary studies have indicated that SBA interferes in a sugar-specific manner with the resistance of wheat against *Puccinia graminis*, it is possible that MGDG and DGDG are involved in the host-parasite interaction.

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