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Full Length Research Paper

A plasma membrane H^+ATPase gene is germination-induced in wheat embryos

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The expression pattern of a germination specific plasma membrane H^+-ATPase was analyzed by RT-PCR and *in situ* RNA hybridization methods. RT-PCR results revealed that germination specific plasma membrane H^+-ATPase accumulation was detectable in all organs and tissues of germinating wheat embryos. H^+-ATPase expression was not observed in dry wheat embryos and in immature wheat embryos. *In situ* RNA hybridization indicated that germination specific plasma membrane H^+-ATPase gene expression was associated with all organs of germinating wheat tissues. The accumulation of H^+-ATPase mRNA was more heavily on the cells of vascular bundles and epidermal cells of coleoptiles. Since germination specific plasma membrane H^+-ATPase gene was identified as a growth related gene, interest was focused on the activity of growth regulators (GA, IAA, ABA) and stress factors, NaCl and Mannitol, on H^+-ATPase gene expression. The results indicated that there were not any dramatic changes in the accumulation of germination specific plasma membrane H^+-ATPase gene in any case. More rigorous analysis is necessary to evaluate the effect of growth regulators on germination specific plasma membrane H^+-ATPase.

Key words: Biosynthesis, cereals, DNA, enzymes, physiology, plant cell culture.

INTRODUCTION

Cereal embryo development results in the formation of a fully differentiated, immature embryo within 10-12 days following fertilization. The earliest period of germination is characterized by rapid water uptake, resulting in cell expansion which leads to protrusion of the root and the shoot primordia from within the enveloping tissues of the embryo and caryopsis. Extensive vacuolation of cells in the postmeristematic zones of root and shoot accounts almost entirely for the growth observed during the first 24 h of germination. DNA replication and subsequent mitotic divisions in the meristem thereafter contribute to subsequent root, coleoptile and leaf development. Although the transition from the dormant embryo to the germinating embryo necessarily entails a dramatic change in the underlying genetic programme, comparatively few genes have been identified which are characteristic of the onset of germination (Cuming, 1993; Caliskan, 2000).

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Subtractive screening of a cDNA library corresponding to germinating wheat embryos resulted in the isolation of a sequence initially called pSB5 and then called germination specific plasma membrane H^+-ATPase following a search of the EMBL and databases revealing that wheat germination specific pSB5 sequence corresponded to a plasma membrane H^+-ATPase gene family (Caliskan et al., 2003).

The H^+-ATPase constitutes an extremely important component of the plant cell, being involved in a variety of process. H^+-ATPases of the plant plasma membrane act as primary transporters by pumping protons out of the cell, creating pH and electrical potential differences across the plasmalemma (Michelet and Boutry, 1995).

The H^+-ATPase is a single polypeptide that has been predicted to be anchored in the plasma membrane by 10 membrane-spanning regions (Wach et al., 1992).

H^+-ATPase transports one proton per molecule of ATP hydrolyzed, with a pH optimum of about 6.6. It is not difficult to envisage a role for such a transporter in the germination and explosive growth of embryos. The elongation of stem and coleoptile cells has been demonstra-

ted to occur in response to auxin treatment by promoting cell wall loosening via intrusion of H^+ ions (Rayle and Cleland, 1977), giving rise to the "acid growth theory" (Hager et al., 1991).

H^+-ATPase also appears to be involved in the control of cell turgor (Curti et al., 1993). It was shown that osmotically stressed cultured *Arabidopsis thaliana* cells responded by activating H^+-ATPase-mediated H^+ efflux, allowing the uptake of more K^+ ions, result in osmotic adaptation (Curti et al., 1993). Proton-pumping ATPase is also involved in the regulation of stomatal opening (Kurkdjian and Guern, 1989). It appears that a number of H^+-ATPase genes is present within the plant, probably expressed in a tissue specific manner. Ewing and Bennet (1994) identified at least 7 genes in tomato and Harper et al. (1994) identified 10 genes in *Arabidopsis thaliana*, indicating the presence of large families of H^+-ATPase genes.

In this report, we determine and localize the expression pattern of germination specific plasma membrane H^+-ATPase in germinating wheat organs and tissues and suggest a role for this sequence in mediating the process leading to germinative cell expansion.

MATERIALS AND METHODS

Plant material

Wheat grains (*Triticum aestivum* L. var. Chinese Spring) were obtained from the John Innes Centre for Plant Science Research, Norwich, UK. Grains were surface-sterilized for 10 min using a solution of domestic bleach (1% free Cl_2), washed extensively with sterile water and imbibed at 25°C in darkness on two layers of water-soaked filter paper. For the isolation of immature embryos, plants were grown in compost, in a controlled environment chamber with a 16 h light (25°C) 8 h dark (18°C) photoperiod.

Stress applications

The expression pattern of H^+-ATPase was analyzed in response to a variety of treatments. The treatments chosen were osmotic stress (Mannitol at a concentration of 20% w/v), ionic stress (NaCl at 200 mM), indoleacetic acid (IAA) at 10^{-5} M, abscisic acid (ABA) at 10^{-4} M and gibberellic acid (GA_3) at 10^{-6}. To examine early responses, dry wheat grains were imbibed on sterile 3 MM papers soaked with water for 24 h at 25°C in darkness. Embryos were then dissected and placed on sterile 3 MM paper soaked with MS medium with or without the chosen agent. Embryos were placed at 25°C in darkness for a further 24 h after which they were harvested and RNA was extracted. To examine in older seedlings, grains were imbibed at 25°C in darkness for 5 days. Seedlings were then placed at 25°C in darkness for a further 24 h after which the roots, coleoptiles and leaves were harvested and RNA was extracted.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was used for the detection of gene expression in a variety of tissues as described by Wang et al. (1989) with alterations. The initial reverse transcription was carried out with the addition of 1 µg total RNA in a solution containing 1x PCR buffer (provided with Taq polymerase), 1.5 mM $MgCl_2$, 300 µmol dNTP mix, 1.125 pmol random hexamer primer, RNA-guard (12 u), M-MLV Reverse Transcriptase (200 u), made to a final volume of 20 µl and incubated at 23°C for 10 min, followed by 42°C for 60 min and 95°C for 5 min. The solution was chilled to 4°C. To the reaction mix, the following reagents were added: 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 µCi [a ^{32}P]-dCTP, T3 primer (20 pmol), T7 primer (20 pmol), Dynazyme Taq polymerase (1 u). Amplification was performed for the appropriate number of cycles at [94°C, 1 min; 55°C, 30 s; 72°C, 30 s] then maintained at 72°C for 5 min and chilled to 4°C. Samples were resolved on 6% acrylamide gels and products were autoradiographically visualized by placing the gel on X-ray film between two glass plates for overnight exposure. Control reactions were carried out on all samples exactly as described above with the absence of reverse transcriptase.

In situ RNA hybridization

PSB5 mRNA was detected in coleoptile sections by hybridization, *in situ*, with transcripts of a "pSB5" cDNA sequence, kindly provided by S. Bashiardes (University of Washington). 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. 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 (Cox et al., 1984). A modified procedure of Caliskan and Cuming (1998) 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, 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-1x Denhardts solution, pH 6.8 for 30 min.

Sections were then incubated with buffer containing the DIG-labelled probe at a final concentration of 3 µg/ml and incubated overnight at 50°C. Sections were washed in 2xSSC-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, 1mM Na_2EDTA at 37°C for 30 min. After a final wash in PBS, 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

Analysis of the expression patterns of H^+-ATPase in a variety of tissue of the growing wheat seedling was carried out in the current study. Analysis was carried out on immature embryos (13 day post-anthesis); dry embryos, 16 h germinating embryos and root, coleoptile and leaf tissues dissected from 6 day germinated embryo. Analysis of pattern of expression in a variety of wheat tissues, by RT-PCR, revealed H^+-ATPase mRNA presence in all growing tissues of wheat embryos (Figure 1). Immature embryos and dry embryos before their imbibition did not

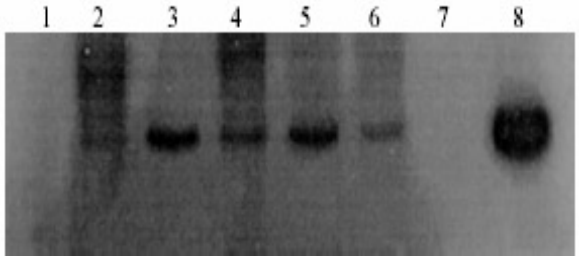

Figure 1. Analysis of expression of H^+-ATPase by RT-PCR in wheat tissues. Analysis was carried out on immature (13 day post anthesis) embryos (lane 1), dry embryos (lane 2), 16 h germinated embryos (lane 3), 6 day roots (lane 4), 6 day coleoptiles (lane 5), 6 day leaves (lane 6). A negative control containing no template was used (lane 7) and a positive control containing 1 ng H^+-ATPase was included (lane 8).

show any accumulation of germination specific H^+-ATPase mRNA (Figure 1 lane 1, 2). However, dry wheat embryos following 16 hours imbibition, the root, coleoptile and leaf tissues dissected from 6 day germinated seedlings were the side of germination specific H^+-ATPase mRNA accumulation (Figure 1 lane 3, 4, 5 and 6). A negative control, containing no template (Figure 1 lane 7) and a positive control, containing 1 ng H^+-ATPase (Figure 1 lane 8) indicated the correct insert size and the trueness of the signals.

Following the determination of germination specific H^+-ATPase gene products in wheat organs and tissues (Figure 1), *in situ* RNA hybridization was employed for the spatial localization of germination specific H^+-ATPase gene products in these tissues. In this method, the localization pattern of germination specific H^+-ATPase mRNA was aimed in the tissue of germinating wheat embryos. Firstly, the localization of germination specific H^+-ATPase gene products was determined on the whole germinating embryo's tissues. As seen in Figure 2A, the horizontal section of embryos was characterized with primary root and secondary roots. All of these organs are surrounded by some protective and supportive cells. In these sections, it was not possible to localize the shoot apex and leaf primordium. Following *in situ* RNA hybridization, it was found that, germination specific H^+-ATPase gene products were localized on all the tissues of germinating embryo including primary and secondary roots (Figure 2A). In control section (Figure 2B); there was not any indication of hybridization product. This is expected because in control experiment, sense probe was used instead of anti-sense probe. The expression of H^+-ATPase gene products were determined to be more conspicuous on the shoot section of germinating embryos (Figure 2C). Although, germination specific H^+-ATPase gene products were on the all tissues and cells, the cells surrounding coleoptile (epidermal cells) and the cells within vascular bundles were more prominent in respect to H^+-ATPase gene products accumulation (Figure 2C). Apparently, the

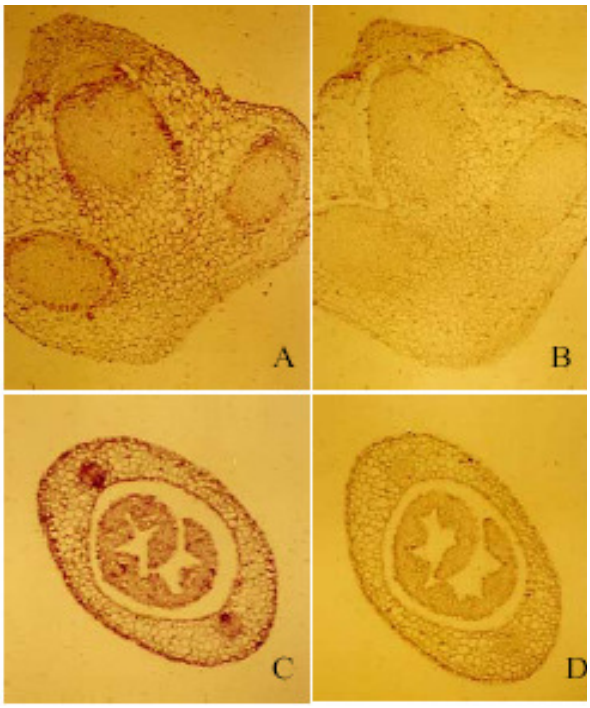

Figure 2. Localization of H^+-ATPase gene products by *in situ* RNA hybridization. Embryo section reacted with DIG-labeled anti-sense probe (A), embryo section reacted with DIG-labeled sense probe (B), coleoptile section reacted with DIG-labeled anti-sense probe (C), coleoptile section reacted with DIG-labeled sense probe (D).

leaf primordium which is localized at the center of coleoptile was one of the sites for the expression of H^+-ATPase gene products (Figure 2C). The sequencel section was reacted with sense probe for control experiment and as expected no signal formation was observed on this section (Figure 2D).

The effects of various stress applications on the synthesis of H^+-ATPase is given in Figure 3. The control experiment (no stress) was shown in Figure 3A. There is a relatively abundant level of germination specific H^+-ATPase mRNA synthesis in 48 hours germinated embryos, 6 day roots and 6 day coleoptiles (Figure 3A). Among the organs of 6 day-old germinated embryos, the leaves appeared to express low level of H^+-ATPase comparing to roots and coleoptiles (Figure 3A). All subsequent analysis carried out to determine the effects of stresses on H^+-ATPase were related to the results obtained from this (control) experiment. In response to 200 mM NaCl, levels of H^+-ATPase mRNA remained unchanged in the embryo whereas among the organs of 6 day-old germinated embryos the level of mRNA appeared at a low equal level in each case except for leaves which expressed elevated level of H^+-ATPase (Figure 3B). The extreme osmotic stress imposed by mannitol appeared to have a greater effect on H^+-ATPase. Although there appeared to be no change in the level of expression in the 48 h embryos and 6 day coleoptiles, in the root there appeared to be a

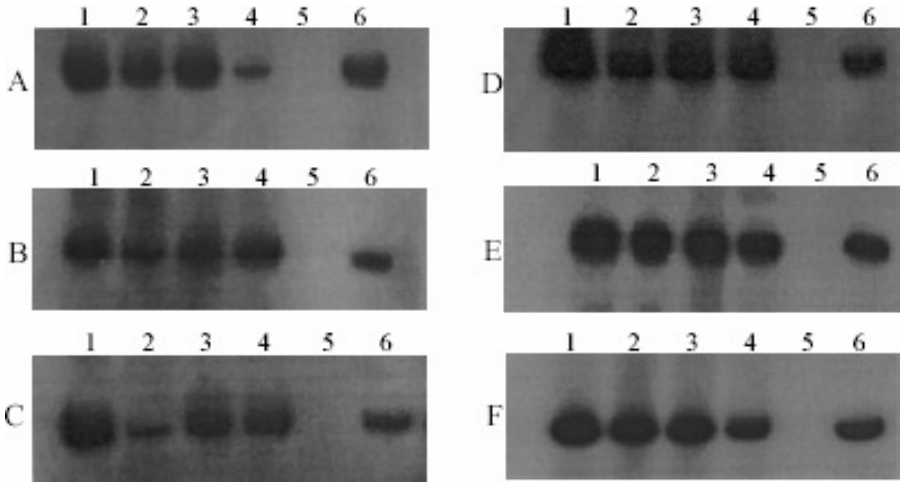

Figure 3. The effect of a variety of stresses on the expression pattern of H^+-ATPase analyzed by RT-PCR. The stress imposed were 200 mM NaCl (B), 20% mannitol (C), 10^{-4} M ABA (D), 10^{-4} M GA3 (E), and 10^{-6} M IAA (F). A control was prepared where no stress was imposed (A). In each case, the tissues analyzed were 48 h germinated embryos (lane 1), 6 day roots (Lane 2), 6 day coleoptiles (lane 3), 6 day leaves (lane 4), a negative control that contained no template (lane 5) and a positive control that contained 1 ng H^+-ATPase were included (lane 6).

down regulation and in leaves there was a high regulation comparing to control (Figure 3C). Treatment with ABA appeared to produce a high regulation of H^+-ATPase in all samples (Figure 3D). Treatment with GA_3 and IAA caused visible differences in H^+-ATPase expression only in the 6 day leaves comparing to control (Figure 3E and F).

DISCUSSION

Once the pattern of germination specific H^+-ATPase expression was determined as differential in dry and germinating embryos (Caliskan et al., 2003), it was interesting to establish whether expression only occurred as a short term effect during early germination or if it persisted in older differentiated tissues. The results obtained revealed that H^+-ATPase gene products expressed in all tissues of growing wheat embryos. Such a pattern of expression was expected since H^+-ATPase activity would be needed by cells within all germinating tissues. Germination entails rapid cell elongation, in which cell wall loosening is mediated by proton extrusion from the cell (as in the auxin-mediated “acid-growth” response) (Hager, 2003). Accumulation of proton pumping ATPase in the plasma membranes of the cells newly generated by post-germinative mitosis will clearly be required for such processes to operate.

Since germination specific plasma membrane H^+-ATPase was identified as a growth related gene; interest was focused as to whether conditions affecting wheat growth (GA, IAA, Mannitol, NaCl and ABA) would have an effect on its expression pattern. This analysis was carried out by the technique of RT-PCR. As seen in results, dramatic change was noticed only in 6 day leaves in which upon treatment the expression level of H^+-ATPase were raised. It was observed that in the case of NaCl and mannitol application the expression of H^+-ATPase was decreased particularly in 6 day roots. It should be noticed that both of these application are defined as osmotic stress factors. However, more rigorous quantitative ana-lysis will be necessary to determine whether the indi-cation given by this experiment represents a true case.

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