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Two Novel Intrinsic Annexins Accumulate in Wheat Membranes in Response to Low Temperature

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Four immunologically related proteins that belong to the annexin family were identified in cold acclimated wheat (*Triticum aestivum*). Two soluble forms with molecular masses of 34 and 36 kDa were found to bind phospholipid membranes in a calcium-dependent manner. These two forms are similar to the previously reported doublet in several plant species. The other two forms, with molecular masses of 39 and 22.5 kDa, were found associated with the microsomal fraction. Biochemical analysis showed that both forms are intrinsic membrane proteins and their association with the membrane is calcium independent. This is, to our knowledge, the first report of the presence of these annexin forms in plants. Membrane purification by two phase partitioning demonstrated that the p39 form is localized to the plasma membrane. Immunoblot analysis showed that the protein level of both p39 and p22.5 increases gradually reaching a maximum level after one day of low temperature exposure. The protein accumulation was similar in both hardy and less hardy cultivars, suggesting that the accumulation is not correlated with freezing tolerance. The results are discussed with respect to the possible role of these new intrinsic membrane annexins in low temperature signal transduction pathway.

Key words: Annexin — Calcium — Cold acclimation — Plasma membrane — Signal transduction — *Triticum aestivum*.

Cold acclimation (CA) is the process that allows hardy plants to develop freezing tolerance (FT) for winter survival. During this period, numerous physiological, biochemical and molecular changes occur. These include increased levels of proteins, sugars, osmolytes such as betaine and proline, lipid modifications, and the appearance of new enzyme isoforms (Guy 1990). It is also associated with complex modulations of energy production within the chloroplasts and remodelling of many membrane-associated processes such as endo/exocytosis, cytoskeleton architecture and water and ionic transport (Guy 1990, Hughes and Dunn 1996, Huner et al. 1998, Mazars et al. 1997).

It is not fully understood how the induction of these cellular changes is triggered by low temperature (LT). However, strong evidence supports the role of calcium as a second messenger in the early events leading to these changes. Previous research showed that the cytosolic calcium concentration increased following plant exposure to LT (Minorsky 1989, Knight et al. 1991, 1996, 1998). Studies employing calcium chelators and ionophores have demonstrated that cytosolic Ca^{2+} modulates LT-induced nuclear gene expression (Monroy et al. 1993, Monroy and Dhindsa 1995). It is believed that activation of Ca^{2+}-binding proteins such as calmodulin, calcium-dependent protein kinases (CDPK) and protein kinases C homolog (PKC), triggers a series of phosphorylation events that result in the expression of cold-regulated genes (Monroy et al. 1993, Monroy and Dhindsa 1995, Vazquez-Tello et al. 1998).

In spite of these advances, a major question remains concerning the identity of the LT sensor or receptor, responsible for the increased concentration of cytosolic calcium (Minorsky 1989, Knight et al. 1996, Ding and Pickard 1993). Since CA and induction of some LT-responsive genes depend on the influx of extracellular calcium (Knight et al. 1996, Monroy et al. 1993), it can be speculated that the sensor-receptor for LT may be associated with the plasma membrane. This led Ding and Pickard (1993) to propose that the LT sensor may be a calcium channel. Based on these studies it can be hypothesized that proteins such as annexins, which are calcium and phospholipid binding proteins capable of forming calcium channels in vitro, may be involved in the LT signal transduction pathway.

Annexins are a large family of calcium and phospholipid binding proteins. They are usually considered as soluble calcium dependent phospholipid binding proteins but recent reports have suggested that they could also be tightly associated with cellular membranes (Sheets et al. 1987, Valentine-Braun et al. 1987, Campos-Gonzales et al. 1989, Pula et al. 1990, Bianchi et al. 1992, Boustead et al. 1993, Futter et al. 1993, Tagoe et al. 1994, Böhm et al. 1994, Trotter et al. 1994, 1995, Blanchard et al. 1996, Liu

Abbreviations: CA, cold acclimation; DTT, dithiotreitol; FT, freezing tolerance; IPTG, isopropyl-1-thio-β-D-galactopyranoside; LT, low temperature; LT_{50}, temperature required to kill 50% of the plants; PEG, polyethylene glycol.

The nucleotide sequence reported in this paper has been submitted to Genbank under accession number AF006197.

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et al. 1997, Harder et al. 1997, Jost et al. 1997, Turpin et al. 1998). Annexins have a common structure consisting of a core domain of four or eight repeated sequences containing calcium and phospholipid binding sites. Mammals possess at least 10 different annexins that in addition to their core domain contain N-terminal tails of variable length (6 to 191 amino acids). This N-terminal region contains several regulatory regions such as phosphorylation and protein binding sites. A generally accepted observation is that each mammalian cell type expresses a specific subset of annexins that can be viewed as a cellular fingerprint (Raynald and Pollard 1994, Gerke and Moss 1997). Biochemical and molecular analysis of mammalian annexins have provided some insight to their diverse biological activities. These include their function as calcium channels and as inhibitors of protein kinase C and phospholipase A_2. They are also involved in phospholipid metabolism, fusion of membrane vesicles, regulation of exocytosis and receptor endocytosis, mitotic signalling and DNA replication (Raynald and Pollard 1994, Gerke and Moss 1997).

In plants, at least seven distinct annexin subfamilies have been identified (Clark and Roux 1995, Delmer and Potikha 1997, Morgan and Fernandez 1997). Molecular and cellular studies suggest that plant annexins, like their animal counterparts, are diverse in function, gene structure, expression and subcellular interactions (Clark and Roux 1995, Delmer and Potikha 1997, Morgan and Fernandez 1997, Kovacs et al. 1998). Given this diversity, it would not be surprising to find some plant annexins with unique functions in plant cells (Clark and Roux 1995).

In this study, we describe the identification and characterization of two new isoforms of wheat annexin proteins with molecular mass of 39 and 22.5 kDa. The level of both proteins increased rapidly in response to LT. Biochemical analysis demonstrates that both annexins are intrinsic membrane proteins that cannot be released from membranes when EDTA removes Ca^{2+}. We discuss in this report, the possible function of these proteins in LT signal transduction.

Materials and Methods

Plant material, growth and stress conditions—Wheat (*Triticum aestivum* L.) cultivars were germinated in water-saturated vermiculite for 7 d. Cool white fluorescent and incandescent lighting was combined to provide an irradiance of 250 μmol m^{-2} s^{-1}. The temperature was maintained at 20±1°C with a 15 h photoperiod under a relative humidity of 70±5%. After germination, control plants were maintained for specific periods under the same conditions of light and temperature. CA was performed by subjecting germinated seedlings to a temperature of 4°C with a 12 h photoperiod for different periods of time as specified for each experiment.

Salt stress was performed by incubating seedlings for 18 h in a nutrient solution containing 300 or 500 mM NaCl. ABA treatment was done by incubating seedlings for 48 h in a nutrient solution containing 0.1 mM abscisic acid. Polyethylene glycol-induced osmotic stress was performed by removing seedlings from vermiculite and incubating them in a solution containing 50% (w/v) of polyethylene glycol (average molecular mass of 8,000) for 24 and 48 h. Control plants were kept in water. Salicylic acid (1 mM) and hydrogen peroxide (10 mM) treatments were obtained by spraying the seedlings each day once for two days with a solution containing the product in 0.01% (v/v) Triton X-100 for better leaf adsorption. Control plants were sprayed with Triton X-100 only.

Production and purification of AnxLt1 proteins and antibodies—The open reading frame of *AnxLt1* which encodes an annexin from *Lavatera thuringiaca* (GenBank accession number: AF006197) was subcloned into the *Bam*HI/*Kpn*I site of pTRCHisA (Invitrogen). Expression of the recombinant annexin as a fusion protein containing six histidines at the N terminus (HIS-ANXLT1) was induced in *E. coli* XL1Blue by the addition of 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) for 4 h at 37°C. To evaluate protein expression, 100 μl of culture was harvested and the proteins were analysed by SDS-PAGE.

To produce large quantities of recombinant proteins, 2 liter of culture were prepared as described above. The cells were harvested by centrifugation at 5,000 × *g* for 5 min and resuspended in a minimal volume of binding buffer (5 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl, pH 7.9). The cells were sonicated using a Fisher ultrasonic probe BP-2 (Blackstone Ultrasonic Inc) on ice in short bursts until the solution turned clear. Cell debris were removed by centrifugation and the supernatant was incubated in one volume of His-Bind resin (Novagen) for 5 min on ice. Resin and bound proteins were pelleted by centrifugation, washed three times with binding buffer and then twice with the washing buffer (60 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl, pH 7.9). Recombinant proteins were eluted with 1 M imidazole, 0.5 M NaCl and 20 mM Tris-HCl, pH 7.9.

The recombinant proteins were further purified by preparative SDS-PAGE. The expressed protein of 41 kDa was excised, electroeluted for 3 h, precipitated with 4 volumes of acetone, resuspended in 0.85 M NaCl and stored for further experiments. The anti-HIS-ANXLt1 antibodies were raised in rabbit by injecting the purified HIS-ANXLT1 protein. The antibodies were purified on HIS-ANXLT1 coupled to Affi-gel-10 beads (Bio-Rad) at 3 mg ml^{-1} of bed resin in 0.1 M HEPES buffer, pH 7.5 containing 80 mM of $CaCl_2$. Free sites were saturated with 1 M Tris-Glycine for 1 h. The coupled resin was washed with phosphate-buffered saline (PBS) containing 0.1% NP-40. The immune serum was passed several times on a small column containing the beads. After washing with PBS alone, the bound antibodies were eluted with 0.3 M glycine, pH 2.0 and immediately neutralized with Tris base and stored in small fractions at −20°C.

Immunoblot analysis—Proteins in SDS loading buffer were separated on a 10% SDS-polyacrylamide gel (Laemmli 1970) and transferred electrophoretically for one hour to a 0.45 μm nitrocellulose membrane (MSI) without SDS in the transfer buffer. The membrane was blocked in a 4% (w/v) solution of reconstituted skimmed milk powder prepared in PBS containing 0.2% (v/v) Tween-20, and then probed with the anti-HIS-ANXLT1 antibodies at a 1 : 1,000 dilution overnight at room temperature. After washing with PBS-Tween, the proteins recognised by the primary antibodies were revealed with a horseradish peroxydase-coupled anti-rabbit IgG (Jackson Immunoresearch Inc.) at a 1 : 25,000 dilution. The complexes were visualised using the ECL chemiluminescent detection system (Amersham) and X/OMAT-RP film (Eastman-Kodak, Rochester, NY).

Extraction of calcium dependent phospholipid binding proteins—Calcium-dependent phospholipid binding proteins were purified as described by Blackbourn et al. (1992). Briefly, one week cold acclimated wheat shoots were ground in a chilled mortar with 5 volumes (per gram of fresh weight) of cold buffer (0.15 M NaCl, 10 mM HEPES, 10 mM EDTA pH 7.4, 2 mM DTT and 0.25 mM PMSF). The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 30,000 × *g* for 30 min at 4°C. Ca^{2+}-dependent phospholipid binding proteins were precipitated from the supernatant, containing the soluble proteins and the microsomal membranes, by the addition of bovine brain lipid (Sigma B-3635) at 1 mg g^{-1} of starting material and $CaCl_2$ to a final concentration of 15 mM. After 30 min on ice, the suspension was centrifuged at 30,000 × *g* for 30 min at 4°C. The pellet containing the calcium dependent phospholipid binding proteins and the microsomal membranes was washed twice with the initial buffer except that EDTA was replaced by 1 mM $CaCl_2$ to enrich the fraction in calcium dependent phospholipid binding proteins. Each wash was followed by centrifugation at 30,000 × *g* for 30 min at 4°C. The final pellet was resuspended in a minimal volume of the initial buffer containing 10 mM EDTA and centrifuged at 100,000 × *g* for 1.5 h at 4°C. Proteins from the supernatant of the EDTA wash were precipitated with 5 volumes of acetone and resuspended in SDS loading buffer. The proteins of the washed pellets containing the calcium-independent phospholipid binding proteins were extracted by the phenol extraction procedure (Hurkman and Tanaka 1986).

Isolation of microsomal and plasma membranes—Wheat shoots were ground in a mortar with the following extraction medium (50 mM MOPS-KOH, pH 7.6, 0.5 M sorbitol, 10 mM EGTA, 2.5 mM potassium metabisulfite, 4 mM salycylhydroxamic acid, 1 mM PMSF and 5% (w/v) PVP-30 soluble) (Uemura and Yoshida 1983). The homogenate was squeezed through two layers of Miracloth (Calbiochem) and then subjected to two successive centrifugation at 14,000 × *g* for 15 min and 156,000 × *g* for 30 min. For plasma membrane purification, the crude microsomal pellet was washed once with a solution containing 0.25 M sucrose and 10 mM KH_2PO_4, pH 7.8 and partitioned in an aqueous two-phase polymer system (Zhou et al. 1994). After phase partitioning, the upper phase (enriched in plasma membranes) and the lower phase (containing a mixture of other membranes) were diluted with 0.5 M sorbitol in a buffer containing 5 mM MOPS-KOH, pH 7.3, 1 mM EDTA, 0.1 mM PMSF, 10 mM KCl, and 2 mM DTT. Both suspensions were centrifuged at 156,000 × *g* for 30 min and membrane pellets resuspended in the same buffer. Enrichment of plasma membrane vesicles was determined by measuring the activity of the vanadate-sensitive ATPase as the marker enzyme (Uemura et al. 1995). Proteins from different fractions were extracted by the phenol extraction procedure for imunoblot analysis (Hurkman and Tanaka 1986).

Treatments with sodium carbonate and proteinase K—The crude microsomal fractions containing 200 μg protein were diluted with 4.9 ml of Na_2CO_3 0.1 M, pH 11 or water, incubated on ice for 30 min and centrifuged at 200,000 × *g* for 60 min at 4°C (Fujiki et al. 1982). Proteins from the pellet fraction were extracted by the phenol extraction procedure (Hurkman and Tanaka 1986). For the proteinase K treatments, the crude microsomal fractions were resuspended in 0.25 M sucrose and 10 mM KH_2PO_4, pH 7.8. Proteinase K (200 μg ml^{-1}) and/or Triton X-100 (w/v) were added as indicated. After incubation for 1 h on ice, proteinase activity was blocked by the addition of PMSF to a final concentration of 2 mM and the proteins extracted as described above.

Results

Identification of annexin-like proteins in wheat—To identify annexins from wheat tissues and to characterize the effect of LT and other stimuli on their accumulation, we produced polyclonal antibodies against a *Lavatera thuringiaca* annexin (ANXLT1). To this end, the *anxLt1* cDNA was subcloned in a procaryotic expression vector and the ANXLT1 protein was expressed in *E. coli* as a fusion product with a short histidine tag at its amino-terminal (HIS-ANXLT1). This recombinant protein migrates as a 41 kDa polypeptide on a SDS polyacrylamide gel (Fig. 1, lanes a–e). The anti-HIS-ANXLT1 rabbit polyclonal antibodies were affinity-purified and used for immunoblot analysis. This antiserum cross-reacted with the 41 kDa recombinant HIS-ANXLT1 whereas the preimmune serum did not. These polyclonal antibodies were further tested and were shown to cross-react positively with the common annexin protein doublet in the 10,000 × *g* soluble fraction in *Lavatera* and alfalfa suspension cells (Fig. 1, lanes f and g).

In the present work, we focused on the identification of immunologically-related annexin proteins from wheat. Immunoblot analysis showed that annexin proteins were undetectable in the 10,000 × *g* soluble fraction from different wheat tissues (shoots, crown and roots) of one week old seedlings. To circumvent the detection problem, we prepared annexin-enriched protein fractions that were selectively precipitated by the addition of calcium and a mixture of phospholipids (Blackbourn et al. 1992). The resulting pellet was washed with EDTA and both the EDTA-supernatant and washed-pellet were used for immunoblot analyses. Using this procedure, four immunologically-related

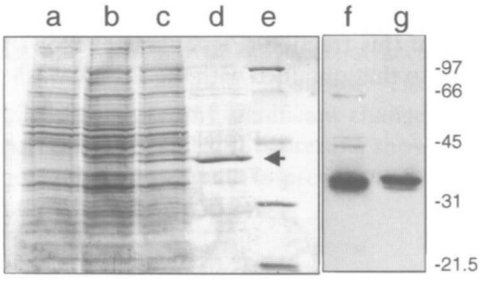

Fig. 1 Purification of the recombinant His-tagged annexin ANXLT1. Proteins were extracted and separated on a 10% SDS-polyacrylamide gel. Lanes a to e: the gel was stained with Coomassie blue R-250. Lane a, untransformed *E. coli*; lane b, transformed before induction with IPTG; lane c, transformed after induction with IPTG; lane d, purified recombinant his-tagged annexin; lane e, molecular mass markers (kDa). The arrow indicates the position of the 41 kDa fusion protein. Immunoblot detect band at the same position. Lane f: Immunoblot analysis of total proteins (10 μg) from *Lavatera thuringiaca* suspension cells; lane g: Immunoblot analysis of total proteins (10 μg) from alfalfa suspension cells.

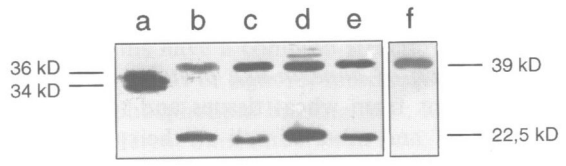

Fig. 2 Identification of annexin-like proteins in wheat shoots. Proteins were separated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the anti-ANXLT1 antibody. Lane a, proteins from the EDTA wash fraction extracted from 7 d cold acclimated shoots as described in the Materials and methods; lane b, proteins from the pellet fraction after the EDTA wash; Lanes c, d and e, crude microsomal fractions (10 μg) of shoot, crown and root respectively; Lane f, plasma membrane enriched upper phase fraction (10 μg) isolated from wheat shoots.

proteins were identified in 7 d cold acclimated wheat shoots. Two annexin-like proteins of molecular mass of 34 and 36 kDa (p34 and p36, respectively) were recovered in the EDTA wash-out (Fig. 2, lane a). This observation indicates that both p34 and p36 annexins are soluble proteins of low abundance in wheat at this growth stage. Their release from the pellet by EDTA suggests that their association with phospholipids is calcium dependent. The molecular mass of these proteins are similar to the previously reported annexin-doublet from several plant species (Clark and Roux 1995, Delmer and Potikha 1997). In addition, the antibodies detected two more annexin proteins of 39 and 22.5 kDa in the washed pellet fraction (Fig. 2, lane b). These annexins are probably associated to the EDTA washed-pellets containing the phospholipid precipitated microsomal membranes. To determine whether these two forms are localized in the microsomal fraction, a crude microsomal membrane fraction (156,000 $\times g$) from wheat shoots was prepared in the presence of EDTA. Immunoblot analysis of this fraction (Fig. 2, lane c) shows an identical pattern to that obtained with the EDTA-washed pellet (Fig. 2, lane b) confirming that indeed the p39 and p22.5 annexins are Ca^{2+} independent and are present in the crude microsomal membrane fraction. The relative abundance of p39 and p22.5 forms in the crude microsomal fractions of leaf, crown and roots tissues were similar (Fig. 2, lanes c–e). Furthermore, immunoblot analysis of plasma membrane enriched fractions from wheat shoots indicates that the p39 annexin is located in the plasma membrane (Fig. 2, lane f). This protein was not removed by subsequent EDTA washes.

The p39 and p22.5 annexins are intrinsic membrane proteins—To determine whether these proteins are extrinsic or intrinsically embedded in the membrane, we treated crude microsomal membrane preparations (156,000 $\times g$) with sodium carbonate. This treatment converts closed vesicles into open membrane sheets and causes the release of trapped and peripheral proteins ionically linked to the membrane. The immunoblot shown in Figure 3, lane b, indicates that this harsh treatment did not remove efficiently the p39 and the p22.5 annexins from the microsomal membranes, suggesting that both proteins are not ionically or loosely attached. In addition, we treated the crude microsomal and the purified plasma membrane fractions with proteinase K, which degrades peripheral proteins and protruding peptides from the membranes. The proteinase K treatment alone had very little effect on both proteins (Fig. 3, lanes c and g). However, in the presence of increasing concentrations of Triton X-100, a detergent that causes the disruption of the membranes, the p39 and p22.5 proteins were extensively degraded (Fig. 3, lane d, e and h).

Based on these results, we have classified the wheat annexin proteins into two groups. The soluble group is present in the cytosol and requires calcium for phospholipid association. This group includes both the p36 and the p34 forms. The other group is represented by annexins p39 and p22.5 that are intrinsically embedded in the microsomal membranes in a calcium independent manner.

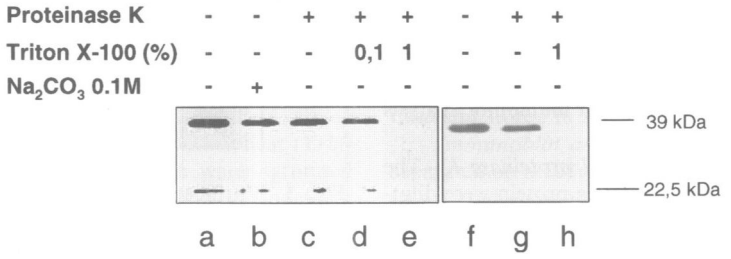

Fig. 3 Biochemical characterization of Ca^{2+}-independent annexins p39 and p22.5 in the microsomal and plasma membrane fractions of wheat shoots. Proteins were separated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the anti-HIS-ANXLT1 antibody. Lanes a to e: sodium carbonate treatment and proteinase K digestion of the crude microsomal fraction. Lane a, control microsomal fraction (10 μg); lane b, treated with 0.1 M-Na_2CO_3, pH 11; lane c, treated with proteinase K alone; lane d, treated with proteinase K in the presence of 0.1% (v/v) Triton X-100; lane e, treated with proteinase K in the presence of 1% (v/v) Triton X-100. Lanes f to h: proteinase K digestion of the plasma membrane enriched fraction. Lane f, control, the plasma membrane enriched upper phase fractions (20 μg); lane g, treated with proteinase K; lane h, treated with proteinase K and 1% (v/v) Triton X-100.

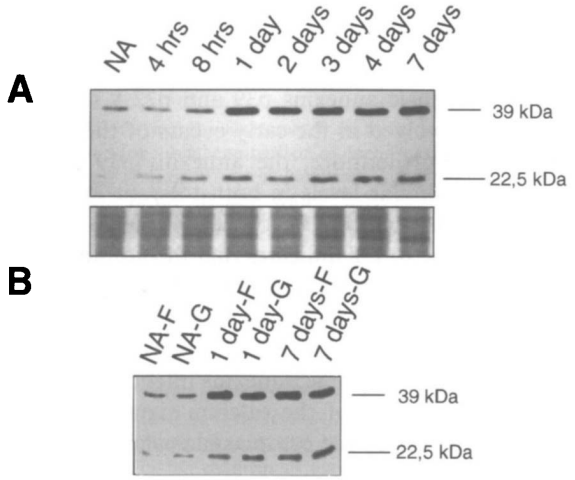

Fig. 4 Accumulation of annexin-like proteins in wheat shoots during cold acclimation. A, an equal amount of proteins (10 μg) from the crude microsomal fraction isolated from the winter wheat cv. Fredrick were separated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the anti-HIS-ANXLT1 antibody. NA, non-acclimated plants. Hours and days of cold acclimation at 4°C. Lower panel indicates the protein loads. B, accumulation of annexins in the cold tolerant winter wheat cv Fredrick (F) (LT_{50}, −15.6°C) and the less tolerant spring wheat Glenlea (G) (LT_{50}, −5.5°C). Proteins (10 μg) from the microsomal fraction were separated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the anti-HIS-ANXLT1 antibody.

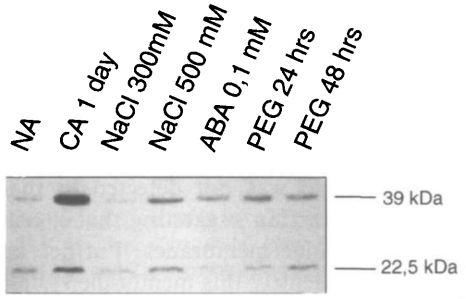

Fig. 5 Effect of different stresses on the accumulation of annexin-like proteins in wheat shoots. Equal amount of proteins (10 μg) from the microsomal fraction were separated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with the anti-HIS-ANXLT1 antibody. NA, non-acclimated; CA, cold acclimated; NaCl, salt stress; ABA, abscisic acid; PEG, polyethylene glycol.

However, we cannot rule out the possibility that calcium may be involved early in the integration of both p39 and p22.5 into the membrane.

Accumulation of p39 and p22.5 during cold acclimation—The accumulation of p39 and p22.5 was determined by immunoblot-densitometry in wheat seedlings grown at 20°C and at 4°C for different periods of time. Upon exposure to 4°C, both annexins accumulate gradually reaching a maximum level after one day, and then remained at a high level throughout the 7 d of the treatment (Fig. 4A). After one day of cold exposure, the protein level was 22 fold higher compared to the control plants grown at 20°C. This LT-induced protein accumulation occurs in a similar manner in the three tissues examined (shoots, crown and roots). Addition of calcium to the extraction buffer did not result in any increase of the level of both p39 and p22.5 in the micrososmal fraction. This suggests that there is no pool of free p39 and p22.5 that can bind to the membrane in a Ca^{2+} induced manner. On the other hand, we were unable to precisely determine the effect of LT on the relative accumulation of soluble p34 and p36 annexins. This was due to their very low abundance in the soluble extracts and the lack of quantitative recovery of these proteins by the calcium-phospholipid enrichment technique.

To determine if the LT induced accumulation of p39 and p22.5 is correlated with the capacity of wheat plants to develop FT, we examined two wheat cultivars differing in their acclimation capacity. The results shown in Figure 4B indicate that the proteins accumulate to similar levels during CA in both the hardy cultivar Fredrick (LT_{50}, −15.5°C) and the less hardy Glenlea (LT_{50}, −5°C). This demonstrates that the accumulation of annexins p39 and p22.5 is not correlated with FT. However, the rapidity of their accumulation during LT exposure suggests an important role for p39 and p22.5 during CA.

Accumulation of annexins during different stresses—To determine if annexins are induced by other treatments, we measured their levels after subjecting the plants to stresses such as NaCl, PEG, hydrogen peroxide and the application of the hormones ABA and salicylic acid. Results in Figure 5 indicate that osmotic stress induced by PEG and NaCl resulted in only partial accumulation of both p39 and p22.5 annexins in shoots. The other treatments failed to induce any significant changes in the protein levels. Taken together, these results show that the accumulation of both proteins is preferentially induced by cold treatments.

Discussion

Four different forms of annexin proteins have been identified in wheat using the polyclonal antibody anti-HIS-ANXLT1. Two annexins, p36 and p34, were found to be soluble and to bind phospholipid membranes in a calcium-dependent manner (reversible with EDTA). These two forms were also detected in *L. thuringiaca* and alfalfa. Annexin doublets of similar molecular mass (in the range of 32 to 36 kDa) have been previously identified in several other species (Clark and Roux 1995, Delmer and Potikha 1997). The other two annexin proteins of 39 and 22.5 kDa

were detected in the wheat microsomal fraction. Membrane purification by two phase partitioning demonstrated that p39 annexin is associated with the plasma membrane. Recently, a 39 kDa annexin have been microsequenced in a large proteome analysis of plasma membrane protein from *Arabidopsis thaliana* (Santoni et al. 1998). On the other hand, the p22.5 form was not detected in the purified plasma membrane fraction suggesting that it may associated with other cellular membranes. Further analysis is needed to precisely identify this membrane system.

Biochemical analysis of crude and purified plasma membranes demonstrated that p39 and p22.5 are intrinsic membrane proteins and their association with the membrane was calcium independent. Therefore, they are novel annexins that differ from wheat p34 and p36 and from other plant annexins, which are dissociated from membranes when calcium is removed with EDTA. This is, to our knowledge, the first report describing EDTA resistant intrinsic annexins in plants. Several animal annexins, similar to those found in wheat, have been identified. Annexin subtypes I, II, V and VI, the most studied annexins, were initially classified as soluble calcium dependent phospholipid binding proteins. However, they were found recently to be present in a second state, where EDTA cannot reverse their association to the membrane (Sheets et al. 1987, Valentine-Braun et al. 1987, Campos-Gonzales et al. 1989, Pula et al. 1990, Bianchi et al. 1992, Boustead et al. 1993, Futter et al. 1993, Tagoe et al. 1994, Böhm et al. 1994, Trotter et al. 1994, 1995, Blanchard et al. 1996, Liu et al. 1997, Harder et al. 1997, Jost et al. 1997, Turpin et al. 1998). Characterization of some of these membrane bound annexins revealed that they behave, as wheat p39 and p22.5, like intrinsic proteins (Bianchi et al. 1992, Harder et al. 1997). Based on these observations, it is plausible that p39 and p22.5 could also exist as soluble calcium-dependent phospholipid binding proteins.

Several studies have revealed that a pH of around 6.0, a high calcium concentration, specific protein-protein interactions or the phosphorylation state are important factors that regulate the integration of animal annexins into the membrane (Johnston et al. 1990, Bianchi et al. 1992, Hoekstra et al. 1993, Trotter et al. 1997, Harder et al. 1997, Jost et al. 1997, Köhler et al. 1997, Rosengarth et al. 1998, Langen et al. 1998). In our experiments the pH was buffered at a physiological level and because the inclusion of calcium in the microsomal extraction buffer had no effect on the recovery of p39 and p22.5, we believe that the intrinsic state of p39 and p22.5 may be induced by a specific protein-protein interaction or by phosphorylation.

Possible function of p39 annexin during cold exposure—In our studies on LT gene regulation in wheat, we became interested with the possible involvement of annexin proteins in the LT-signal transduction pathway. Several lines of evidence indicate that calcium binding proteins play an important role in LT signal transduction (Knight et al. 1991, 1996, 1998, Monroy et al. 1993, Monroy and Dhindsa 1995). The rapid and high LT-induced accumulation of the intrinsic annexins p39 and p22.5 suggest that both may be involved in the early events of the LT signal transduction. Furthermore, the annexins p39 and p22.5 accumulate to similar levels in both tolerant and less tolerant wheat cultivars used in our experiment. This similarity of response to LT indicates that the accumulation of these annexins is not correlated with FT. It may represent a general response to LT, usually associated with the first phase of CA. Based on these observations, we speculate that the intrinsic membrane annexins might play a role, as sensors or transducers of the calcium signal triggered by LT or as regulators of the cytoplasmic calcium concentration.

In animal systems, several of the extracellular signals are first perceived by membrane-associated receptor proteins. Annexins are known to be major phosphorylation substrates of many of these receptors (Rothhut 1997). In plants, a class of receptors with unknown function named RLK for receptor like kinases has been identified (Lease et al. 1998). These receptors have the structural features of a transmembrane protein kinase, with an intracellular kinase domain and an extracellular ligand-binding domain. These features are highly reminiscent of the animal receptor kinase family, such as the large class of tyrosine receptor kinases and a few serine-threonine receptor kinases that are involved in transmembrane signal transduction. The transcript of one of these receptors like kinases (RPK1) which encodes a putative serine/threonine kinase is known to be induced by cold, dehydration, abscisic acid and high salt treatments in *Arabidopsis thaliana* (Hong et al. 1997). It is thus conceivable that phosphorylation of p39 by an RLK may activate its translocation to the plasma membrane in a Ca^{2+} independent intrinsic state. At this location, annexin p39 may act as a calcium channel as suggested by several electrophysiological and crystal structure data using animal annexins (Raynald and Pollard 1994, Gerke and Moss 1997). Additional experiments are required to verify this hypothesis.

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