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Monitoring of Germin Synthesis by Pre–Absorbed Anti–Germin Serum

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Abstract: The transition of a quiescent plant embryo into a vigorously growing embryo revealed some nascent synthesis of gene products, among them germin has been characterized. Germin is a water soluble homopentameric glycoprotein whose synthesis and accumulation appear to be closely related to early wheat embryo development.

Germin synthesis was monitored by employing an anti–germin serum which had been pre–adsorbed with wheat extract and optimized in order to decrease nonspecific binding. Germin accumulation was shown to occur in germinating embryos from 16h up to 6 day post–imbibition. Germin as a rare protein in wheat seedlings was detected as a dark band on X–rays in amounts as small as 20 µl protein extracts. Neither germin in mature wheat plant organs nor germinated embryos were detected prior to 16 hours of imbibition.

Key Words: Early Wheat development, Germin, Western blotting.

Germin Sentezinin Spesiyalitesi Artırılmış Germin Antikorları ile İzlenmesi

Özet: Bitki embriyoları dormant halden aktif bir büyüme dönemine girdikleri zaman, bazı yeni gen ürünleri sentezlerler. Bu ürünlerden germin proteini karakterize edilmiştir. Germin, suda çözünen, homopentamerik bir glikoproteindir. GermiN sentezi ve birikimi buğday embriyosunun ilk büyüme evreleri ile yakından ilgilidir.

Germin sentezi spesifik olmayan bağlanmaları önlemek için buğday–ekstraktı ile on muamele edilmiş anti–germin antikoru kullanılarak analiz edilmiştir. Germin proteini 16 saat ile 6 gün arası tüm filizlenen buğday embriyolarında bulunmuştur. Germin az bulunan bir protein olmasına rağmen, 20 µL protein ekstraktında bile X–Ray filmi üzerinde koyu bir band olarak antikorlar tarafından belirlenmiştir. Germin olgun buğday bitkisi organlarında ve 16 saatten önceki filizlenen embriyolarda bulunamamıştır.

Anahtar Sözcükler: İlk buğday gelişimi, Germin, Western blotting.

Introduction

Germination is a critical period in plant development in which the rapid growth of the embryo is driven by water uptake. The water content of a mature wheat embryo in an ungerminated grain is less than 5%; upon germination it rises to about 60% in less than 1 hour. between 1 and 5 hours of imbibition there is no further increase in fresh mass or water content. A “secondary water uptake” phase then raises the water content from 60% to 85% by 24 hours post–imbibition (1). Biochemical analysis of wheat embryo germination indicates that there is

only a limited accumulation of new gene products during germination (2) and until recently, only germin had been observed to signal the onset of early plant development (3). Germin is a water–soluble pepsin–resistant homopentameric glycoprotein whose monomer molecular mass is ~ 25 kDa and oligomer molecular mass is ~ 125 kDa (4). Germin was first detected in germinating cereals (5), but subsequently, germin–like proteins were also identified in a protist (6), dicotyledonous angiosperms (7–9), and gymnosperms (10).

The germination–related "germins" of cereal embryos have been found to have oxalate oxidase activity (EC 1.3.3.4)–an activity which utilizes oxalate to generate hydrogen peroxide (11, 12). The cereal proteins differ from those identified, by DNA sequence homology, in dicotyledonous plants, both in their physicochemical and their enzymatic properties. Whereas the cereal "germins" are physically resilient, exhibiting extreme resistance to proteolysis, a high degree of thermostability and retention of both their oligomeric structure and enzymatic activity in the presence of SDS (11, 13), their dioctyledonous counterparts do not appear to share these properties.

A full understanding of the contribution of germin synthesis and activity to the processes which occur during the initial burst of growth that comprises wheat embryo germination requires a greater knowledge of the quantitative and temporal specificity of germin expression. To this end, we undertook an analysis of germin accumulation in germinating embryos by employing an anti–germin serum.

Materials and Methods

Plant Material

Grains of spring wheat (*Triticum aestivum* L. var. Tonic) were obtained from Kenneth Wilson Grain, Leeds, UK. Dry grains were surface sterilised by incubation in a 10% solution of domestic bleach (ca. 1% free Cl_2) and five washes with sterile distilled water. Grains were germinated by incubation on two layers of water–soaked Whatmann 3MM chromatography paper at 25°C.

Pre–adsorption of Anti–germin Serum with Wheat Germ Protein Extract

Anti–germin serum was pre–adsorbed with wheat germ extract (which lacks germin) in order to increase its specificity. The following protocol was used: 5g wheat germ was homogenised in 10 ml extraction buffer (50 mM KCl, 5 mM $MgCl_2$, 5mM β–Mercaptoethanol and 25 mM Tris–HCl pH: 7.5) and the extract was centrifuged at 12000 x g for 10 minutes. The supernatant was recovered and recentrifuged at 12000 x g for 10 minutes. The second supernatant was collected and a nitrocellulose membrane (9cm x 9cm) was shaken in this solution for 20 minutes at room temperature. Wheat–germ proteins were allowed to dry on the membrane. Meanwhile 50 µl crude antiserum was diluted 1/100 with PSB/0.3% Tween 20. Nonspecific binding sites on the membrane were blocked with 5% milk powder/PBS–0.3% Tween 20 for at least 2 hours. The membrane was washed with PBS/0.3% Tween 20 for 3x10 minutes. The membrane was incubated with diluted antiserum for 2 hours, following which the antiserum was collected and stored at–80°C.

Western Blotting of Protein Gels

The preparation of soluble proteins from wheat embryos was denatured by boiling in SDS buffer and loaded onto a 12% polyacrylamide gel. Resolution of proteins was carried out according to the method described by Laemmli (14) at 40 mA for 4 hours. After separation by electrophoresis, proteins were transferred onto a nitrocellulose membrane by Western blotting in a semi–dry transfer apparatus as explained by Towbin et al. (15). The transfer membrane was blocked in PBS solution containing 5% (w/v) milk powder, overnight. Following washing in PBS, the membrane was incubated for one hour in a 1/4000 diluted anti–germin antibody. The membrane was then washed several times in PBS solution prior to incubation with horseradish peroxidase–linked goat anti–rabbit antiserum which was diluted 1/2000 with PBS for one hour. Following this incubation the membrane was thoroughly washed with PBS. The position of germin proteins was identified by the Enhanced Chemiluminesence system (ECL, Amersham UK) as described by the suppliers.

Results and Discussion

Pre–adsorption of Anti–germin Serum and Monitoring the Purification Process

Anti–germin serum (raised in rabbits) was provided by Prof. B.G. Lane (University of Toronto) as a crude serum preparation containing polyclonal antibodies. It was found in preliminary experiments that this preparation was far from monospecific. In a Western blot carried out to identify germin protein in homogenates from 48 hour wheat seedlings, the crude anti–germin serum was used and the result showed the anti–germin serum bound to a broad spectrum of embryonic polypeptides (Fig. 1–A). This lack of specificity may relate to the nature of the germin protein used as the immunizing antigen. Germin is known to be a glycoprotein and also to be closely associated with adventitious (non–covalently–bound) carbohydrate species–chiefly GGAX (16). Carbohydrate side–chains of plant glycoproteins are known to be highly immunogenic [for example many of the well characterised "JIM–series" monoclonal antibodies used to identify cell–specific markers during plant development (17, 18) recognise the glycoside moieties of glycoproteins, rather than their apo–protein components] and it is probable that the anti–germin serum contains many antibodies recognising carbohydrate groups which are also found in a range of other cereal glycoproteins.

In order to overcome the problem posed by nonspecificity, the crude serum was pre–adsorbed with a preparation of proteins from dry wheat embryos, immobilised on a nitrocellulose filter. It is known that the population of proteins present in dry (ungerminated) embryos is virtually identical with that present in germinated embryos, but that one of the principal differences is that germin, present in germinated embryos, is not present in ungerminated embryos. It was reasoned that the bulk, if not all of the nonspecific antibodies in the crude serum would thus become bound to immobilised antigens in the dry embryo preparation, leaving behind a selection of antibodies highly enriched for the germin protein itself. When the Western blot was repeated for a protein homogenate from 48h germinated wheat seedlings using pre–adsorbed anti–germin, a monospecific reaction was obtained (Fig.

1–B). It is clear, therefore, that pre–adsorption is a very efficient method to remove irrelevant antibodies which cause background "noise".

The dilution factor of the antiserum is another contributor to background "noise". It is likely that a high concentration of antiserum will increase the possibility of nonspecific binding. An experiment was designed to indicate the relationship between pre–adsorption and dilution factor with background. As seen in Fig. 2–C, the crude antiserum exhibited a high degree of nonspecific binding, even at a high dilution factor (1/4000). However, the pre–adsorbed antiserum gave a very specific reaction, although some nonspecific binding was apparent at the lower dilution factor of 1/2000 (Fig. 2–B and A). Clearly, pre–adsorption and the appropriate dilution factor eliminated nonspecific binding and achieved monospecific binding of crude polyclonal antibodies.

Quantitative Detection of Germin

The germin is a rare germination related protein which accounts for about 0.1% of the total protein in a soluble extract of germinated wheat embryos and it cannot be detected by dye staining (Coomassie blue) after SDS–PAGE separation of the proteins (5). In order to obtain quantitative detection of germin, different amounts of protein extracts from 48 h germinated wheat embryos (100 µg, 50 µ g and 20 µg) were loaded on an SDS–PAGE gel and analyzed by Western blotting using pre–adsorbed anti–germin serum. The result revealed that germin accumulation was easily detectable in 100 µg and 50 µg total protein extracts from 48 hour germinated wheat embryos with Western blotting (Figs 3–1 and 2). Furthermore, germin

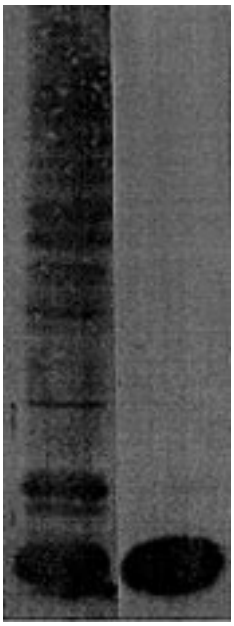

Figure 1. Application of crude and pre–adsorbed anti–germin: Protein extracts from 48h germinated wheat seedlings were resolved by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane and then reacted with crude (A) and pre–adsorbed (B) anti–germin serum.

acumulation was sufficiently abundant to be determined in amounts as small as 20 µg protein extracts (Fig. 3–3).

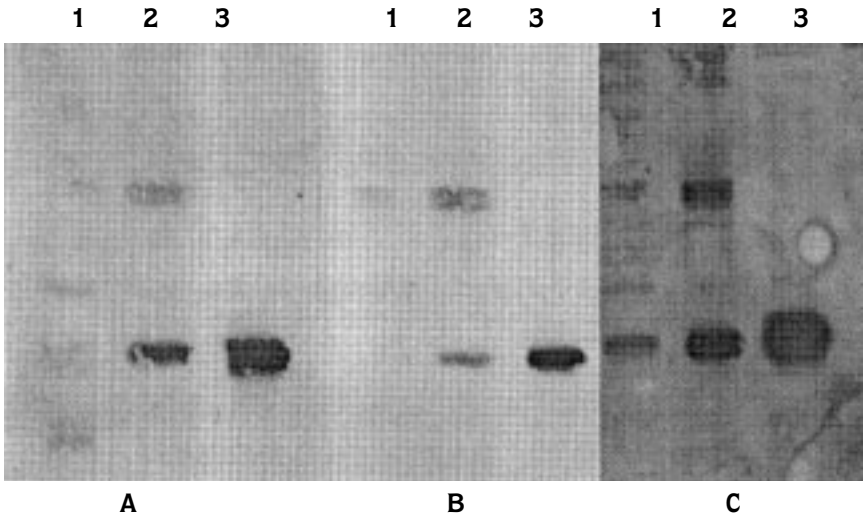

Figure 2. Optimization of anti–germin serum: Following SDS–PAGE, the nitrocellulose membrane was treated with: A: Pre–adsorbed anti–germin serum (1/2000 diluted). B: Pre–adsorbed anti–germin serum (1/4000 diluted). C: Crude anti–germin serum (1/4000 diluted).
Lane 1– Protein extracts from ungerminated, mature embryos, Lane 2– Protein extracts from 24h germinated embryos and lane 3– Protein extracts from 48h germinated embryos.

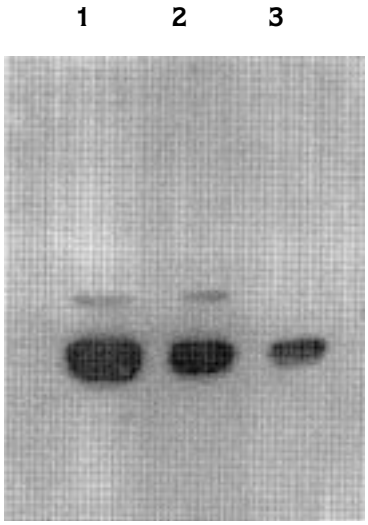

Figure 3. Quantitative detection of germin: The quantitative relationsihp between signal intensity and germin protein was demonstrated with (1) 100µg, (2) 50µg, (3) 20µg protein extracts from 48h germinated wheat seedlings for electroblotting using pre–adsorbed anti–germin serum.

Temporal Accumulation of Germin during Germination

Germination is a critical period in plant development and in wheat it represents a transition from a quiescent, dry embryo to a vigorously growing seedling. Germination is triggered by massive water uptake by the mature embryos. The appearance of germin mRNA is coincident with this period of water uptake and the accumulation of germin reaches its highest level between 24 and 48 hours post–imbibition (19). Further investigation of the temporal induction of germin, with gel blot analsis carried out with embryos at different stages of development: 6, 16, 24, 48 hours, 6 days post–imbibition and in mature tissues (stem, flowers and leaves). While germin was not detected in mature organs and 6 hour–germinated embryo protein extracts, it was detected in extracts of 16, 24, 48 hour–germinated embryos and of 6 day–old seedlings (Fig. 4). This correlates well with the results of Lane et al. (19) who observed germin accumulation apparent at ca. 15 hours post–imbibition with maximal accumulation occuring when the growth and development of embryos was distinguished by obvious cell enlargement (20).

As a result, crude polyclonal antigermin serum which caused nonspecific binding was optimized for Western blotting analysis of germin. The germin temporal and quantitative accumulation in wheat was determined with this pre–adsorbed antiserum. At this point we do not have sufficient data to suggest a possible function for germin, apart from saying that it is involved in early wheat development. However, determining the cellular and tissue specificity of germin by employing pre–adsorbed anti–germin antibody could give further clues about possible functions of germin in early wheat development.

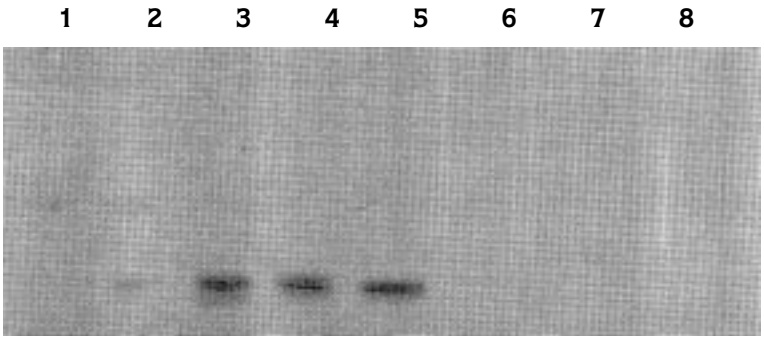

Figure 4. Temporal accumulation of germin: Protein extracts from (1) 6h, (2) 16h, (3) 24h germinated embryos, (4) 48h germinated wheat seedlings, (5) 6 day germinated wheat seedlings, (6) mature leaves, (7) mature flowers and (8) mature stem were resolved by SDS–PAGE and germin accumulation was determined by electroblotting with pre–adsorbed anti–germin serum.

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