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Temporal and Spatial Determination of Germin Biosynthesis in Wheat Tissues

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Abstract: In the current study, the biosynthesis of germin was determined in the organs of wheat embryos during germination. The temporal and spatial determination of germin protein was carried out by employing an anti-germin sera in gel-blot analysis. Germin was detected in the coleoptile, roots and vascular transition region of and 6-day-old germinated wheat seedlings. It was shown that the leaf tissue acquires germin accumulation only at the late stage of germination. The pellet fraction of protein homogenate obtained from embryos at different ages revealed higher germin accumulation than the supernatant fraction from the same sources.

Key Words: Early wheat embryo development, Germin, Western blotting.

Germin Biyosentezinin Buğday Dokularında Yersel ve Zamansal Tespiti

Özet: Bu çalışmada germin proteininin çimlenen buğday embriyo organlarındaki biosentezi belirlenmiştir. Germin protein sentezinin yersel ve zamansal analizi germin antikor serumu kullanılarak gel-blot tekniği ile belirlenmiştir. Germin proteini 2, 3, ve 6 gün süreyle çimlendirilmiş olan buğday fidelerinin koleoriza, koleoptile ve kök bölgelerinde belirlenmiştir. Detaylı çalışmalar germin protein sentezinin yaprak dokusunda ancak çimlenmenin ileri devresinde olduğunu göstermiştir. Değişik yaşlardaki embriyolardan alınan protein homowenatları içinde pellet fraksiyonlarının supernatant fraksiyonlarına göre daha çok germin içerdiği belirlenmiştir.

Anahtar Sözcükler: Erken buğday embriyo gelişimi, Germin, Western blotting.

Introduction

In plants embryogenesis is a developmental stage which covers the time period beginning with the formation of the zygote and ending with the formation of a mature embryo within a seed. When the mature embryo, which is a structurally and functionally organised miniature of the adult plant, is provided with ample water and oxygen at an appropriate temperature, water is taken up by imbibition to initiate germination. Upon germination an embryo in a quiescent stage turns into a very active stage of development which is characterised by vigorous metabolic processes. In quiescent seeds, the germination period comprise; three stages: (i) activation of metabolic processes (ii) preparation for elongation, and (iii) preparation for seedling growth (1). The boundary between stages (i) and (ii) is the termination of so-called phyical imbibition, whereas the seedling emergence (radicle protrusion) due to cell elongation manifests the transition from the second to the third stage which is characterised by the appearance of a

nascent protein which is called germin (2). Germin is a water soluble homopentameric glycoprotein with 125 kDal polymeric weight. Germin revals unusual resistance to dissociation by various agents (e.g. SDS) and to degradation by proteases including pepsin (3). Although germin was first detected in germinating ceraeals (4), the proteins showing homology to germin have been shown to occur in other organisms as well (5-9).

Germin accumulation was shown to occur in wheat embryos from 16 hours up to 6 days post-imbibition. Germin has neither been detected in mature wheat plant organs nor in germinated embryos until 16 hours of imbibition (10). The germination-related germins of cereal embryos have been found to have oxalate oxidase activity (EC 1.2.3.4)-an activity which utilizes oxalate to generate hydrogen peroxide (11,12).

In early development wheat seedlings exhibit extensive root and shoot growth. The primary and lateral roots rupture the coleorhiza, which has limited growth, and extend up to a few centimetres in length. Similarly, the shoot-which remains sheathed with the rapidly elongating çoleoptile-extends up to 2 cm in length by 48 hours of imbibition; whereas tissues like coleorhiza and coleoptile, the tissues surrounding roots and leaf primordium respectively, have limited growth in plant life. Thus the development of the seedling growth can be characterised in terms of distinct populations of cells undergoing proliferation or expansion growth in a temporally and spatially co-ordinated manner Thus the aim of the current study was to establish the tissue specificity of germin biosynthesis at various stages of wheat development.

Materials and Methods

Plant Material

Spring wheat seeds (*Triticum aestivum* L. var. Tonic) were obtained from Kenneth Wilson Grain, Leeds, UK. Dry seeds were surface sterilised by incubation for 15 minutes in a 10% solution of domestic leach (ca. 1% free Cl_2) and washed five times with sterile distilled water. Seeds were germinated by incubation on two layers of water-soaked 3MM chromatography paper (Whatman) at 25°C.

Protein Isolation From Wheat Embryos

Embryos (5 gm) were homogenised with 10 ml extraction buffer [Tris-HCl (pH: 7.5) 25 mM, KCl 50 mM, $MgCl_2$ 5 mM and B-mercaptoethanol 5 mM] in a Eppendorf tube. To get the supernatant fraction, the total protein extract was centrifuged at 12000 x g for 5 minutes at RT. Supernatant was taken and recentrifuged at 12000 x g for another 5 minutes at RT. After this, the supernatant was transferred into a fresh tube and stored at -20°C until needed for the analysis. To get pellet fraction, the total protein extract was homogenised in extraction buffer and centrifuged at 12000 x g for 5 minutes at RT. The supernatant was discarded and 150 µl sample buffer was added [Tris-HCl (pH: 6.8) 7.5 g/l, gycerol 100 g/l, SDS 23 g/l and B -mercaptoethanol added to final solution as 5%] to further homogenise the extract. The extract was centrifuged at 12000 x g for another 5 minutes at RT and the supernatant was discarded. At this stage the pellet was boiled for 5 minutes and centrifuged at 12000 x g for 5 minutes at

RT. The supernatant was discarded and 150 µl sample buffer was added to the pellet. The centrifugation was repeated. The final supernatant was collected to use in the determination of cell wall bond germin proteins. The protein concentration was estimated by the dye-binding assay (13).

SDS-PAGE electrophoresis

The preparation of supernatant and pellet fraction of wheat embryos was denatured by boiling for 2 minutes is 30 ml SDS sample buffer [Tris-HCl (pH: 6.8) 7.5 g/l, gycerol 100 g/l, SDS 23 g/l and B-mercaptothanol added to final solution as 5%] and loaded onto a 12% polyacrylamide gel. Resolution of proteins was carried out according to Laemmli (14) at 40 mA for 4 hours. After separation by electrophoresis, proteins were either stained in the gel with Coomassie blue or transferred onto nitrocellulose membrane by Western blotting.

Western Blotting of Protein Gels

A modified method of Towbin et al. (15) was used for the electrophoretic transfer of proteins from gels to nitrocellulose sheets. After electrophoresis, the polyacrylamide gel was washed in 200 ml transfer buffer [mix: 100 ml 10x electrophoresis buffer (glycine 141 g/l, tris base 30 g/l, SDS 10g/l), 200 ml methanol and 700 ml distilled water] for 30 minutes. Proteins were electrophoretically blotted onto nitrocellulose membrane for 1 hour at 2 mA/cm^2 in a semi-dry transfer apparatus. After electroblotting, the membrane was washed in PBS solution [Disodium hydrogen orthophosphate 0.08 M, Sodium dihydrogen orthophosphate (pH: 7.5) 0.02 M, Sodium Chloride 0.1 M] and air dried.

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 that was diluted 1/2000 with PBS for one hour. Following this incubation the membrane was throughly 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

Determination f Germin in Wheat Tissue Extracts

IIn this study it was found that at 48 hours of germination, the roots, shoot (coleoptile plus leaf perimordium) and vascular transition region (the part remaining when the shoot and root are dissected from the embryo) retain germin protein. Although germin is a relatively rare protein (ca. 0.1%) in the germinating embryos (4), in this study it was found to be abundant enough to be detected in 100 µg and 25 µg (not shown) soluble protein extracts obtained from wheat embryos at 48 hours post-imbibition (Figure 1.). The accumulation of the germin protein was determined to be the highest in the vascular transition region among the organs of wheat embryos. The gel blot indicated that the roots retain higher germin expression than the shoot (Figure 1). This result is in accordance with that of Hurkman and Tanaka (16) who also

demanstrated the highest levels of germin accumulation in the vascular transition region in wheat seedlings. Inconstrast, these authors found the root to be the organ with the greatest germin accumulation in barley seedlings. Grzelczak et al. (4) reported that in early wheat seedling development (35 hours post-imbibition) the "stem" had the highest levels of germin expression among roots, stem and leaves. However, at a later stage of development (7 days) when root growth is greater than stem growth, the roots showed the highest levels of accumulation of the germin protein.

At a later stage of wheat seedling development (6 days post-imbibition) where root growth is more conspicuous than shoot growth, the immunoblot results indicated that germin was approximately equally distributed between the roots and the vascular transition region (Figure 2). A further analysis of the shoot indicated that germin was equally expressed in the different parts of the shoot when extracts taken from the shoot tip, central region and the region nearest to the vascular transition region were analysed (Figure 2). However, the overall germin content of the shoot was not as great as that in the roots or in the vascular transition region.

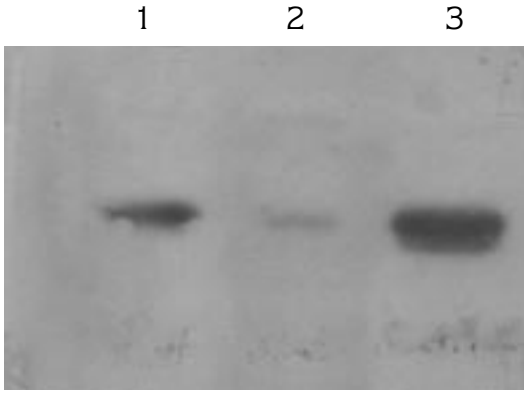

Figure 1. Distribution of germin among 48-hour-old wheat seedling organs. Equal quantities of protein from 48 hours wheat seedlings (1) roots, (2) shoot, and (3) vascular transition regions were analysed for germin accumulation by electroblotting using antigermin serum.

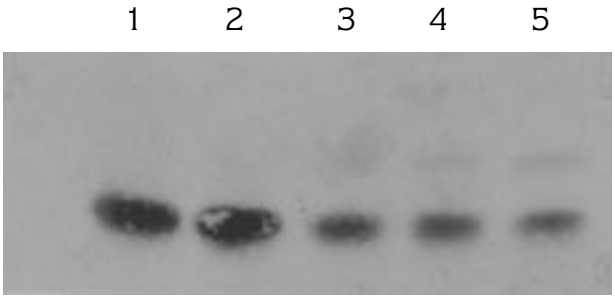

Figure 2. Determination of germin in 6-day-old wheat seedling organs. Equal quantities of protein from 6 day old wheat seedlings (1) roots, (2) vascular transition regions, (3) shoot tips, (4) shoot middle regions, (5) shoot basal regions were analysed for germin accumulation by elelctroblotting using antigermin serum.

Germin Detection in Pellet vs. Supernatant Fractions of Homogenates of Wheat Tissues

Germin is a water-soluble homopentameric glycoprotein. Further investigation by electron microscopy localisation indicated that some germin was cell wall-bound and that this wall-associated germin accounted for about 40% of the total germin in germinating wheat embryos (17). It was therefore important to compare the wall-bound form of germin (associated with the pellet fractions of homogenates) with the soluble from, detected in soluble protein extracts (in the supernatant fractions) among the tissues of wheat embryos at different stages of development.

The pellet fraction of 3-day-old germinated wheat embryo hologenates was found to contain greater quantities of germin than the supernatant fractions of the same tissues. In all organ systems, the pellet fractions (from coleoptiles, roots and vascular transition regions) had a higher germin content than the corresponding supernatant fractions from the same tisssues (Figure 3). When the germin was investigated in the pellet and supernatant fractions of protein extracts from the wheat seedlings (6-day post-imbibition) and the later stage of development, the pallet fractions showed a higher germin expression than the corresponding supernatant fractions from the same tissues (Figure 4). The only organs in young wheat seedlings in which germin accumulation was not detected were the leaves. Germin was not detected either in the pellet fraction or in the soluble fraction of leaves from 3 or 6-day-old germinated seedlings (Figure 3 and 4).

Temporal Analysis of Germin Synthesis in Wheat Leaves

It has been reported that germin-like proteins are expressed in cereal leaves in response to infection by the powdery mildew fungus (18-20); therefore it is clear that the leaves retain the potential for germin gene expression although no germin was detected in 3 and 6-day-old wheat

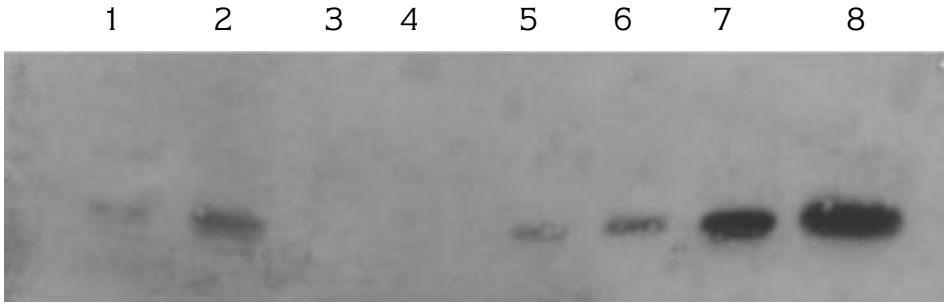

Figure 3. Determination of germin in pellet *vs* supernatant fractions of homogenates of 3-day-old wheat tissues. Equal quantities of protein from 3-day-old wheat seedlings (1) coleoptile supernatant, (2) coleoptile pellet, (3) leaf supernatant, (4) leaf pellet, (5) root supernatant, (6) root pellet, (7) vascular transition region supernatant and (8) vascular transition region pellet were analysed for germin accumulation by electroblotting using antigermin serum.

seedling leaves (Figure 3 and 4). In some species it was shown that germin-like proteins are responsive to photoperiodic treatments (21). An experiment was set up to find out whether darkness or light induces germin accumulation in leaves of germinating wheat embroys. The shoots of 4 and 7-day-old seedlings grown in darkness and light were dissected to coleoptile and leaves. The immunoblot indicated that at this stage of development, the leaf tissue does not retain germin proteins in wheat seedlings grown in darkness or light (Figure 5). Following that a more extensive survey of leaf development was therefore undertaken to determine when the leaves first gain germin accumulation. Leaf protein extracts from seedlings of increasing age (3, 7, 9 and 13 days post-imbibition) were analysed by immunoblotting, and it was found that

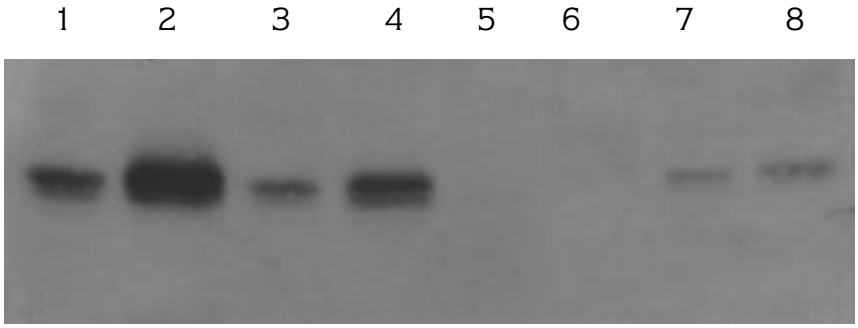

Figure 4. Determination of germin in pellet *vs* supernatant fractions of homogenates of 3-day-old wheat tissues. Equal quantities of protein from 6-day-old wheat seedlings (1) vascular transition region supernatant, (2) vascular transition region pellet, (3) coleoptile supernatant, (4) coleoptile pellet, (5) leaf supernatant, (6) leaf pellet, (7) root supernatant annd (8) pellet were analysed for germin accumulation by electroblotting using antigermin serum.

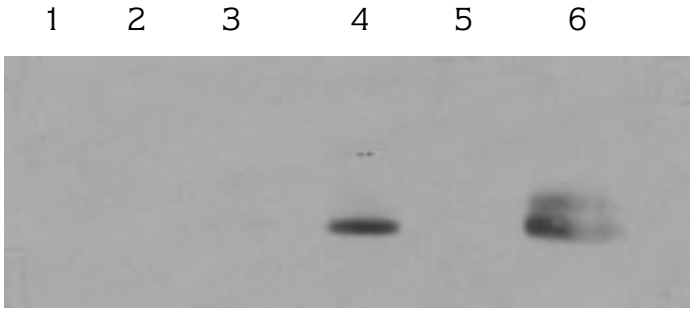

Figure 5. Analysis of germin synthesis in leaves of wheat seedlings grown in darkness and light. Equal quantities of protein from (1) Leaves of 4-day darkness, (2) Leaves of 4-day light, (3) Leaves of 7-day light, (4) Coleoptiles of 7-day light, (5) Leaes of 7-day darkness and (6) Coleoptiles of 7-day draknss were analysed for germin accumulation by electroblotting using antigermin serum.

germin accumulation could first be detected in leaves after 9 days of germination (Figure 5). Prior to this, the only detectable germin protein in the soot parts of the seedling occurred within the coleoptile. While in 3 and 7-day-old wheat seedlings leaves germin was not detected, in 9 and 13-day-old seedlings, leaves germin protein accumulation was determined (Figure 5).

In early wheat embryo development, the vascular transition region was shown to have the highest germin accumulation among the organs. The vascular transition region, which is a protective tissue of embryonic roots, has limited growth in early wheat embroy development. Like the vascular transition region, the coleoptile has limited growth and is characterised by germin accumulation. The coleoptile is a protective tissue of the leaf primordium in the early shoot development. The leaf tissue gains germin expression comparatively late during gerimantion. It is known that in seedlings grown in darkness there is extensive etiolation of the coleoptile and relatively little leaf expansion. Since germin was found to be greatly associated with the cell wall bound fraction, it could be speculated that its role in early wheat embryo development might be a contribution to the cell wall modification.

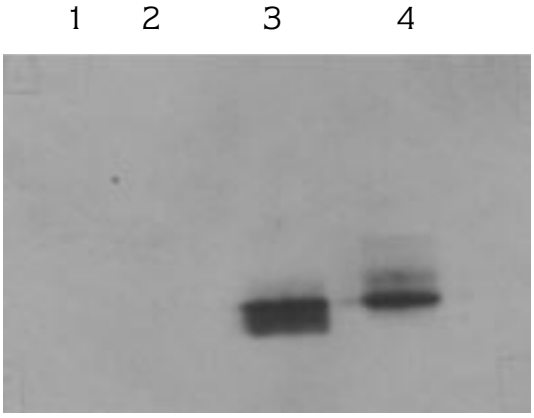

Figure 6. Temporal analysis of germin accumulation in leaves. Germin accumulation was investigated in (1) 3-day seedlings' leaves, (2) 7-day seedlings' leaves, (3) 9-day seedlings' leaves and (4) 13-day seedlings' leaves by electroblotting using antigermin serum.

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