Expression of a Lipid Transfer Protein in *Escherichia coli* and Its Phosphorylation by a Membrane-Bound Calcium-Dependent Protein Kinase

M.L. Martin*, E. Espinosa Vidal and L. de la Canal

FINAL

Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Casilla de Correo 1245, Mar del Plata (7600), Argentina

Abstract: Ha-AP10 is a basic antifungal peptide from sunflower seeds (*Helianthus annuus* antifungal peptide of 10 kDa) belonging to the family of plant lipid transfer proteins. We report here its expression in *E. coli* [Glutathione S-transferase (GST) system] and its phosphorylation by endogenous membrane-bound calcium-dependent protein kinases.

Keywords: Calcium-dependent protein phosphorylation – GST expression system - *Helianthus annuus* - Lipid Transfer Protein

INTRODUCTION

Plant lipid-transfer proteins (LTPs) are small (91 to 95 residues), basic polypeptides that were first characterized in potato in the mid-1970s [1]. They share several structural features, the most striking being eight strictly conserved cysteine residues engaged in four disulfide bridges [2]. Cellular localization of LTPs is complex, although the great majorityes are localized extracellularly, there are some intracellular LTPs. For example the intracellular LTP in Vigna unguiculata seeds are localized in protein storage vacuoles and in lipid-containing vesicles [3] as well as the LTP localized in the glyoxysome matrix of castor been cotyledon cells [4]. Despite efforts of numerous research groups, the role of these proteins is still a matter of debate. Based on experimental results, several hypotheses have been suggested. The *in vitro* lipid transfer capacity shown by Kader [1] suggested that these proteins were involved in the intracellular traffic of phospholipids. However, the extracellular location of the majority of the LTPs and the fact that all the sequences known to date exhibit a secretion signal in the N-terminal part of the peptide seem to rule out such a role for LTPs. Several results suggested a role for LTPs in plant defense mechanisms; however LTPs have been implicated in many other functions in plants [2]. Initially, the defense function for LTPs was proposed taking into account the ability of certain members of this family to inhibit the growth of fungal and bacterial pathogens *in vitro* [5,6] but this role has further been supported by other experimental evidence. First, their expression patterns and apoplastic localization are consistent with a defense role [2]. Secondly, transgenic Arabidopsis and tobacco plants with constitutive overexpression of an antimicrobial LTP gene displayed a reduction in the symptoms caused by the bacterial pathogen Pseudomonas syringae [7]. These observations suggest that at least certain LTPs may control the growth of microbial pathogens. However, two recent reports have demonstrated the possible existence of novel roles for LTPs in plant defense. Particularly, it has been reported that LTPs from wheat [8] and tobacco [9] bind to a plant receptor involved in the control of defense responses (the elicitin receptor) and it has been postulated that LTPs may be involved in the signaling pathways leading to the hypersensitive response triggered by elicitins [8, 9]. In addition, an LTP-like gene from Arabidopsis is involved in the production/transmission of the mobile signal in the systemic acquired resistance response [10]. Taking into account these reports, LTPs could act either as antimicrobial proteins of direct effect or as signaling molecules.

LTPs have been reported to be substrates for calcium-dependent protein kinases (CDPKs) *in vitro*. LTPs isolated from wheat [11,12], barley [11] and petunia [13] are phosphorylated by a calcium-dependent protein kinase (CDPK) isolated from wheat embryos. These works describe the CDPK-catalyzed phosphorylation sites on LTPs. However there is no evidence that LTPs are phosphorylated in *vivo*. In addition, the physiological relevance and the functional consequences of phosphorylation of LTPs are actually unknown.

Ca^{2+}-dependent protein kinases (CDPKs), which are only found in plants, green algae, and certain protists, play important roles regulating downstream components of calcium signaling. The basic structural features of CDPKs are conserved. Within a single polypeptide chain, these kinases contain three functional domains: catalytic, autoinhibitory, and calcium binding [14-17]. CDPKs exhibit diverse subcellular localization patterns, suggesting that they participate in a wide variety of signaling pathways. CDPKs have been localized in the cytosol, nucleus [18], cytoskeleton [18,19], oil bodies of endosperm cells [20], endoplasmic reticulum [18,21], plasma membrane [18,22-24], and peroxisomes [18]. A subset of these kinases contains a src homology domain (SH4) at the N-terminal portion of the molecule that targets them to the membrane fraction through lipid modifications [25].

We previously isolated and characterized a basic antifungal peptide, Ha-AP10 (*Helianthus annuus* antifungal peptide of 10 kDa), belonging to the family of plant LTPs [26]. Molecular characterization of its cDNA clone [27] shows that it is homologous to ltp4 from *Arabidopsis thaliana* [28] and

*Address correspondence to this author at the Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Casilla de Correo 1245, Mar del Plata (7600), Argentina; Tel: (011) 0223-475-3030; Fax: (011) 0223-475-3150;
E-mail: marimart@mdp.edu.ar

0929-8665/07 $50.00+.00

presents a signal peptide that directs it to the secretory pathway. We have shown that Ha-AP10 behaves as a typical antimicrobial peptide demonstrating its ability to induce the permeabilization of fungal spores [29]. This was the first demonstration of fungal membrane damage by an LTP, giving a clue to elucidate the basis of its antimicrobial properties.

This work describes the recombinant expression of Ha-AP10. In addition, the present paper reports the phosphorylation of Ha-AP10 by a membrane-bound calcium-dependent protein kinase.

MATERIALS AND METHODS

Plant Material

Seeds (*Helianthus annuus* line PSC8) were provided by ADVANTA SEMILLAS, Centro Biotecnológico Balcarce, Argentina. Sunflower seeds were imbibed in sterile water during 16 hours at 25 °C in the dark to trigger the germination process.

GST-Ha-AP10 Fusion Protein Expression and Purification

For the production of the sunflower LTP Ha-AP10, an inducible expression system using the expression vector pGEX was chosen. PCR mutagenesis of the plasmid pGEM-T-Ha-AP10 [27] was used both to delete the sequence encoding this signal peptide, and to introduce restriction enzyme recognition sites to allow the sequence encoding the Ha-AP10 mature to be cloned into the chosen expression vector. Plasmid pGEM-T-Ha-AP10 was used as template in PCR reactions in the presence of the following primers: LTP-6 (5'-CGCGGATCCATTACTTGCAATGATG-3') primer containing *BamHI* site (in underscore) and LTP-8 (5´-CGCGAATTCTCAAGGAATGGTGTTACAG-3´) primer containing *Eco*R1 site (in underscore) to amplify mature Ha-AP10. The amplified PCR product was cloned into *Bam*H1/*Eco*R1 sites of pGEX-4T-3 pLysS (Amersham Pharmacia). The resulting recombinant vector (pGEX-Ha-AP10) was sequenced to verify that no mutation had been introduced within the coding sequence of the Ha-AP10 during amplification or cloning. Then the expression vector pGEX-Ha-AP10 was introduced into *E. coli* BL21 (DE3) strain. Bacteria containing the recombinant expression vector was grown at 37 °C in 2X YTA medium supplemented with 100 µg/mL ampicillin until OD_{600} 0.8 . The bacteria were induced by addition of 0.1 or 0.5 mM IPTG to the culture medium. After different times cells were harvested by centrifugation 20 min at 7 700 g and resuspended in lysis buffer (PBS 1X containing a protease inhibitors mix: 140 µM pepstatin A, 210 µM leupeptin, 140 µM antipain, 280 µM bestatin, 25 mM EDTA, 50 µM PMSF). The *E. coli* cells were sonicated in portions of 10 mL for 5 pulses of 30 s at 60 W output with the broad tip of a High Intensity Ultrasonic Processor. To solubilize 1 % Triton 100X was added and shaken for 30 min at 4 °C. The suspension was centrifuged at 12 000 g for 10 min at 4 °C to separate the insoluble material from the soluble fraction. The pellet, containing the inclusion bodies, was resolubilized by gentle shaking using 6 M urea.

Purification of the glutathione S-transferase fusion protein was carried out according to protocols supplied by the manufacturer, using a glutathione Sepharose matrix (Amersham Pharmacia). Protein preparations were analyzed for purity and correct size on SDS-PAGE following the manufacturer's procedures and visualized by Coomassie Blue staining.

Protein concentration was determined by the bicinchoninic acid assay with BSA as standard and according to the manufacturer's instruction (Sigma).

Preparation of Homogenates of CDPKs from Soluble and Microsomal Membrane Fractions

All procedures were performed at 4 ºC. Germinating seeds (0.5-2 g) imbibed for 16 h were homogenized in three volumes of extraction buffer (50 mM Tris-HCl pH 7.5, 2 mM DTT, 0,1 mM EDTA, 5 mM NaF, 1 mM Na_2VO_4, 20 mM β-glycerophosphate and protease inhibitors) and extracted for 10 min at 4 ºC. The extracts were filtered through cheesecloth and centrifuged twice at 10 000 g for 10 min to remove cell debris. The homogenate was centrifuged at 100 000 g for 1 h obtaining a soluble fraction (kept for protein kinase activity assays) and the pellet. The pellet was washed in extraction buffer and the microsomal membrane fraction was obtained by resuspending the pellet during one hour at 4 ºC in the same buffer containing 1 % (v/v) Nonidet P40.

In Silico Analysis of Potential Phosphorylation Sites on Ha-AP10

Complete sequences of the sunflower Ha-AP10 was aligned with homologous sequences of the phosphorylable LTPs from wheat (WPB1A and WBP1B [11, 12]), barley (LTP1 [12]), petunia (PET1 and PET2 [13]) and the consensus sequence for LTPs by using Clustal format for T-Coffee Version 1.41 [39].

Phosphorylation of Ha-AP10 *In Vitro* Using Pull Down Assays

GST or GST-HaAP10 from the fraction purified from the *E. coli* culture supernatant bound to glutathione-Sepharose beads (approximately 5 µg) were used as substrate. Aliquots of 20 µL (containing 80 µg of soluble proteins or 20 µg of membrane proteins, see above) were assayed in a final volume of 40 µL of reaction mixture containing: 50 mM Tris-HCl pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 10 µM ATP, 5 µCi [γ-^{32}P]ATP (6000 Ci.mmol^{-1}, NEN Dupont), 5 mM NaF, 1 mM Na_2VO_4, 20 mM β-glycerophosphate, protease inhibitors (140 µM pepstatin A, 210 µM leupeptin, 140 µM antipain, 280 µM bestatin, 25 mM EDTA, 50 µM PMSF) and $CaCl_2$ or EGTA at the indicated concentration. Reactions were incubated with gentle shaking for 15 min at room temperature and stopped by adding EDTA to a final concentration of 25 mM. The glutathione-Sepharose beads were pelleted and washed three times with PBS buffer containing protein phosphatases inhibitors (5 mM NaF, 1 mM Na_2VO_4, 20 mM β-glycerophosphate) and proteases inhibitors (140 µM pepstatin A, 210 µM leupeptin, 140 µM antipain, 280 µM bestatin, 50 µM PMSF). GST and GST-Ha-AP10 proteins were eluted from the matrix by boiling in SDS-PAGE loading buffer fractionated by SDS-PAGE and stained by Coomassie Brilliant Blue R250. The stained gel was dried and autoradiographed.

Protein Gel Blot Analysis

Aliquots of homogenates, soluble and membrane fractions were resolved in 15 % SDS-PAGE and electroblotted onto nitrocellulose membranes. Molecular weight markers were identified by staining the membrane with Red Ponceau S [30]. We used a primary antibody against the Ha-AP10 protein (dilution 1:8000) [26]. The LTP was visualized after incubation with a secondary antibody using the 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate system according to the manufacturer's directions (Sigma).

RESULTS AND DISCUSSION

Production of the Recombinant GST-Ha-AP10 Protein

The heterologous production of LTPs in prokaryotic and eukaryotic systems has previously been reported [31-34]. In this regard, a soluble wheat LTP has been produced in cytoplasm of *E. coli* fused to maltose binding protein (MBP). However, the purification yield was low and the purified protein was found to be degraded after the cleavage by the factor Xa [31]. When produced as an unfused protein with the pET expression system the wheat LTP accumulated in inclusion bodies [34]. The existence of four disulfide bridges in LTPs could be responsible for the improper folding of the wheat LTP in *E. coli*. The use of eukaryotic system such as *P. pastoris* was used to overcome this problem. However the LTP was improperly processed and the protein carried three extra amino acids [33].

Taken into account these evidences, we choose the glutathione S-transferase (GST) gene fusion system for the production of the sunflower LTP Ha-AP10. The system is based on inducible high-level expression of genes as fusion with GST. This expression system has important advantages: 1) Simple and fast purification, fusion proteins could be rapidly purified by affinity in a single chromatographic step; 2) Avoid toxicity of the recombinant protein. This is an inducible system, and this characteristic prevented problems due to possible toxicity of the recombinant protein when expression is not tightly controlled within *E. coli* cells; 3) Improve solubility of the recombinant protein; and 4) GST-fusion proteins can be used as a vehicle to determine the crystal structures of the attached small peptides [35].

The construction of the expression vector carrying the sequence encoding Ha-AP10 was realized as described in Materials and Methods. The resulting plasmid, pGEX-Ha-AP10, was used to transform *E. coli* BL21 (DE3). Expression conditions were optimized by using different inductor (IPTG) concentrations (0.1 and 0.5 mM) during different times of induction (0, 1, 3 and 16 hours). Bacterial pellets were harvested, disrupted directly in the gel loading buffer and the proteins were analyzed by SDS-PAGE. After the Coomassie staining of the gel, the accumulation of a polypeptide with the expected electrophoretic mobility for the fusion protein (39 kDa) was detected in induced bacteria carrying the pGEX-Ha-AP10 plasmid (+IPTG) but was absent from uninduced control (-IPTG) bacteria (Fig. **1A**). We found both IPTG concentrations tested to be equally effective for the induction of the 39 kDa protein so, for further assays the lowest concentration of IPTG (0.1 mM) was chosen. The expression of GST-Ha-AP10 increased during the time of induction reaching the highest levels at 16 hours (Fig. **1A**).

In addition to the 39 kDa protein, peptides with an estimated molecular weight between 24 and 37 kDa were also detected upon induction. In order to avoid the presence of these peptides culture conditions were optimized. The relative proportions of these peptides increased with the time of induction (Fig. **1A**). Moreover, the addition of protease inhibitors just before disrupting cells was unable to avoid the presence of these peptides (data not shown) suggesting they are not a result of protein degradation and probably they could be abortive peptides. There are evidences that bacterial systems could not recognize efficiently codons more regularly used by the eukaryote translational machinery producing delays and interrupting protein synthesis.

In conclusion, the best condition to obtain a high production of fusion protein with the lowest amount of low molecular weight peptides (24-37 kDa) was 0.1 mM IPTG and one hour of induction (Fig. **1A**).

A protein of the expected molecular mass for GST (29 kDa) was detected in lysates of cells transformed with plasmid pGEX as a control (Fig. **1A**). In addition, a higher production of GST than GST-Ha-AP10 was obtained.

It is known that recombinant proteins overproduced in bacteria often form inclusion bodies to store insoluble proteins. To determine the solubility of the recombinant protein GST-Ha-AP10, the lysates obtained from induced bacterial culture were centrifuged to separate soluble from insoluble proteins. The resulting supernatant and pellet were analyzed by SDS-PAGE. As seen in Fig. **1B**, a relatively high amount of the recombinant GST-Ha-AP10 fusion protein was in soluble form (70 % by densitometric analysis), while some amount of the fusion protein was also found in inclusion body. As Ha-AP10 is a low-molecular-mass cystein-rich protein the production of a fusion with GST probably improved its solubility. The GST control was also detected mainly in the soluble fraction (Fig. **1B**).

Purification of the GST-Ha-AP10 fusion protein was carried out using a glutathione-Sepharose chromatography. To control the homogeneity of the eluted fraction and to confirm its identity, the purified protein was analyzed by SDS-PAGE (Fig. **2A**) and protein gel blotting followed by immunodetection with antiserum against Ha-AP10 isolated from sunflower seeds (Fig. **2B**). The purified fusion protein showed one clear band of the expected molecular mass (39 kDa) and additional bands of lower intensity corresponding to peptides of 24-37 kDa in SDS-PAGE. A GST-sized (29 kDa) protein consistent with that encoded by the parent vector without the insert was purified as a control (Fig. **2A**). Antibodies developed against Ha-AP10 purified from sunflower seeds recognized specifically the GST-Ha-AP10 fusion protein, while the control GST and polypeptides of 24 and 37 kDa were not immunodetected (Fig. **2B** right panel). As a control of protein transference the nitrocellulose membrane was stained with Red Ponceau (Fig. **2B** left panel).

A sufficient quantity of recombinant GST-Ha-AP10 (13.72 $mg.L^{-1}$ bacterial culture) was obtained for biochemical and functional studies. A similar production was obtained for a wheat LTP [31].

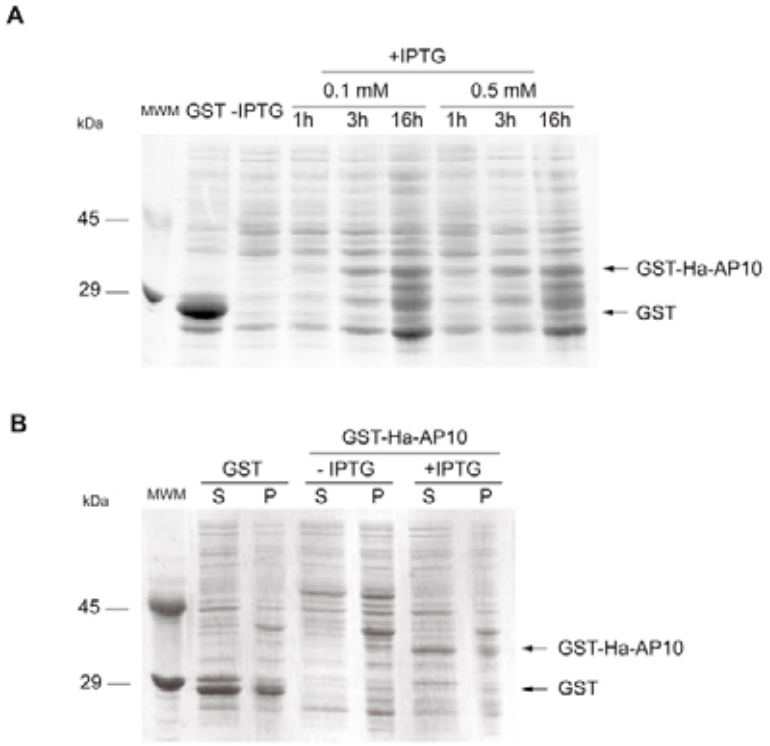

Figure 1. *Expression of the recombinant GST-Ha-AP10 protein*. *E. coli* containing the plasmid pGST-Ha-AP10 (GST-Ha-AP10) and the empty plasmid (GST control) were cultivated at 37 °C and when the growth OD600 reached 0.8, IPTG (0.1 and 0.5 mM) was added and cultivation continued for 1, 3 and 16 h. Bacterial pellets were harvested and disrupted directly in the gel loading buffer. A) Coomassie-stained SDS–PAGE from uninduced control cell lysate (-IPTG) and IPTG induced cell lysates (+IPTG). B) Total cell lysates induced with 0.1 mM IPTG 1h (+IPTG) and uninduced control lysate (-IPTG) were centrifuged (12 000 g, 10 min, 4°C) yielding a soluble fraction and an insoluble pellet. Soluble fraction (S) and pellet solubilized with 6M urea (P) were analyzed by SDS-PAGE stained with Coomassie blue. The arrows indicate the GST control protein and the GST-Ha-AP10 protein. MWM: Molecular weight markers.

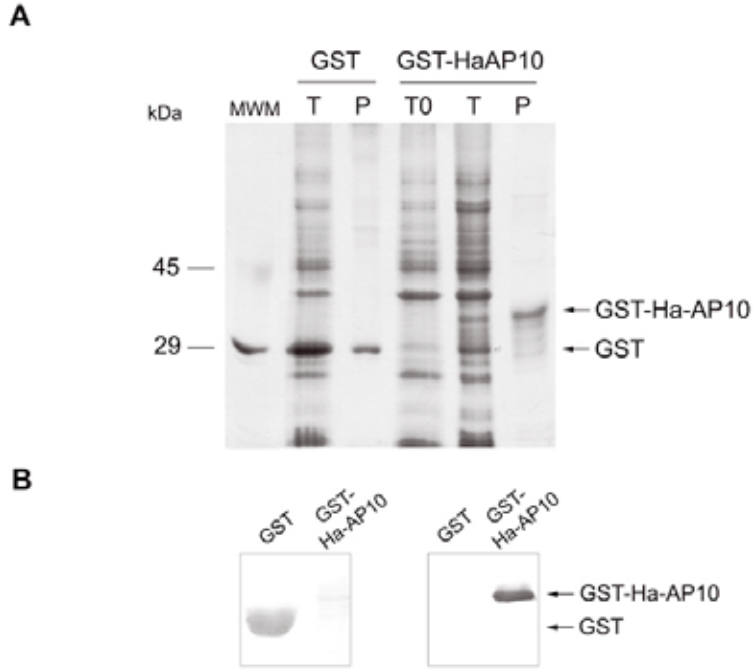

Figure 2. *Purification and immunoblot analysis of the recombinant GST-Ha-AP10 protein.* A) SDS-PAGE analysis stained with Coomassie blue of the total cell lysate (T) and the purified fraction (P) for the recombinant GST-HaAP10 and the GST control. t0: uninduced cell lysate. MWM: Molecular weight markers. B) Immunodetection of the purified recombinant GST-Ha-AP10 and the purified GST control after the incubation with an antiserum raised against sunflower Ha-AP10 (right panel). Proteins stained with red ponceau (left panel). The arrows indicate the GST control protein and the recombinant GST-Ha-AP10.

In Silico Analysis of Potential Phosphorylation Sites in Ha-AP10

To evaluate the presence of a potential phosphorylation site on Ha-AP10, we have looked for phosphorylation site motif found for synthetic peptides substrates of plant CDPKs (Basic-X-X-Ser/Thr) [36] and phosphorylation sites determined for LTPs: wheat WBP1A/B LAPCIS^{17}YAMGR and KQQAS^{59}GI/MGGIK [12]; barley LTP1 PCLT^{17}Y [11]; petunia Pet1 SQAS^{40}TTP and Pet 2 GLPS^{71}TCG [13]. As was reported for others LTPs [11-13], the Basic-X-X-Ser/Thr motif found with many synthetic peptide substrates and various protein substrates of plant CDPKs is absent on Ha-AP10 sequence. An alignment of amino acid sequences of LTPs known to be phosphorylated and Ha-AP10 was performed (Fig. **3**). Wheat WBPs and barley LTP1 are both phosphorylated on Ser and Thr respectively, within homologous sequences, while petunia PET1 and PET2 are both phosphorylated on Ser residues within non-homologous sequence. Phosphorylable Ser/Thr on the LTPs is replaced by P, A, R, Q, K or G residues in other LTPs, including Ha-AP10. Hence, we could not identify a potential phosphorylation site motif on Ha-AP10 sequence. However, the absence of phosphorylation site motifs homologous to those identified in others LTPs does not rule out the possibility that Ha-AP10 could be phosphorylated *in vivo*. In that sense, it is interesting to mention the presence of non-homologous phosphorylation sites in this family of proteins (Fig. **3**). Moreover, the analysis of Ha-AP10 with predictors of phosphorylation sites (Phosphobase and NetPhos) showed the presence of potential phosphorylation sites for others protein kinases (casein kinase I, casein kinase II and protein kinase A).

In Vitro Ha-AP10 Phosphorylation

To examine whether the Ha-AP10 protein is phosphorylated by protein kinases present in sunflower seeds pull down assays were performed. The GST-Ha-AP10 fusion protein from the fraction purified from the *E. coli* culture supernatant bound to glutathione-Sepharose beads was incubated with [γ^{32}P]-ATP and seed extracts as a source of enzyme. The protein was pelleted and eluted by boiling in SDS-PAGE and then autoradiographed. Parallel incubations using GST beads as substrate were also performed to analyze the specificity of the assay. GST-Ha-AP10 was effectively phosphorylated as demonstrated by the visualization of a strong band of phosphorylated protein of 39 kDa (Fig. **4**). However, the control using GST as substrate was also phosphorylated suggesting the presence of protein kinases able to phosphorylate GST protein in homogenates of sunflower seeds. Pull down assays using unbound glutathione-Sepharose beads showed the absence of phosphoproteins bound non-specifically (Fig. **4**). The addition of an excess of free GST protein to the GST-HaAP10 pull down assay reduced but not eliminated fusion protein phosphorylation suggesting that both proteins, GST and Ha-AP10, were phosphorylated (Fig. **4**). Taken into account these evidences, this assay condition can not be used to study Ha-AP10 phosphorylation.

Ha-AP10 is Phosphorylated by Membrane-Bound Protein Kinases Present in Sunflower Seeds

In an effort to distinguish the sources of phosphorylation of the GST and Ha-AP10 moieties, sunflower homogenates were fractionated in a soluble and a microsomal membrane fraction for further use in pull down phosphorylation assays. Membranes were solubilized with a buffer containing 1% (v/v) Nonidet P40 and GST-Ha-AP10 phosphorylation was assayed in both fractions. Results obtained shows that GST-Ha-AP10 and not GST is phosphorylated by a membrane bound protein kinase (Fig. **5A**). It was also observed that in the presence of 1 mM EGTA phosphorylation of the protein was partially blocked. On the other hand, in the soluble fraction both GST and GST-Ha-AP10 were radiolabeled avoiding a detailed analysis of Ha-AP10 phosphorylation (Fig. **5B**). These results suggest that a Ha-AP10-kinase is local-

Figure 3. *In silico* analysis of potential phosphorylation sites on Ha-AP10. Complete sequences of the sunflower Ha-AP10 are aligned with homologous sequences of the phosphorylable LTPs from wheat (WPB1A and WBP1B [11, 12]), barley (LTP1 [12]), petunia (PET1 and PET2 [13]) and the consensus sequence for LTPs. (-) space introduced for alignment of homologous sequences, (X) unknown amino acid, (grey boxes) determined phosphorylation sites, (underlined) phosphorylable amino acid. Clustal format for T-Coffee Version 1.41 [39].

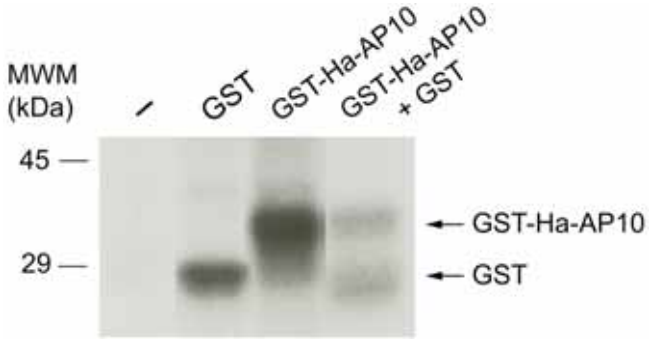

Figure 4. *In vitro* phosphorylation of the recombinant GST-Ha-AP10 using pull down assays. Glutathione-Sepharose beads bound to GST, GST-Ha-AP10 and unbound beads were incubated with [g32P]-ATP and seed extracts. The addition of an excess of free GST protein was assayed (GST-Ha-AP10 + GST). The proteins were pelleted and eluted by boiling in SDS-PAGE and then autoradiographed. The arrow indicates the GST control protein and the recombinant GST-Ha-AP10. Molecular weight markers are indicated on the left.

The *in vitro* phosphorylation of GST by mammalian protein kinase C-α at Ser-93 has already been reported [37] and our results demonstrate that GST is also phosphorylated by protein kinase/s present in soluble fraction of sunflower seeds homogenates.

As shown in Fig. **5B** an additional band of approximately 45 kDa was phosphorylated by a calcium-dependent protein kinase. The presence of this band could be eliminated by preclearing (preincubating homogenates with glutathione-Sepharose 4B, data not shown) suggesting that this ^{32}P-labeled protein of 45 kDa is an endogenous and soluble seed protein that bound non-specifically to the matrix.

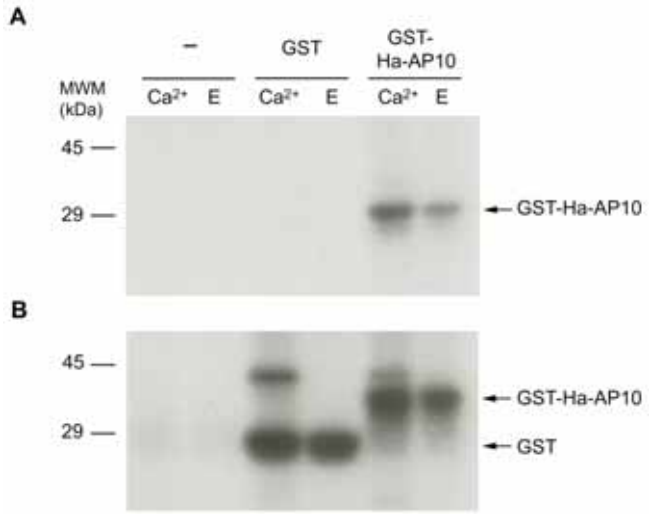

Figure 5. *Phosphorylation of Ha-AP10 by a membrane-bound protein kinase.* Glutathione-Sepharose beads bound to GST, GST-Ha-AP10 and native unbound were incubated with [g32P]-ATP and soluble (A) or microsomal fractions (B) in the presence of 200 mM calcium (Ca2+) or 1 mM EGTA (E). The protein was pelleted and eluted by boiling in SDS-PAGE and then autoradiographed. The arrow indicates the GST control protein and the recombinant GST-Ha-AP10. Molecular weight markers are indicated on the left.

ized in membranes and the GST-kinase is a soluble protein. We could not rule out the existence of a soluble Ha-AP10 kinase.

Properties of the Membrane-Bound Ha-AP10-Kinase

The majority of calcium-stimulated kinases found in plants extracts are associated with CDPKs. These enzymes require micromolar concentration of free calcium and are inhibited by calcium antagonists. To assess whether the Ha-AP10-kinase activity found in membranes from germinating seeds was a CDPK, we measured the kinase activity in presence of micromolar and millimolar calcium concentration. Fig. **6** shows that GST-Ha-AP10 phosphorylation occurred at very low levels of calcium (micromolar) and it is partially inhibited by the presence of 1 mM EGTA. Additional experiments using higher EGTA concentrations (10 mM) still allow us to detect Ha-AP10 kinase activity (data not shown). This result suggests that Ha-AP10 could be phosphorylated at different sites by protein kinases Ca^{2+}-dependent and Ca^{2+}-independent. The presence of multiple phosphorylation sites on LTPs had previously been demonstrated [12].

In addition, the phosphorylating activity was inhibited by H7, an inhibitor of protein kinase C that also affects the activity of CDPKs, but not by calmodulin antagonists: CPZ and W-7 (Fig. **6**).

Figure 6. *Characterization of the membrane-bound Ha-AP10-kinase.* GST-Ha-AP10 bound to glutathione-Sepharose beads was incubated with [g32P]-ATP and microsomal fractions in presence of 1 mM, 200 mM, 1 mM calcium (Ca2+) and 1 mM EGTA (E). To study the effect of protein kinase inhibitors microsomal fractions were pre-incubated 15 min with 1 mM W7, 1 mM H7 and 3 mM CPZ. The protein was pelleted and eluted by boiling in SDS-PAGE and then autoradiographed. The arrow indicated the recombinant GST-Ha-AP10. MWM: Molecular weight markers.

Calcium requirements of the protein kinase that catalyzed the phosphorylation of Ha-AP10 and its response to different inhibitors suggest that the kinase belongs to the CDPK family.

Preliminary results have shown the localization of Ha-AP10 in microsomal membrane fractions from seeds [38]. The co-localization of the LTP and the protein kinase in microsomes support the physiological relevance for Ha-AP10 phosphorylation. The functional consequences of phosphorylation of LTPs are unknown. Possible consequences could relate to folding, processing and targeting of newly-synthesized LTPs, susceptibility to proteolysis and alteration of antifungal function.

This work describes the phosphorylation by endogenous membrane-bound calcium-dependent protein kinases of the recombinant LTP Ha-AP10. These results and previous evi-

dence that shows the binding of LTPs to a plant elicitin receptor [8,9] suggest that LTP's may be involved in signaling pathways.

ACKNOWLEDGMENTS

This work was supported by grants to L.d.l.C from the National Agency for Science and Technology (ANPCyT), the National Research Council of Argentine (CONICET), Fundación Antorchas and the University of Mar del Plata, Argentina. M.L.M and L.d.l.C are members of the Research Career from CONICET, Argentina.

ABBREVIATIONS

CDPK = Calcium-dependent protein kinases

CPZ = 2-Chloro-10-[3-(dimethylamino)propyl]phenothiazine hydrochloride

DTT = Dithiothreitol

EDTA = Ethylenediaminetetraacetic acid

EGTA = Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid

GST = Glutathione S-transferase

H7 = 1-(5-Isoquinolinesulfonyl)-3-methylpiperazine

IPTG = Isopropyl β-D-1-thiogalactopyranoside

LTP = Lipid-transfer proteins

PMSF = Phenylmethanesulfonyl fluoride

PBS = Phosphate Buffered Saline

W7 = N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride

REFERENCES

[1] Kader, J.C. (**1975**) *Biochim. Biophys. Acta, 380*, 31-44.
[2] Kader, J.C. (**1996**) *Annual. Rev. Plant Physiol. Plant Mol. Biol., 47*, 627–654.
[3] Carvalho, O.A. Teodoro, C.E.S. Da Cunha, M. Okorokova-Façanha, A.L. Okorokov, L.A. Fernandes, K.V.S and Gomes, V.M. (2004) *Physiol. Plant., 122*, 328–336.
[4] Tsuboi, S. Osafune, T. Tsugeki, R. Nishimura, M. and Yamada, M. (1992) *J. Biochem. (Tokyo), 111*, 500–508.
[5] Terras, F.R.G. Goderis, I.J. Van Leuven, F. Vanderleyden, J. Cammue B.P.A and Broekaert, W.F. (**1992**) *Plant Physiol., 100*, 1055–1058.
[6] Molina, A. Segura, A. and Garcıa-Olmedo, F. (**1993**) *FEBS Lett., 316*, 119–122.
[7] Molina, A. and Garcıa-Olmedo, F. (**1997**) *Plant J., 12*, 669–675.
[8] Buhot, N. Douliez, J.P. Jacquemard, A. Marion, D. Tran, V. Maume, B.F. Milat, M.L. and Ponchet, M. (**2001**) *FEBS Lett., 509*, 27–30.
[9] Buhot, N. Gomes, E. Milat, M.L. Ponchet, M. Marion, D. Lequeu, J. Delrot, S. Coutos-Thevenot, P. Blein, J.P. (**2004**) *Mol. Biol. Cell., 15*, 5047-5052.
[10] Maldonado, A.M. Doerner, P. Dixon, R.A. Lamb, C.J. and Cameron, R.K. (**2002**) *Nature, 419*, 399–403.
[11] Neumann, G.M. Condron, R. Svensson, B. and Polya, G.M. (**1993**) *Plant Sci., 92*, 159-167.
[12] Neumann, G.M. Condron, R. Thomas, I. and Polya, G.M. (**1994**) *Biochem. Biophys. Acta, 1209*, 183-190.
[13] Neumann, G.M, Condron, R. Thomas, I. and Polya, G.M. (**1995**) *Plant Sci., 107*, 129-145.
[14] Harmon, A.C, Gribskov, M. and Harper, J.F. (**2000**) *Trends Plant Sci., 5*, 154–159.
[15] Harmon, A.C. Gribskov, M. Gubrium, E. and Harper, J.F. (**2001**) *New Phytol., 151*, 175–183.
[16] Hrabak, E.M. (**2000**) *Adv. Bot. Res., 32*, 185–223.
[17] Hrabak, E.M. Chan, C.W.M. Gribskov, M. Harper, J.F. Choi, J.H. Halford, H. Kudla, J. Luan, S. Nimmo, H.G. Sussman, M.R. (**2003**) *Plant Physiol., 32*, 666–680.
[18] Dammann, D Ichida, A. Hong, B. Romanowsky, S. Hrabak, E.M. Harmon, A.C. Pickard, B.G. and Harper, J.F. (**2003**) *Plant Physiol., 132*, 1840–1848.
[19] Putnam-Evans, C. Harmon, A.C. Palevitz, B.A. Fechheimer, M. and Cormier, M.J. (**1989**) *Cell Motil. Cytoskeleton, 12*, 12–22.
[20] Anil, V.S. Harmon, A.C. and Rao, K.S. (**2003**) *Plant Cell Physiol., 44*, 367–376.
[21] Lu, S.X. and Hrabak, E. (**2002**) *Plant Physiol., 128*, 1008–1021.
[22] Baizabal-Aguirre, V.M. and de la Vara, L.E.G. (**1997**) *Physiol. Plant, 99*, 135–143.
[23] Iwata, Y. Kuriyama, M. Nakakita, M. Kojima, H. Ohto, M. and Nakamura, K. (**1998**) *Plant Cell Physiol., 39*, 1176–1183.
[24] Yoon, G.M. Cho, H.S. Ha, H.J. Liu, J.R. and Lee, H.S. (1999) *Plant Mol. Biol., 39*, 991–1001.
[25] Martın, M.L. and Busconi, L. (**2000**) *Plant J., 24*, 1–7.
[26] Regente, M. and de la Canal, L. (**2000**) *Physiol. Plant, 110*, 158–163.
[27] Regente, M. and de la Canal, L. (**2003**) *J. Plant Physiol., 160*, 201–203.
[28] Arondel, V. Vergnolle, C. Tchang, F. and Kader, J.C. (**1990**) *Mol. Cell Biochem., 98*, 49–56.
[29] Regente, M. Giudici, A. Villalaín, J. and de la Canal, L. (**2005**) *Letters Applied Microbiol., 40*, 183–189.
[30] Ausubel, F.M. Brent, R. Kingston, R.E. Moore, D.D. Seidman, J.G. Smith, J.A. and Struhl, K. (eds.). (**1999**) *Short Prot. Mol. Biol.*, pp. 10-44.
[31] Dieryck, W. Lullien-Pellerin, V. Marion, D. Joudrier, P. and Gautier, M.F. (**1995**) *Protein Expr. Purif., 6*, 597-603.
[32] Gel, X. Chen, J. Sun, Ch. and Cao, K. (**2003**) *Protein Eng., 16*, 387-390.
[33] Klein, C. Lamotte-Guery, Gautier, F. Moulin, G. Boze, H. Joudrier, P. Gautier, M.F. (**1998**) *Protein Expr. Purif., 13*, 73-82.
[34] Lullien-Pellerin, V. Devaux, C. Ihorai, T. Marion, D. Pahin, V. Joudrier, P. Gautier, M.F. (**1999**) *Eur. J. Biochem., 260*, 861-868.
[35] Zhan, Y. Song, X. and Zhou, G.W. (**2001**) *Gene, 281*, 1–9
[36] Cheng, S-H. Willmann, M.R. Chen, H-Ch. and Sheen, J. (**2002**) *Plant Physiol., 129*, 469-485.
[37] Rodríguez, P. Mitton, B. and Kranias, E.G. (**2005**) *Biotechnology Letts., 27*, 1869–1873.
[38] Martin, M.L. and de la Canal, L. (**2003**) *Biocell, 27*, 132 Abstract TS-P24.
[39] Notredame, C. Higgins, D. and Heringa, J. (**2000**) *J. Mol. Biol., 302*, 205-217.

Received: September 23, 2007 Revised: February 19, 2007 Accepted: February 28, 2007