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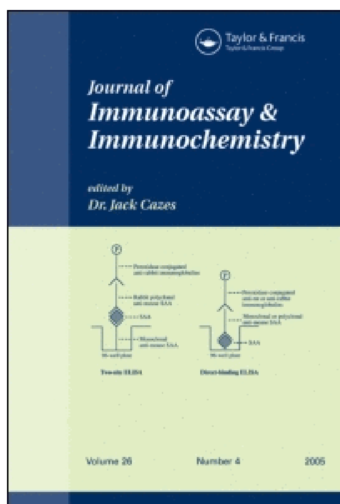

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Use of the Dot-Blot Immunogold Assay to Identify a Proliferative Antigen in the Initial Cells of a Wheat Stem Meristem

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USE OF THE DOT-BLOT IMMUNOGOLD ASSAY TO IDENTIFY A PROLIFERATIVE ANTIGEN IN THE INITIAL CELLS OF A WHEAT STEM MERISTEM

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ABSTRACT

We have devised a protocol for the isolation and identification of a proliferative antigen of the initial cells of wheat stem meristems (termed PAI). We have carried out a variety of immunochemical and immunocytochemical methods, using colloidal gold (CG) complexed with monospecific antibodies to PAI as the marker for the detection of PAI. We have been able to determine the effectiveness of immunoaffinity chromatography in isolating PAI from plant tissues and have shown the advantages of CG over enzyme labels for identification of the antigen. Finally, we have obtained a purified preparation of PAI and have determined its molecular mass (~83 kDa). (KEY WORDS: Immunogold Labeling; Immunoperoxidase Staining; Dot-Blot Immunoassay; Stem Meristem; Proliferative Antigen; Initial Cells).

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INTRODUCTION

An antigen (Ag) which is characteristic of actively dividing cells has previously been found in the apical meristem of the wheat stem (1, 2). The Ag has been termed a 'proliferative antigen of initials' (PAI). Using an immunochemical assay system (1), we have shown that the cellular PAI level in the stem apex is correlated with growth processes in the entire plant and reflects the functional activity of meristematic cells during the post-stress repair (2). Up to now, however, the reliable identification of PAI and its obtainment in ample quantity for preparative purposes have been hampered by the general difficulty of isolating individual Ags from plant cells.

This paper communicates the use of the dot-blot immunogold assay to refine present recipes for the isolation and identification of PAI.

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MATERIALS AND METHODS

Plant Materials

The shoot apical meristem and the youngest leaf of seedlings of wheat (*Triticum aestivum* L.) were used. The total meristem protein fraction was obtained by extracting the homogenized tissue for 1 h at 4°C in 50 mM Tris-HCl buffer of pH 7.8, which contained 0.02 M 2-mercaptoethanol and 0.001 M phenylmethylsulfonyl fluoride. The resultant extract was centrifuged at 20,000 *g* for 30 min, and the supernatant containing total water-soluble proteins was subjected to differential salting out with ammonium sulfate and used for subsequent immunization.

Preparation of Antisera

Antiserum to the total water-soluble proteins from the wheat stem apices was developed in rabbits by giving lymph node injections as described (3). Antibodies (Abs) with narrow specificity were prepared by depleting the initial antiserum with differentiated-leaf Ags. The efficiency of the purification was evaluated by double radial immunodiffusion in agarose gel, by using PAI as the Ag.

The resulting precipitate, after removal of unbound Ags and Abs by electroelution, was cut out, homogenized, and used for a second rabbit immunization, which was carried out in an identical manner. An immunoglobulin fraction was isolated from the resultant serum by using caprylic acid (4). The purified monospecific Abs were conjugated to colloidal gold (CG) (see below).

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Immunoaffinity Chromatography

Sepharose CL-6B (LKB-Pharmacia, Sweden) was activated with 1,1'-carbonyldiimidazole (5), by using Abs against PAI as the ligand. The water-soluble protein fraction from the wheat stem apices, which was obtained after salting out with ammonium sulfate, was applied onto an affinity column for 12 h under continuous circulation. Elution of PAI was carried out with 0.1 M glycine buffer of pH 2.0. The resultant protein was precipitated with a 10% trichloroacetic acid solution and studied by disk electrophoresis in polyacrylamide gel.

Polyacrylamide Gel Electrophoresis and Blotting Analysis

Proteins were subjected to electrophoresis according to Laemmli (6) in a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate.

Semi-dry electroblotting was carried out as described (7) for 1 h at a constant current of 0.8 mA cm^{-2}. Protein transfer to a BA-85 nitrocellulose membrane (Bio-Rad, USA) was performed from both unstained and Coomassie brilliant blue R 250-stained gels (8).

Preparation of Immunogold Markers

CG (mean particle size, 20 nm) was synthesized according to Frens (9) by reducing tetrachloroauric acid (Aldrich, USA) with sodium citrate (Fluka, Switzerland). Conjugation of CG to the Abs obtained against PAI was accomplished at pH 9.0, with polyethylene glycol (M_r 20 000) as a secondary stabilizer (10).

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Dot-Blot Immunoassay

The direct dot-blot assay was carried out on nitrocellulose membranes (0.45 μm pore size; BioRad, USA), as described (11-13). To block sites of nonspecific adsorption, the filters, after sample application, were incubated for 30 min at room temperature in a blocking buffer of 1% bovine serum albumin (Sigma, USA), 150 mM NaCl, and 20 mM Tris-HCl; pH 8.2. Two methods were employed for detection of PAI: the most-commonly-used horseradish-peroxidase (HRP)-based enzyme-immunoassay and the dot-blot immunogold assay as an alternative.

RESULTS

Following the method described above, we obtained a crude extract of soluble proteins from the apical meristematic tissues of wheat. Figure 1A depicts the electrophoretic pattern for these proteins, isolated after the differential salting out

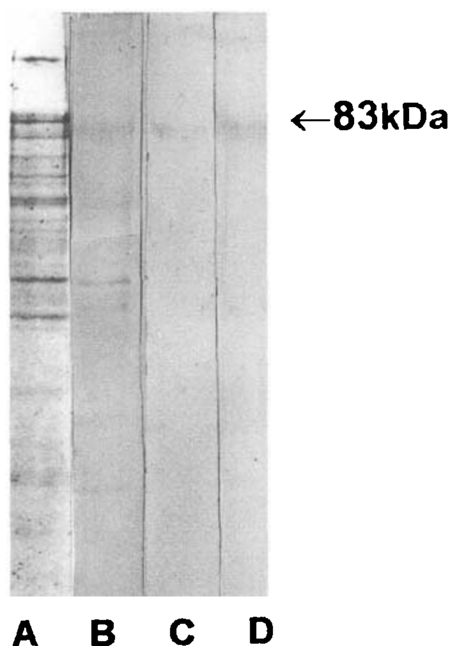

Figure 1. Immunochemical identification of PAI in wheat stem meristems:
A: The electrophoretic pattern of a crude extract of the meristem proteins after salting out with ammonium sulfate.
B: Blot assay of PAI in a crude extract of the meristem proteins, by using a HRP-labeled monospecific antiserum.
C: Blot assay of PAI in a crude extract of the meristem proteins, by using a CG-labeled monospecific antiserum.
D: Blot assay of the Ag, eluted from the affinity column, by using CG-labeled Abs against PAI.

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with ammonium sulfate. PAI was visualized by means of a blot assay using the monospecific antiserum from the second immunization with the immunoprecipitate from the serum with narrow specificity to PAI.

Using HRP-labeled Abs against PAI, we detected a series of protein bands on the nitrocellulose replicas (Figure 1B). Yet, immunogold-blot assay with CG coupled to

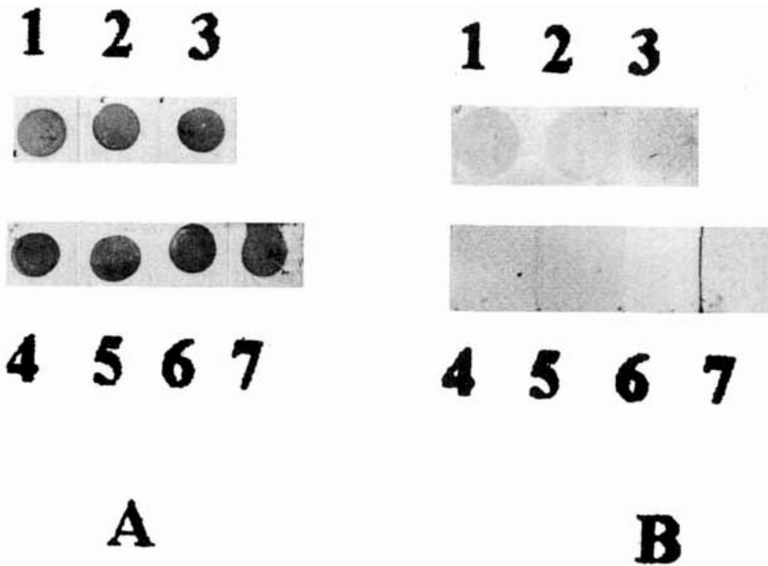

Figure 2. Use of Abs conjugated to HRP (A) and CG (B) to identify PAI by dot assay in various tissues and organs of wheat: seed embryo (1), stem apex (2), callus (3), mature leaf (4), stem (5), root (6), and endosperm (7).

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the same Abs showed that, out of the entire pattern for the total meristem protein fraction, this marker stained only one band, corresponding to a polypeptide of approximately 83 kDa (Figure 1C). A protein with the same electrophoretic mobility was eluted by us from the affinity column. Figure 1D depicts the interaction outcome between this protein and the CG-labeled Abs against PAI in the blot assay.

The dot assay method was exploited further to identify PAI in a variety of tissues and organs of wheat. Figure 2A depicts the results of a dot-ELISA with HRP-labeled Abs against PAI. As viewed in this figure, the Ab-HRP conjugate interacted with the Ag from both proliferous (stem apex, seed embryo, and callus) and

differentiated tissue cells of the root, leaf, and stem of wheat. To evaluate the interaction specificity of the immunoenzyme marker, two controls were performed: (i) by using a non immune serum and (ii) by omitting Abs. A positive response was obtained in either case. However, after the membrane had been pretreated with a solution of 0.3% hydrogen peroxide and 0.1% sodium azide (14) to block endogenous peroxidase activity, no specific or nonspecific staining was observed.

Alternatively, after the immunogold staining of the test samples, a positive response was obtained only during interaction of the marker with the Ag from the proliferous cells (Figure 2B) (in which PAI had, in fact, been identified originally (1, 2)).

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DISCUSSION

The protocol devised in this study for the isolation of PAI from wheat stem meristems, which involves extraction of soluble proteins, differential salting out, and affinity chromatography, allowed us to isolate this Ag from the total protein fraction and to determine its molecular mass, which is approximately 83 kDa.

The HRP-labeled Abs to PAI were of no value in the direct immunoenzyme dot-blot assay because of the nonspecific staining of the plant material. We believe that this staining may have come from the presence of endogenous peroxidases in the plant tissue extract (15), which is what has produced several bands (Figure 1B). Use of the previously suggested means of blocking peroxidase activity with hydrogen peroxide and sodium azide (which is known as being the mildest toward antigenic determinants and the nitrocellulose membrane) (14) did not have any positive effect

since it led to the complete disappearance of staining. Such treatment might have led in our case to substantial alterations in the antigenic structure of PAI.

We succeeded in obviating these problems by using, as the marker, CG conjugated to the monospecific Abs raised by us against PAI (the direct labeling variation). Furthermore, based on our observations and reports in the literature (16, 17), we conclude that, in terms of sensitivity, simplicity, rapidity, and economy, the dot-blot immunogold assay gives superior results as compared with the enzyme-immunoassay method.

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