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Abstract: Wheat embryogenic callus was induced by treating immature wheat embryos with 2,4-D. Digoxigenin-labeled germin sense and anti-sense RNA probes were prepared by *in vitro* transcription. By using these probes it was revealed that there was a striking induction of germin gene expression prior to visible callus formation. Although no germin gene expression was localized on the sections of immature embryos which were not incubated in callus induction medium, following 4 hours incubation in callus induction medium, germin mRNA was localized on the sections of immature embryos. Germin gene expression more conspicuous and widespread on the sections which were incubated for 6 hours in callus induction medium. It was suggested that germin genes induced during callus formation were related to the remodeling of the cell wall to accomplish the transition from precocious germination to callus formation.

Key Words: Callus, Germin, Auxin, Embryogenesis

Oksin'in Oluşturduğu Kallus Dokusunda Germin Gen Ekspresyonunun Belirlenmesi

Özet: Olgunlaşmamış buğday embriyoları 2, 4-D ile muamele ettirilerek embriyonik kallus oluşturulmuştur. Germin'in digoxigenin ile işaretlenmiş anlamlı ve anlamsız RNA probları laboratuvar koşullarında hazırlanmıştır. Bu problar kullanılarak, oksinle muameleye tabi tutulan embriyolarda, belirgin kallus oluşumundan önce önemli oranda germin gen ekspresyonunun olduğu anlaşılmıştır. Oksinsiz ortamda bulunan embriyolarda germin gen ekspresyonu belirlenmemesine rağmen, oksin'li ortamda 4 saatlik bir inkübasyon sonucunda embriyolarda, germin gen ekspresyonunun olduğu belirlenmiştir. Germin gen ekspresyonunun, oksinli ortamda 6 saatlik bir inkübasyon sonunda, çok daha yaygın ve belirgin olduğu gözlemlenmiştir. Germin gen ekspresyonlarının, olgunlaşmamış buğday embriyolarının erken çimlenme aşamasından kallus oluşumu aşamasına geçişi gerçekleştirmek için gerekli olan, hücre çeperinin yeniden şekillenmesi ile ilgili olabileceği önerilmiştir.

Anahtar Sözcükler: Kallus, Germin, Oksin, Embriyogenez

Introduction

Wheat embryo development is initiated by fertilization of the ovum, following which the formation of a viable embryo passes through several distinct stages. Following a period of cellular proliferation and differentiation, during which the primordial organs are formed, the embryo achieves a state of functional maturity such that its dissection from the developing grain permits the onset of precocious germination (1,2). If left within the grain, the embryo remains in a dormant state; a stage during which the accumulation of a discrete set of maturation-associated gene products occurs (3,4). The embryogenic phase of development is brought to a close by the programmed desiccation of the grain, causing a cessation of metabolic activity in the embryo. Desiccation causes a change to occur in the developmental potential of the embryo so that upon rehydration, instead of resuming an anabolic embryogenic pathway of development, the embryonic metabolism switches into a catabolic germinative mode utilizing embryonic and endosperm reserves to fuel the explosive growth of root and shoot meristems.

The programming of embryonic development is, in part, regulated by plant growth substances such as auxin, abscisic acid (ABA), gibberellic acid (GA), and ethylene (5-9). This also is associated with a change in the genetic program. For instance, successful germination requires the transcription of new genes (10). Conspicuous amongst the population of germination associated gene products is the remarkable homopentameric, extra cellular glycoprotein called "germin" (11,12). Germin genes have been shown to be associated with various aspect of plant development such as salt stress (13,14), defense system (15-18), embryonic development (19), photoperiodic oscillations (20, 21) and hormonal stimuli (22). Germin was suggested to be a member of a "superfamily" which comprises various growth related genes (23-25). Furthermore, wheat germin gene expression was shown to be regulated by biotic (viral infection) and abiotic stress (heavy metal ions) applications (26).

In 1993, it was determined that cereal germin proteins had strong oxalate oxidase activity (27,28). Oxalate oxidase (EC 1.2.3.4) is an oxidoreductase which degrades oxalic acid to produce one mole of H_2O_2 and two moles of CO_2. The principal product of the degradation of oxalate is hydrogen peroxide, which is a highly reactive compound known to be involved in several metabolic processes in higher plants. It was reported that hydrogen peroxide might act as a signaling molecule at low concentrations (29) or a component of cell wall modifications at high concentrations (30). The involvement of oxalate, germin genes and hydrogen peroxide in plant development was reviewed by Caliskan (31,32). The role of germin genes and their enzymatic activity during wheat embryo germination have been studied (33-35). Our previous results revealed that germin expression was associated with the tissues have caused whose growth was restricted during germination. It was reported that germin might have caused the growth restriction by inducing cross-linking of cell wall polymers prompted by H_2O_2 accumulation, which is a product of germin action (33).

Culture of immature wheat embryos, isolated from developing grains, in the presence of 2,4-D caused scutellar tissue to undergo unchecked proliferation, as callus (36). Callus has the capacity to undergo subsequent regeneration via somatic embryogenesis, a process of some significance for the potential genetic manipulation of this important cereal (36,37). Since sequence analysis of a wheat germin gene indicated the presence of potentially auxin responsive *cis*-acting elements in the promoter region (38), the expression pattern of germin genes in callus formation has been investigated in the current study in order to characterize the processes inherent in the capacity of embryonic wheat tissues for dedifferentiation and redifferentiation.

Materials and Methods

Plant material

Grains of spring wheat (*Triticum aestivum* L. var. Tonic) obtained from Kenneth Wilson Grain, Leeds, UK, were grown in a controlled-environment chamber with a 16h light (25°C) - 8 h, dark (18°C) photoperiod at 60% relative humidity. Immature grains were harvested at 12 days after fertilization (12 daf). For callus induction, grains were surface sterilized with sodium hypochlorite solution (1% free Cl_2) for 10 minutes and washed with sterile water prior to dissection of the embryos. Embryos were washed in sterile water to remove adhering liquid endosperm and transferred to 0.6% agar medium containing Murashige-Skoog medium (MS) supplemented with 2% (w/v) sucrose with or without 2mg l^{-1} 2,4-diclorophenoxyacetic acid (2,4-D; Sigma, UK) (36). Cultures were incubated at 25°C in darkness until use. Sections were prepared from cultures following 0-hour (immediately), 4-hour and 6-hour incubation periods in callus induction medium.

In situ hybridization

"Germin" mRNA was detected in sections by hybridization, *in situ*, with transcripts of a "germin" cDNA, kindly provided by Prof. Dr. B. G. Lane (University of Toronto). This cDNA was subcloned in the plasmid vector pBluescript (Stratagene, La Jolla, CA) for the production of digoxygenin-labeled riboprobes (DIG system: Boehringer Mannheim, FRG). Labeled anti-sense and sense probes were produced with T3 and T7 RNA polymerases (Figure 1). Probes were subjected to mild alkaline hydrolysis by incubation with 40mM $NaHCO_3$, pH 10.2 at 60°C to produce fragments of *ca*. 250 bp (39). Subsequent experiments showed that labeled anti-sense riboprobes specifically hybridized with germin mRNA in tissues, while labeled sense riboprobes gave no hybridization product (data not shown). Therefore, labeled sense riboprobes were used in control experiments in which no signal was expected.

A modified procedure of Jackson (40) was employed for localization of mRNA *in situ*. Samples were prepared for sectioning by fixation in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) at 4°C, dehydrated through ethanol series on ice, and embedded in

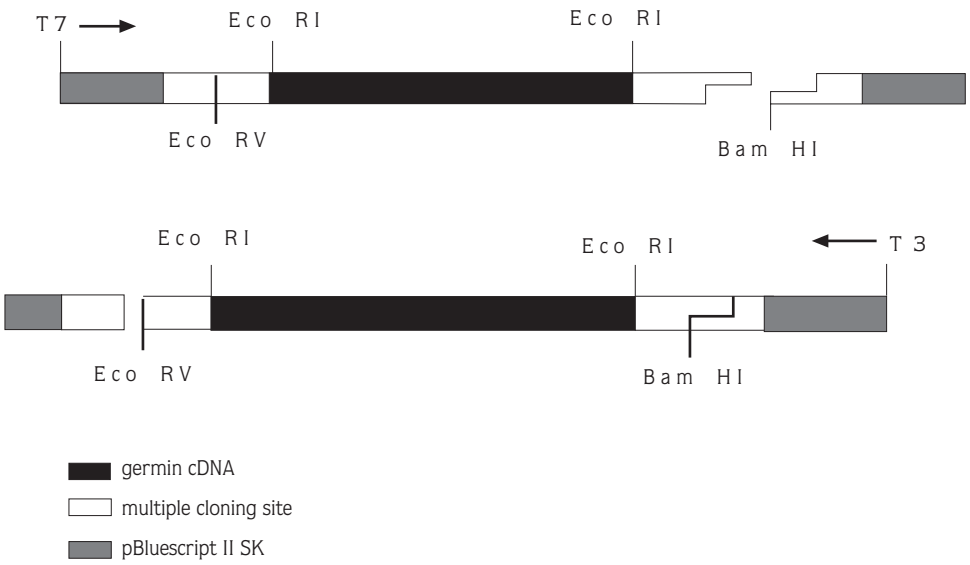

Figure 1. Production of RNA probes. The germin cDNA was cloned in the Eco RI site of pBluescript II SK$^-$. The plasmid DNA was linearized with Bam HI for production of T7 transcripts (sense) and Eco RV for production of T3 transcripts (anti-sense).

paraffin wax using Histolene™ (CellPath, Hemel Hempsted, UK) as the infiltrating solvent. Sections (10µm) were mounted on poly-L-lysine (Sigma, UK) coated slides at 42°C overnight, deparaffinized and rehydrated through ethanol series for hybridization with strand-specific probes. The sections were incubated with pronase (0.125mg ml^{-1} in 50mM Tris-Cl - 5mM EDTA, pH 7.5) and post-fixed with 4% (w/v) paraformaldehyde in PBS. Sections were acetylated with acetic anhydride (0.5% (v/v) in triethanolamine-HCl, pH 8) prior to prehybridization at room temperature with 0.3M NaCl - 10mM sodium phosphate buffer - 10mM Tris-Cl - 5mM EDTA - 50% (v/v) formamide -10% (w/v) dextran sulphate - 0.1% (w/v) tRNA - 1x Denhardts solution, pH 6.8 for 30 minutes. Sections were then incubated with buffer containing the DIG-labeled probe at a final concentration of 3 µg ml^{-1} overnight at 50°C. Sections were washed in 2xSSC-50% (v/v) formamide at 50°C, and then incubated with RNase A (20 µg ml^{-1}) in 0.5M NaCl, 10mM Tris-Cl, pH 7.7, 1mM Na_2EDTA at 37°C for 30 min. After a final wash in PBS, the hybridized probe was detected using anti-digoxygenin antiserum at a 1:3000 dilution, as described in the manufacturer's instructions. Sections were examined by light microscopy and photographed with Kodak Ektachrome Elite II color film.

Results and Discussion

Immature wheat embryos excised from developing grains and cultured in the presence of auxin do not continue their normal pathway of development. Instead, cells of the immature embryos become dedifferentiated and proliferate to form unchecked cell growth that is called callus. Although germin synthesis was not caused by either immature embryos or mature embryos before their germination (34,41), an abundant accumulation of germin gene expression was detected during callus formation from auxin incubated immature wheat embryos (41). The localization pattern of germin gene expression in a spatial and temporal manner during callus formation was the main concern of the current study.

Immature wheat embryos have a conspicuous appearance of primary and secondary roots, scutellum, coleorhiza and coleoptile structures at 12 days post-anthesis (Figs. 2A, 2B). The first two sections were taken from the immature embryos, which were not incubated in callus induction medium, and they were called 0-hour. When these sections were reacted with labeled anti-sense germin riboprobes, no hybridization product was observed (Figure 2A). The sections reacted with labeled sense germin riboprobe also gave no signals as expected (Figure 2B). This was completely in accordance with previous studies, which reported that there was no germin gene expression in immature wheat embryos (41). Following 4-hour incubation in callus induction medium, however, the immature embryo sections revealed some germin gene

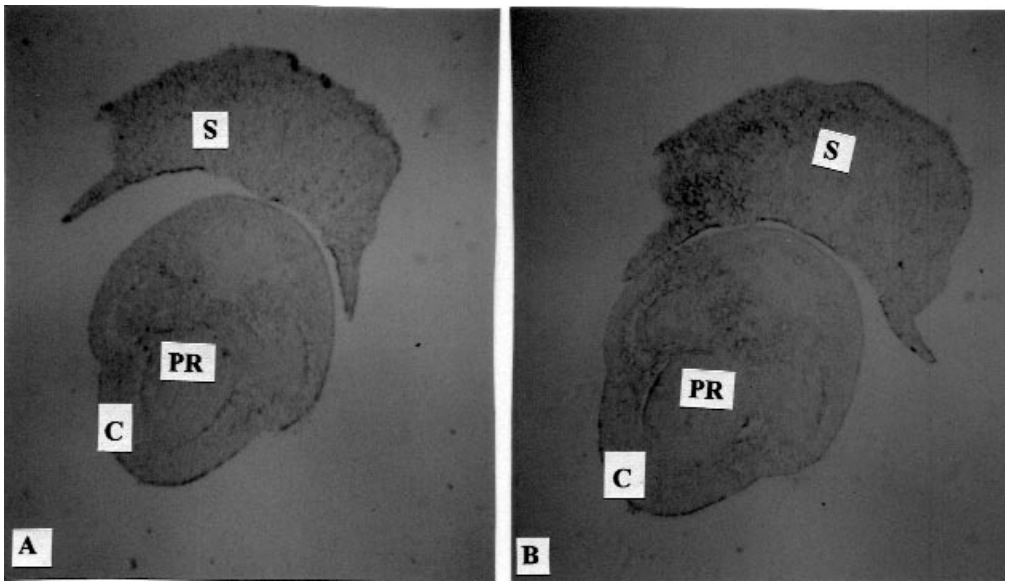

Figure 2. Immature wheat embryo sections treated with germin DIG-labeled (A) anti-sense RNA probes and (B) sense RNA probes (0-hour, no incubation in callus induction medium). S: Scutellum, C: Coleorhiza, PR: Primary root.

expression signals, particularly on the outer cells of the scutellum (Figure 3A). In 4 hours, callus induction medium caused expression of germin genes in the scutellum, which is an embryonic tissue responsible for protecting and supporting the embryo development. The sections taken from the same embryo were reacted with labeled sense riboprobes for the control experiments and no signal was observed (Figure 3B). Therefore, the signals associated with the scutellum were really a product of hybridization between labeled anti-sense germin riboprobes and germin mRNA in cells. Germin gene expression was more conspicuous when immature wheat embryos were incubated for 6 hours in callus induction medium. At that stage of development, germin gene expression was found to be associated with outer scutellum cells, particularly the tips of the scutellum, and the tips of coleorhiza cells (Figure 4A). Although there was some background staining on the primary root in the control section, which was reacted with labeled sense riboprobes (Figure 4B), it is obvious from the figure that primary root cells gave some signals of germin gene expression (Figure 4A). It was observed that there was no visible callus

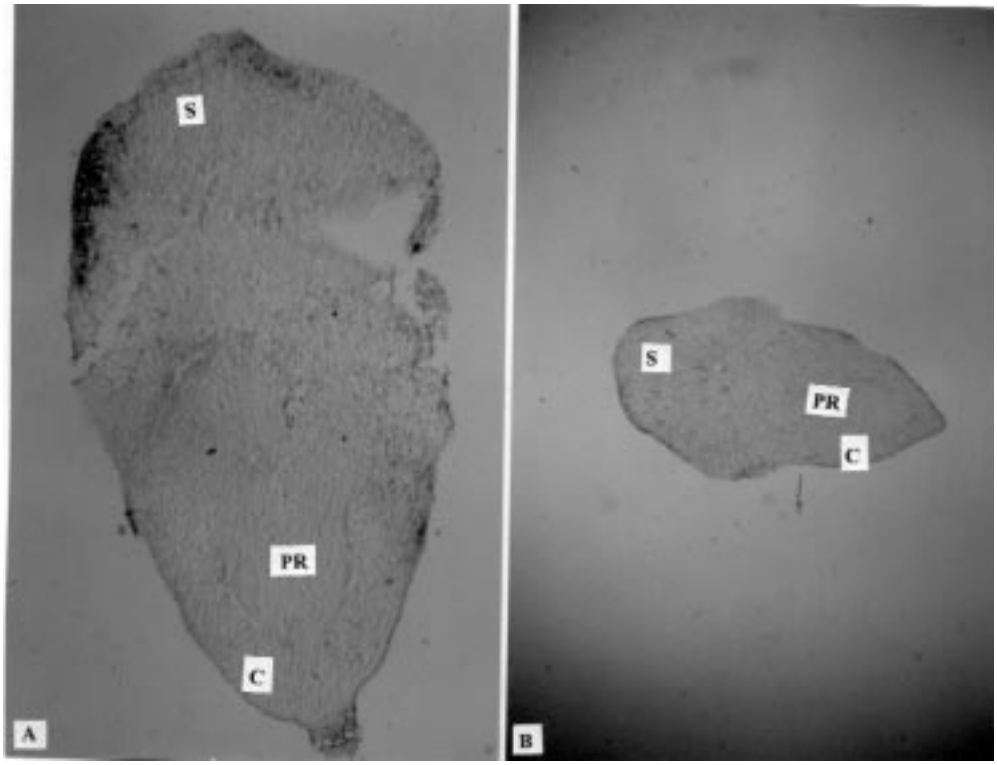

Figure 3. Immature wheat embryo sections treated with germin DIG-labeled (A) anti-sense RNA probes and (B) sense RNA probes (after 4-hour incubation in callus induction medium). S: Scutellum, C: Coleorhiza, PR: Primary root.

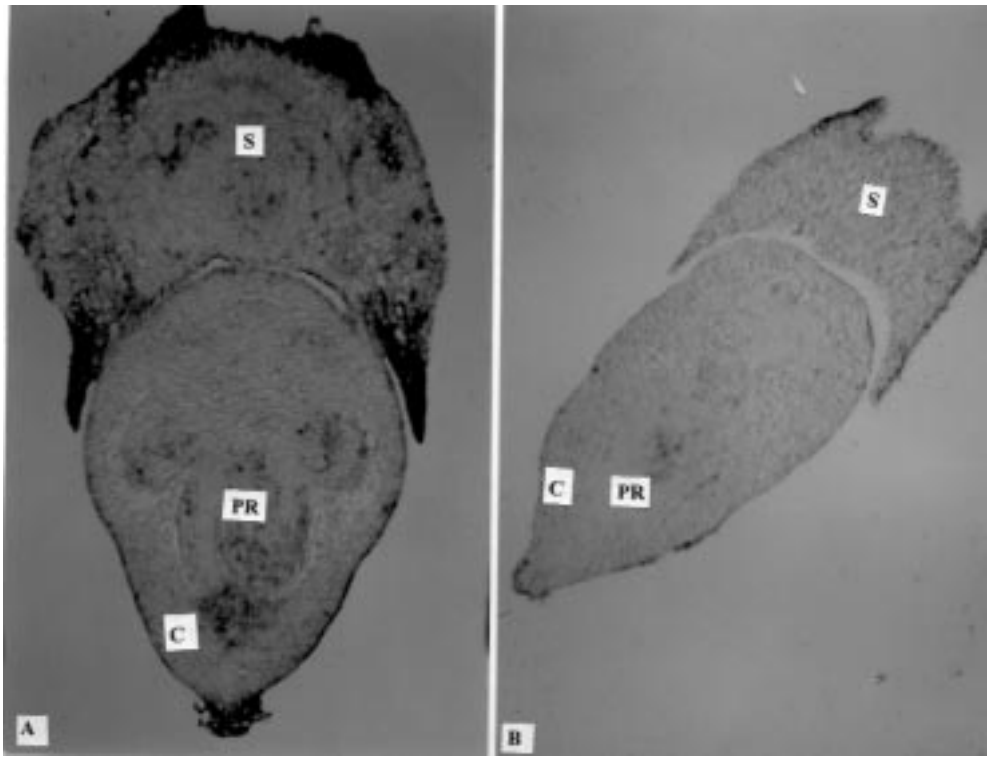

Figure 4. Immature wheat embryo sections treated with germin DIG-labeled (A) anti-sense RNA probes and (B) sense RNA probes (after 6-hour incubation in callus induction medium). S: Scutellum, C: Coleorhiza, PR: Primary root.

formation during these 6-hour incubation period in callus induction medium; however, there was a shift in the quality of genes expressed in immature wheat embryos as the figures indicate (Figs. 3A and 4A).

The current study indicated that germin gene expression was induced during callus formation. *In situ* RNA hybridization, one of the most powerful techniques to characterize genes, revealed that germin genes were associated with the scutellum, coleorhiza and primary root during callus induction. In view of the current results, it seems that accumulation of germin genes during the early hours of callus induction might be related to the arrangement of the reorganization of cells for the transition from normal growth to callus formation in which cells retain their embryonic capacity. Furthermore, the results obtained in this study support Domon (19), who showed that germin accumulation was related to the cells which maintain their embryonic capacity during pine embryogenesis. Obviously, more studies are needed to establish the mechanisms involved in callus formation and embryonic capacity of cells.

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