Presence of Trifoliin A, a Rhizobium-Binding Lectin, in Clover Root Exudate

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Trifoliin A, a Rhizobium-binding glycoprotein from white clover, was detected in sterile clover root exudate by a sensitive immunofluorescence assay employing encapsulated cells of Rhizobium trifolii 0403 heat-fixed to microscope slides. Its presence in root exudate was further examined by immunoaffinity chromatography. The binding of trifoliin A to cells was specifically inhibited by the hapten, 2-deoxyglucose. Significantly higher quantities of trifoliin A were detected in root exudate of seedlings grown hydroponically in nitrogen-free medium than in rooting medium containing 15 mM NO_3^-, a concentration which completely suppressed root hair infection by the nitrogen-fixing symbiont. The presence of trifoliin A in root exudate may make it possible for recognition processes to occur before the microsymbiont attaches to its plant host.

Trifoliin A (previously called trifoliin) is a glycoprotein lectin isolated from seeds and seedling roots of white clover [1]. Certain legume lectins, such as trifoliin A and soybean hemagglutinin, are likely candidates as cellular recognition molecules because they interact selectively with carbohydrate receptors on both their respective nitrogen-fixing rhizobial microsymbiont and root hairs of the host plant [1–4]. Root hairs are the differentiated epidermal cells of the host which are specifically infected by rhizobia.

The presence of lectins in the exudate of intact bean roots [5] raises the question of cellular recognition processes occurring in the rhizosphere before the bacterial symbiont physically contacts the root surface of the legume host. In this report, we document evidence for the presence of trifoliin A in the root exudate of axenically grown clover seedlings. In addition, we show that the levels of this lectin relative to total protein in root exudate are reduced when the roots are grown with NO_3^-, a fixed N ion known to suppress root hair infection and levels of trifoliin A on clover root surfaces.

MATERIALS AND METHODS
Cells and Materials

Rhizobium trifolii strain 0403 was obtained from the Rothamsted Collection, Harpenden, United Kingdom and maintained on Bishop's modified (BIII) agar [6]. Trifolium repens var. Louisiana Nolin (white clover) was obtained commercially. Trifoliin A from seeds, rabbit antiseed-trifoliin A, and immune IgG were prepared by procedures previously described [1, 6]. Fluorescein isothiocyanate (FITC) labeled γ-globulin of goat antirabbit γ-globulin and purified agar were purchased from Difco, Detroit, MI. Solutions were prepared in distilled–deionized water (18 Megohms · cm resistance).

Growth Conditions and Preparation of Clover Root Exudate

Seedling roots were grown in sterile chambers designed to collect root exudate free of contaminating seed coats, cotyledons, and seed exudates [7, 8]. Surface-sterilized seeds (1 gm/assembly) were embedded in nitrogen-free (–N) Fahraeus seedling medium [9] solidified with 1.3% (w/v) purified agar and germinated through stainless-steel wire mesh supports into 50 ml of Fahraeus medium as previously described [7, 8]. The plates were incubated in a growth chamber at 20–22°C, with a 14-hr photoperiod and a light intensity of 21,520 lux. In some cases, modified Fahraeus medium (+N) supplemented with 15 mM NO_3^- (as KNO_3) was used to embed the seeds and support growth of the roots. After 5 days of plant growth, the root exudate was collected, stirred gently for 10 min with acid-washed [1] insoluble polyvinylpyrrolidone (2% w/v), clarified by centrifugation at 4°C and 12,000g for 20 min, concentrated on ice to 4 ml by ultrafiltration through an Amicon PM 10 membrane, and used directly or lyophilized and stored at 4°C. Lyophilized root exudate was reconstituted in M buffer [1] containing 10 mM potassium phosphate, 140 mM NaCl, 1 mM $MgSO_4$, 0.15 mM $MnCl_2$, 0.5 mM $CaCl_2$, pH 7.2, and then clarified by centrifugation at 1,000g for 5 min before use.

Immunofluorescence Assay for Trifoliin A

Trifoliin A was detected by an immunofluorescence assay employing encapsulated cells of R trifolii 0403 heat-fixed to microscope slides. Cells were grown on BIII agar for 5 days at 30°C, conditions which optimize their reactivity with trifoliin A [10]. Cells were harvested in M buffer, centrifuged to wash and enrich for the subpopulation of fully encapsulated cells [1], and transferred to fluorescent antibody slides (Clay Adams, Parsippany, NJ). Slides were dried at room temperature, heat-fixed, rinsed gently with distilled–deionized water, and air-dried. These cells could be stored for several months in slide boxes before use.

Trifoliin A samples being assayed were serially diluted in M buffer, 10 μl was layered on fixed cells, and the slides were incubated in a moist chamber for 1 hr at room temperature, rinsed gently with M buffer followed by water, and air-dried. The smear was then incubated with rabbit antitrifoliin A antiserum (1 mg/ml) for 1 hr, rinsed with 10 mM potassium phosphate, pH 7.2, containing 145 mM NaCl and 1 mM $MgSO_4$ (PBS) followed by water, air-dried, and incubated for 1 hr with FITC goat antirabbit γ-globulin (Difco) diluted 1:5 with PBS. The smears were rinsed as before, air-dried, mounted in glycerol

(pH 9, Difco), and examined for immunofluorescence. Fluorescence microscopy was performed with a Zeiss Photomicroscope I equipped with a 100-W quartz–halogen epiilluminator, two KP 500 and one LP 455 exciter filters, BG 38 red filter, 510 reflector, and LP 528 barrier filter. Photomicrographs were exposed for 20 sec on Tri-X film (Kodak). An "immunofluorescent titer" was determined as the reciprocal of the highest dilution of trifoliin A which produced a positive immunofluorescence. No immunofluorescence of cells occurred with controls which substituted normal preimmune rabbit serum for antitrifoliin A, or if either trifoliin A, antitrifoliin A, or FITC goat antirabbit γ-globulin reagents were deleted from the sequence [1]. The limit of sensitivity of this assay, based on extrapolation from end point titers, is approximately 8 ng of trifoliin A protein as determined by the method of Lowry et al [11]. Hapten inhibition studies were performed by preincubating root exudate with sugars (30 mM) for 30 min prior to assay for trifoliin A.

Immunoaffinity Isolation of Trifoliin A From Root Exudate

Purified rabbit antitrifoliin A IgG was coupled to CNBr-activated Sepharose 4B (Sigma, St. Louis, MO) [12], packed in a column (0.4 × 2 cm), and stabilized with 3 bed vol of M buffer containing 0.5% (v/v) Tween 80 to prevent nonspecific binding of proteins to antibodies [12]. Reconstituted root exudate (40 μg protein/ml) was applied to the column and incubated for 30 min, and the column was then washed with M buffer to remove unbound material and collected in 1-ml fractions. Glycine/HCl buffer (0.1 M, pH 2.5) was then applied and effluent (0.8 ml) was collected in tubes containing 200 μl Tris/HCl (1 M, pH 7.4) for immediate neutralization.

RESULTS

Trifoliin A was detected in root exudate from clover plants grown in −N and +N medium. Encapsulated cells were uniformly fluorescent when incubated for 1 hr with root exudate and stained specifically for trifoliin A by indirect immunofluorescence (Fig. 1). Cells did not fluoresce if the hapten 2-deoxy-D-glucose was added to the root exudate before assaying for trifoliin A (photo not shown). Cells remained immunofluorescent if 2-deoxy-D-galactose or α-D-glucose were substituted. Thus, the binding of trifoliin A from the root exudate to the cells was hapten specific, as it could be inhibited by 2-deoxy-D-glucose, an effective hapten of trifoliin A [1, 6].

Significantly more trifoliin A binding activity was detected in root exudate of plants grown in −N medium than in +N medium (Table I). Since the total protein in −N and +N root exudate was adjusted to the same concentration, titers could be compared directly to examine "specific binding activity" as a relative measure of trifoliin A to total protein. The values from –N grown plants were significantly higher than from +N grown plants in the three separate lots of root exudate (Table I).

Trifoliin A in −N root exudate was examined further. One-fourth of the concentrated root exudate from seedlings produced from 1 gm of seed was applied to the immunoaffinity column. The material in root exudate which washed through the column in M buffer (peak 1 of Fig. 2) was negative in the

immunofluorescence assay for trifoliin A. Approximately 8–9 μg of bound protein from the root exudate was eluted from the matrix by a pH shift (peak 2 of Fig. 2). Therefore, 32–36 μg of trifoliin A protein can be obtained from the root exudate of T. repens var. Louisiana Nolin seedlings when grown under these N-free conditions. The extent to which this estimate varies with different growth conditions or varieties of clover was not examined.
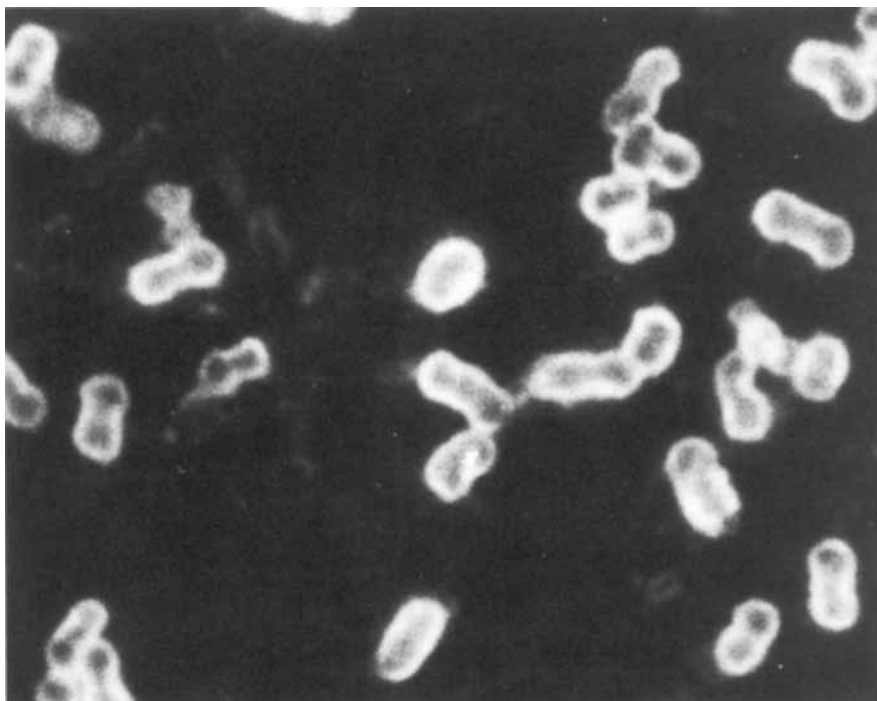

Fig. 1. Immunofluorescent detection of trifoliin A in clover root exudate which has bound to encapsulated cells of Rhizobium trifolii 0403. Fluorescence photomicrograph with antitrifoliin A antibody (9,900 ×).

TABLE 1. Effect of KNO₃ on the Accumulation of Trifoliin A in Clover Root Exudate*

Lot No.[a]	Root exudate protein concentration (μg/ml)[b]	Immunofluorescent titer in root exudate containing	
		No KNO$_3$	15 mM KNO$_3$
1	20	32	2
2	20	64	2
3	20	64	2

*Trifoliin A binding-activity was measured by indirect immunofluorescence on Rhizobium trifolii 0403 heat-fixed to slides.
[a] Lots 1 and 2 were concentrated by ultrafiltration, lyophilized, and reconstituted; lot 3 was used directly after concentration by ultrafiltration.
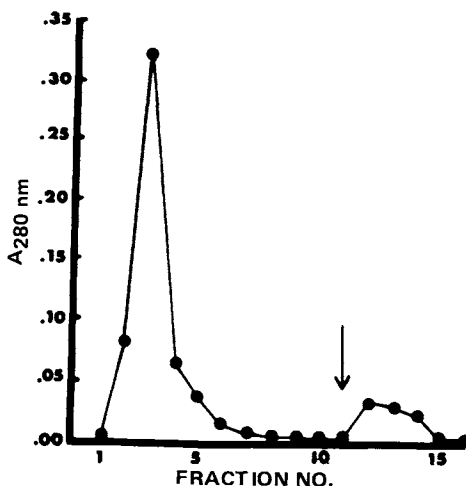[b] Measured by the Folin–phenol method of Lowry et al [11].

Fig. 2. Purification of trifoliin A in clover root exudate by immunoaffinity chromatography. Root exudate was applied to a column of antitrifoliin A IgG coupled to agarose and incubated for 30 min. The unbound material (peak 1) was eluted with M buffer. Trifoliin A was eluted when glycine/HCl buffer was applied to the column (arrow, peak 2) and collected in tubes containing 0.2 ml Tris/HCL buffer for immediate neutralization. Fractions collected in M buffer (peak 1) were 1 ml each; fractions eluted in glycine/HCl buffer (peak 2) were 0.8 ml each.

DISCUSSION

Previous work has shown that trifoliin A, a Rhizobium-recognition lectin, is present in seeds and on root surfaces of white clover [1]. Further evidence is presented here that additional trifoliin A accumulates in the growth medium of intact white clover roots. The mechanism of release of trifoliin A from roots is not known. Significantly higher quantities of trifoliin A were detected immunologically in root exudate of seedlings grown hydroponically in N-free medium than in medium containing 15 mM NO_3^-. Thus, NO_3^- suppresses the accumulation of trifoliin A in the root exudate and on the root surface [7].

There are some advantages of the immunofluorescence assay described here. This assay should theoretically detect monovalent lectin. It is at least 10 times more sensitive and requires less time and volume of sample than do the bacterial agglutination assays previously described [6, 8]. Fixed cells can be stored conveniently on slides and specificity of the antiserum can be easily established [1]. Most importantly, this assay allows direct observation and photographic documentation of the interaction between lectin and receptor sites on symbiotic rhizobial cells.

The presence of trifoliin A in clover root exudate which can bind to receptors on R trifolii provides supporting evidence for a lectin recognition model proposed by Solheim [13]. According to this model, a glycoprotein lectin excreted from the legume root binds to the rhizobia. This active complex then combines with a receptor site on the root. Thus, both partners in the symbiosis could benefit from the discriminatory reaction of a crossbridging lectin which could be either bound to a glycosylated receptor on the root hair cell wall [14]

or released from the root to bind to the rhizobial cell [13]. This event would help to ensure that only the symbiotic bacterium could establish the proper intimate contact required to trigger other recognition events that lead to successful infection. Fixed N (e.g., NO_3^-) would play a role in regulating the recognition process as proposed by Solheim [13].

ACKNOWLEDGMENTS

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