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Localization of Germin Genes and Their Products in Developing Wheat Coleoptiles

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Germination is a process which characterized with nescient synthesis of genes. Among the genes synthesized during the germination of wheat embryos, germin genes, proteins and their enzymatic activity were defined. Germin is a water soluble homopentameric glycoprotein which is remarkable resistant to degradation by a broad range of proteases including pepsin. Germin proteins found to have strong oxalate oxidase activity which produces hydrogen peroxide by degrading oxalic acid. The current study, aimed to localize the germin genes, proteins and enzymatic activities in developing coleoptiles which is a rapidly growing protective tissue of leaf primordium and shoot apex. Non-radioactively labeled germin riboprobes were employed to localize germin mRNAs *in situ*. FITC (Fluorescein isothiocyanate) and alkaline phosphatase linked anti-germin antibodies were used to localize germin proteins under the fluorescence and light microscopy and finally germin enzymatic activity was localized by using appropriate enzyme assay. The results revealed that in coleoptiles germin genes, proteins and their enzymatic activity were predominantly associated with the cells of epidermis and vascular bundle sheath cells.

Keywords: Coleoptile development, Germin genes, Germination, Oxalate oxidase

Introduction

Wheat germin is a relatively rare water-soluble glycoprotein (less than 0.1% of the mass of soluble proteins in germinating wheat embryos) which in homogenates exists as an oligomeric complex even which does not dissociate when analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) unless boiled in the presence of detergent (Grzelczak and Lane, 1984). The most striking characteristics of germin is its resistance to proteolysis and degrading oxalic acid to produce hydrogen peroxide (Dumas *et al.*, 1993). Oxalic acid as a substrate of germin-like oxalate oxidase is one of these molecules which implied take a role in various aspects of plant development (Caliskan, 2000a).

Germin was first detected in germinating cereals, but subsequently, germin-like proteins were also identified in a protist (Lane, 1991), dicotyledonous angiosperms (Delseny *et al.*, 1994; Heintzen *et al.*, 1994), and gymnosperms (Domon *et al.*, 1995). Germins and germin-like proteins constitute a large family of proteins ubiquitously distributed in the plant kingdom. They are part of a superfamily of proteins now called "cupins" that also includes seed storage globulins, sucrose binding proteins as well as microbial and animal proteins (Dunwell *et al.*, 2000). Germin mRNAs were localized in wheat immature embryos which were incubated in callus induction medium. The localization patterns of germin mRNAs were mainly on scutellum, coleorhiza, and primary root (Caliskan and Cuming, 1998; Caliskan, 2001). Recently germin and germin-like proteins were shown to respond to pathogen infection, environmental and hormonal stimuli (Caliskan, 2000b; Bernier and Berna, 2001; Lane, 2002).

Wheat embryo germination is characterized with nescient accumulation of genes (Caliskan *et al.*, 2003). When mature wheat embryos imbibed for 2-days, they were distinguished with extensive root and coleoptile elongation. Wheat coleoptile which encloses leaf primordium and shoot apex is a hollow cylindrical structure. It functions chiefly as a protective cover to the young foliage leaves during their upward growth. The tissues of the coleoptile are of a simple character. Outside and inside the cylindrical structure are covered with epidermal cells. The rest of the cells are cortical cells which comprise two vascular bundles on opposite sides of the coleoptile. Coleoptile is a tissue which derives its rapid growth from

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elongation of cells other then division of cells. The mechanism of this elongation is a matter of debate (Fry, 1989; Schopfer, 1993; Kutschera, 1994; Hohl and Schopfer, 1995). A few genes were considered to be involved in the elongation of coleoptiles (McQueen-Mason *et al.*, 1992; McQueen-Mason, 1995). Since germin genes were determined in the tissues of germinated wheat embryos (Caliskan and Cuming, 2000), the current study was carried out to localize the germin genes, proteins, and their enzymatic activity in a tissue-specific manner to have a clue about their biological importance.

Materials and Methods

Plant material Grains of spring wheat (*Triticum aestivum* L. var. Tonic) were obtained from Kenneth Wilson Grain (Leeds, UK). Mature grains were surface sterilized 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 3 MM chromatography paper (Whatman, ent, UK) at 25°C. Wheat grains were germinated for 48 h. Following that the coleoptile was excised by cutting from the embryo and fixed in paraformaldehyde.

***In situ* RNA hybridization** Germin mRNA was detected in coleoptile sections by hybridization, *in situ*, with transcripts of a "germin" cDNA sequence, kindly provided by B.G. Lane (University of Toronto). This sequence was sub-cloned in the plasmid vector pBluescript (Stratagene, La Jolla, CA) for the production of digoxygenin-labeled transcripts with T_7 (sense transcripts) and T_3 (anti-sense transcripts) RNA polymerases (Fig. 1). Probes were subjected to mild alkaline hydrolysis by incubation with 40 mM $NaHCO_3$, pH 10.2 at 60°C to produce fragments of ca. 250 bp (Cox *et al.*, 1984). A modified procedure of Jackson (1991) was employed for localization of mRNA *in situ*. Tissue was prepared for sectioning by fixation in 4% (w/v) paraformaldehyde in PBS at 4°C dehydrated through an ethanol series on ice, and embedded in paraffin wax using HistoleneTM (CellPath, Hemel Hempsted, UK) as the infiltrating solvent. Sections (10 µm) were mounted on poly-L-lysine (Sigma, Gillingham, UK) coated slides at 42°C overnight, deparaffinized and rehydrated through an ethanol series for hybridization with strand-specific probes. The sections were incubated with pronase (0.125 mg/ml in 50 mM Tris-Cl, 5 mM 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 0.3 M NaCl, 10 mM sodium phosphate buffer, 10 mM Tris-Cl, 5 mM EDTA, 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) tRNA, 1× Denhardts solution, pH 6.8 for 30 min. Sections were then incubated with buffer containing the DIG-labeled probe at a final concentration of 3 µg/ml and incubated overnight at 50°C. Sections were washed in 2 × SSC, 50% (v/v) formamide at 50°C, then incubated with RNase A (20 µg ml^{-1}) in 0.5 M NaCl, 10 mM Tris-Cl, pH 7.7, 1 mM Na_2EDTA at 37°C for 30 min. After a final wash in PBS, hybridized probe was detected using antidigoxygenin antiserum at a 1 : 3000 dilution, as described in the manufacturers instructions. Sections were examined by light microscopy and photographed with Kodak Ektachrome Elite II color film.

Histochemical detection of oxalate oxidase activity Oxalate oxidase activity *in situ* was detected using the procedure of Dumas *et al.* (1995). Germinated wheat embryo coleoptile was dissected and sectioned at –20°C after freeze-embedding in OCT resin: plant material was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), then washed with PBS, prior to embedding. Sections were mounted on poly-lysine coated microscope slides and incubated with a 15 µl assay buffer (25 mM succinic acid, 3.5 mM EDTA, 2.5 mM oxalic acid, 0.6 mg/ml 4-chloro-1-naphthol, pH 4.0) at 25°C until color development occurred. Control samples were incubated in assay buffer lacking oxalic acid.

Immunocytochemical detection of germin Germin proteins were localized, *in situ*, in coleoptile sections using a monospecific anti-germin antiserum kindly provided by Prof. B.G. Lane (University of Toronto). Tissue was fixed overnight in 4% (w/v) paraformaldehyde in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7) prior to embedding in OCT resin. Tissue sections were prepared by freeze-sectioning at –20°C and mounted on poly-L-lysine coated slides. Sections were incubated with 2% (w/v) powdered milk in PBS for 5 min, and then with anti-germin serum (1 : 500 dilution). After washing with PBS, the detection of germin proteins was carried out either with an alkaline-phosphatase-conjugated second antibody under a light microscopy or FITC (Fluorescein isothiocyanate)-linked second antibody under fluorescence microscopy. Control sections were incubated with non-immune serum as the primary antibody.

Results

In the current study, wheat embryos were imbibed for 2-days in the dark, following that coleoptile was dissected from embryos and fixed as explained. Then, coleoptile sections were prepared by using a microtome. To determine germin mRNA localization pattern, coleoptile sections were reacted

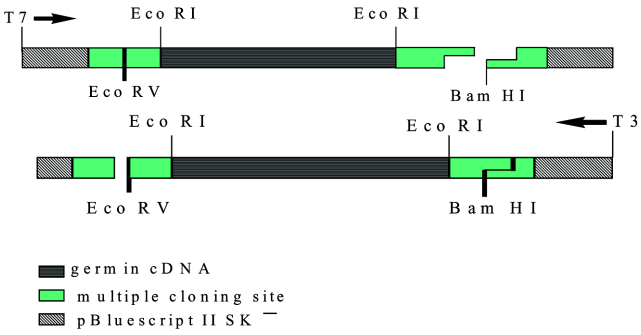

Fig. 1. Production of RNA probes by *in vitro* transcription. The germin cDNA was cloned in the *Eco*RI site of pBluescript II SK^-. The plasmid DNA was linearized with *Bam*HI for production of T_7 transcripts (sense probe) and *Eco*RV for production of T_3 transcripts (anti-sense probe).

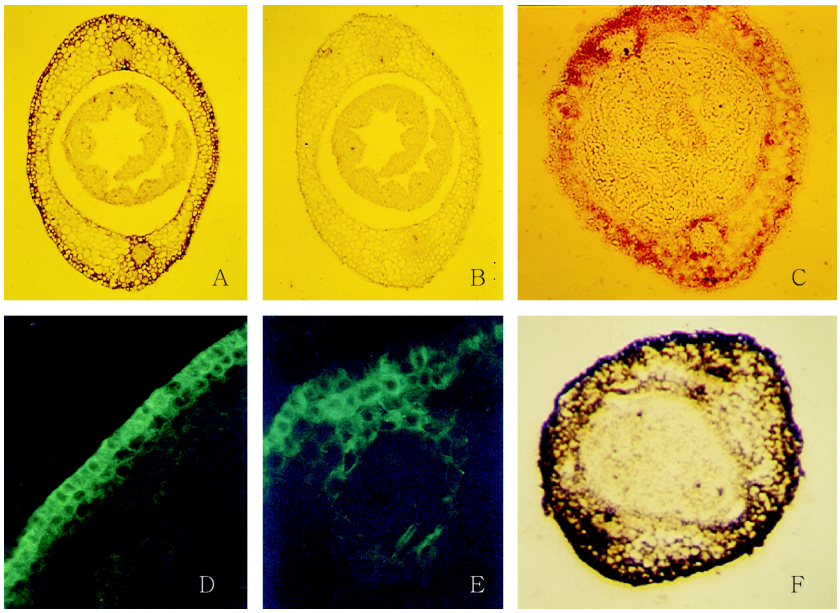

Fig. 2. Localization of germin mRNA by anti-sense probes (A), control section reacted with sense RNA probes (B), localization of germin proteins with alkaline phospatase linked antibody (C) localization of germin proteins with FITC-linked antibody (D, E), and localization of germin enzymatic activity (F).

with DIG-labeled anti-sense riboprobes which were prepared by *in vitro* transcription as explained by manufacturer (Fig. 1). In this system, the anti-sense probes that are expected to hybridize with germin mRNAs were produced from a T_3 promoter and sense probes that are expected not to hybridize any sequences were produced from T_7 promoter. While labeled anti-sense probes were used to localize germin mRNAs, the labeled sense probes were used as control probes.

The coleoptile sections reacted with labeled anti-sense riboprobes indicated that germin mRNA expression sides were outer epidermis and vascular bundle sheath cells (Fig. 2A). Epidermal cells are a kind of cells which surround coleoptile tissue. Similarly, vascular bundle sheath cells surround the vascular tissue in both sides of the coleoptile tissue. There was no expression of germin mRNAs on foliage leaves, cortex and inner epidermis (Fig. 2A). The control sections, which were reacted with the DIG-labeled sense riboprobes did not give any signal as expected (Fig. 2B).

Following the localization of germin mRNAs we moved to localize their products, proteins, by employing an anti-germin antiserum. Germin proteins were localized under light microscopy by using an alkaline phosphatase based detection system which was expected to give a red color formation. As seen in Fig. 2C, the germin proteins were associated with all the coleoptile cells except vascular bundle cells. A coleoptile section reacted with pre-immunization serum which is lack of germin antibodies gave no signal of germin proteins as expected (data not shown). The florescence-labeled antibodies are more sensitive than enzyme labeled antibodies. Therefore, FITC-labeled anti-germin serum was used to have a more specific localization pattern of germin proteins in coleoptiles. When germin proteins were localized with the FITC-linked antibodies under fluorescence microscopy which is more sensitive comparing to light microscopy, germin proteins were localized on epidermis (Fig. 2D) and vascular bundle sheath cells (Fig. 2E). These localization patterns were in complete agreement with the localization pattern of germin mRNAs (Fig. 2A).

It is known that wheat germin proteins have oxalate oxidase activity. This activity utilizes oxalic acid to generate hydrogen peroxide. In this study, we localized germin enzymatic activity by a method which was explained by Dumas *et al.* (1995). The dark paint which was an indication of germin enzymatic activity was associated with almost all of the coleoptile cells; however, it was more conspicuous with the outer epidermis (Fig. 2F). When oxalic acid was omitted from the enzyme assay, no signal was observed on coleoptile sections (data not shown).

Conclusions

Germins are remarkably stable oligomeric glycoproteins of apoplast that have oxalate oxidase activity. The current information on germins and germin-like proteins allows us to consider that all germin-like proteins are somehow associated with extra cellular matrix. Even though the details of germins and germin like proteins functions in the plant are still missing, accumulating evidence suggested several ways in which germins might act. The current study indicated that the germin genes, proteins and enzymatic activity were mainly

associated with the outer epidermis and vascular bundle sheath cells. Coleoptile is a rapidly growing protective tissue of germinating wheat embryos. The extensive growing of coleoptiles is due to cell enlargement other than cell division. The localization of germin proteins and enzymatic activity specifically on epidermal cells may imply a function in cell wall remodeling during cell enlargement or a function in the protection of the tissue. Similarly germin mRNAs were localized on the immature embryo's protective tissues like scutellum and coleorhiza in callus induction medium (Caliskan 2001).

Germin proteins and enzymatic activity were localized on the cells surrounding the vascular bundles (Fig. 2A and 2E). These cells were reported to be rich in calcium oxalate accumulation (Ward *et al.*, 1979). The localization of an oxalic acid degrading enzyme in these cells is worth to pay attention. Obviously, more studies are needed to establish the mechanisms in which germin and germin like proteins act.

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