Extracellular Sunflower Proteins: Evidence on Non-classical Secretion of a Jacalin-Related Lectin

Marcela Pinedo[1], Mariana Regente[1], Mercedes Elizalde[1], Ivana Y. Quiroga[1], Luciana A. Pagnussat[1] Jesús Jorrín-Novo[2], Ana Maldonado[2] and Laura de la Canal[1]*

[1]*Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata-CONICET, Funes 3250, 7600-Mar del Plata, Argentina;* [2]*Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, Córdoba, España*

Abstract: Extracellular proteins from sunflower seedlings were analyzed by electrophoresis followed by peptide mass fingerprinting. Tentative identification revealed novel proteins for this crop. A significant number of those proteins were not expected to be extracellular because they lacked the typical signal peptide responsible for secretion. *In silico* analysis showed that some members of this group presented the characteristic disordered structures of certain non-classical and leaderless mammalian secretory proteins. Among these proteins, a putative jacalin-related lectin (Helja) with a mannose binding domain was further isolated from extracellular fluids by mannose-affinity chromatography, thus validating its identification. Besides, immunolocalization assays confirmed its extracellular location. These results showed that a lectin, not predicted to be secreted in strict requirement of the N-terminal signal peptide, occurs in a sunflower extracellular compartment. The implications of this finding are discussed.

Keywords: Agglutinin, *Helianthus annuus*, jacalin, peptide mass fingerprinting, Secretome P, signal sequence, unconventional secretion.

INTRODUCTION

The extracellular matrix of plant tissues (apoplast) is a dynamic compartment that contains the cell wall, air and a variety of molecules including proteins. Since it has not only a structural role but acts like the boundary between cells and the environment it is associated to biological functions as diverse as cell growth, defense and cell-tissue communication [1-4]. In plants, the identification of extracellular proteins first emerged from genomic approaches that detected in databases the presence of sequences considered signal peptides, an amino acid stretch required for entering the ER/Golgi mediated secretory pathway. Alternatively, a database named MAIZEWALL originated after an extensive cell wall-related keywords search [5]. Novel proteins were also detected by proteomic analyses of cell culture media, intercellular fluids or isolated cell walls of model species, mainly *Arabidopsis thaliana* and rice [6-10]. Altogether these extracellular proteins define the plant secretome. However, the assignment of a reliable apoplastic location is not an obvious task, since the occurrence of proteins not expected to be extracellular on the base of the absence of a signal peptide has been reported not only in plants but also in other eukaryota (2,10-13]. They are referred as "*non classical*" and reach as much as half of the proteins identified in the *A. thaliana* and rice secretomes [6,9]. Indeed, besides the classical secretory pathway involving recognition of a signal peptide in the ER, alternative translocation across the plasma membrane was shown for nuclear and cytoplasmic mammalian proteins and the mechanisms are beginning to emerge [11]. Hence, Secretome P, a prediction tool designed to identify these extracellular proteins in bacteria and mammals was developed [14]. However, in plant sciences, the identification of reliable unconventionally secreted proteins is still lacking. Recently, evidence of the secretion of a normally symplastic mannitol dehydrogenase through an uncharacterized non-Golgi mechanism has been obtained [15], while apoplastic exosome-like vesicles, with proposed function in alternative secretion mechanisms have been reported in sunflower seeds [16]. Thus, the identification of "*non classical"* proteins will help to unravel not only the specific requirements for this alternative secretory mechanism/s but new functions associated to the plant extracellular matrix and its proteins.

Although sunflower is one of the most important oilseed crops limited information is available on its proteome. This report gives a first insight into sunflower apoplastic proteome and let the identification of previously unknown secreted proteins. Moreover, a significant number of the proteins putatively identified were devoid of signal peptide and are supposed to follow unconventional/non-classical secretion. In addition, the identity and extracellular location of a Secretome P predictible non-classically secreted protein was further validated.

MATERIALS AND METHODS

Collection of Extracellular fluid

Helianthus annuus L seeds (line 10347 Advanta Semillas) were grown 3 days in a Wathman paper embedded in water under sterile conditions and 4 additional days in hy-

*Address correspondence to this author at the Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata-CONICET, Funes 3250, 7600 Mar del Plata, Argentina; Tel: 54-0223-4753030; Fax: 54-0223-4724143; E-mail: ldelacan@mdp.edu.ar

0929-8665/12 $58.00+.00

droponic conditions at 25º C with a 14 h photoperiod and an irradiance of 250 μmoles m^{-2} s^{-1}. The extracellular fluid (EF) from 30 g of 7 days old seedlings was collected by infiltration-centrifugation after a pre-incubation of 45 min in buffer A (50 mM $NaPO_4H_2$, pH 7.2, 0.1% (v/v) 2-mercaptoethanol, 0.15 M NaCl and 0.18 % (v/v) Tween 20). Six pulses of vacuum infiltration of 20 s precede the centrifugation at 400 x g for 50 min. The infiltrated seedlings were recovered, dried on filter paper, placed in glass filters inside centrifuge tubes and centrifuged at 400 x g and 4ºC for 50 min. [17]. A pressure of 45 kPa was applied, generating a soft vacuum, which is a half of the standard condition [18].

Intact seeds were imbibed 1 h in distilled water and then carefully peeled to remove the pericarp previous to the extraction of the seed extracellular fluid fraction (SEF). SEF was obtained through the vacuum infiltration-centrifugation procedure described above. Yields were close to 80-100 μL. g^{-1} FW^{-1} for both EF and SEF. These fractions were loaded on centrifugal filter device centricon YM 3 to allow a six-fold concentration of proteins. The concentrated EF and SEF were evaluated for intracellular contamination as described [19] using a dilution 1/25.000 of polyclonal antibodies against the cytosolic proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Total Seedling Extracts (TE)

Five g of seven days old seedlings were homogenized in 10 ml of buffer A, filtered through a nylon cloth and centrifuged for 30 min at 26,000 x g. The supernatant was named total extract (TE). An extract was also obtained by pulverization of two g of decoated seeds previously imbibed for 1 h in distilled water. The resultant powder was immersed in 3 vol. of buffer A and clarified by centrifugation for 30 min at 26,000 x g. The supernatant was named seed total extract (STE).

Protein Analysis

SDS-PAGE was performed in 12% gels and the Protean II system (Bio-Rad). Colloidal Coomassie Blue [20] or Sypro® Ruby protein gel stain were used for protein staining and all the bands detected were recovered in the Investigator ProPic workstation. Trypsin digestion and the MALDI-TOF MS spectra of the products were performed at the Unidad de Proteomica from the Universidad de Córdoba, Spain as previously described [16]. The following default parameters were set: a intensity or S/N threshold of 10; a local noise window width for each peptide of 250 m/z; and a min peak width at full with half Max of 2.9 MS were analyzed in the 800 to 4,000 range of mass to charge ratio (m/z). For calibrating each spectrum the following peaks of trypsin (m/z) were used: 842,51; 1045,56 and 2211,11. All the trypsin peptides were excluded as contaminants. Proteins were identified using the Mascot 1.9 search engine (Matrixscience, UK) on MSDB, NCBI and SwissProt database. A detailed analysis of peptide mass mapping data was performed using GPS ExplorerTM software v 3.5 (Applied Biosystems), allowing the following parameters: specie *Viridiplantae*, one missed cleavage, 100 ppm mass tolerance, as well as cysteine carbamidomethylation and methionine oxidation as possible modifications. The confidence in the peptide mass fingerprinting matches was based on the score level and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum. A threshold of 65 in Mascot score was statistically significant with $p<0.05$. Signal P 3.0 software (www.cbs.dtu.dk/services/SignalP/) and Secretone P (http://www.cbs.dtu.dk/services/SecretomeP/) were used to predict motifs conserved in extracellular proteins. Secretome P is a sequence-based method that uses in the training of their neural networks six different features of secreted proteins. The mean of five resulting output values is the final score for each sequence and non-classically secreted proteins must obtain a score exceeding the normal threshold of 0.5 to be considered Secretome P positive [14]. This criterion was recently used for plant proteins [10].

Jacalin Purification and Antibody Production

EF (4 ml) was loaded on a 1 ml D-mannose-agarose resin (Sigma M6400) equilibrated with buffer B (50 mM HCl-Tris pH 7.5, 100 mM NaCl). Non-bounded proteins were washed exhaustively before the elution of retained proteins with 4 ml of 0.2 M mannose in buffer B. A retained band of 16 kDa was recovered after separation in a 12% SDS-PAGE and 250 μg were used for rabbit inoculation. The antigen was emulsified with complete Freund´s adjuvant and used in an initial antigen injection. Two boosters were given at 2 week intervals using incomplete Freund´s adjuvant. The obtained antiserum was sequentially submitted to ammonium sulfate precipitation and Protein A-Sepharose matrix to purify the immunoglobulin fraction. Pre-immunization blood was collected from the same rabbits to prepare preimmune serum.

Western Blot Analysis

Western blot analysis was performed after separation of the proteins in Total Extracts and extracellular fractions in a 12% SDS-PAGE. The proteins were transferred to 0.45 μm nitrocellulose membranes and incubated in blocking buffer followed by the primary antibodies diluted 1/25.000 or 1/1.000 for anti GAPDH or anti Helja respectively. After washing steps and incubation with alkaline phosphastase conjugated anti rabbit secondary antibodies the membranes were developed [19].

Immunohistochemistry

For the immunohistochemistry, the dry seeds were sectioned in 2 mm slices. They were vacuum infiltrated in 10% dimethyl sulfoxide (DMSO) and fixated in 0.05 M phosphate pH 7.4, 10% DMSO, 4% paraformaldehyde, 1% glutaraldehyde and 0.06 M sucrose at 4 °C for 4 h. Dehydration through series of 30%; 50%; 70%; 90% and 96% ethanol in phosphate buffer (PBS) was followed before an overnight embedding step in the low melting point Steedman's wax (PEG 9 w/w and 1-hexadecanol 1 w/w) [21]. Sections of 10 μm were cut and collected on polyethylenimine 50% (w/v)-coated slides. They were dewaxed and rehydrated in 96% and 100% ethanol before series of 90% and 50% ethanol in PBS and finally PBS. Sections were blocked with 3% skim milk in PBS and incubated for 12 h at RT with the anti-Helja Immunoglobulins (Ig) or preimmune serum (1: 100 in PBS). Incubations for 2 h with Alexa Fluor 488-conjugated

goat anti-rabbit IgG (Molecular Probes) (1:500) precede the observations with a Nikon C1 confocal laser scanning microscope with a 60X/1.40/0.13 oil-immersion lens (excitation /detection : 488 / 530 nm). The post-processing of images was carried out with the aid of EZ-C1 FreeViewer software version 3.2.

RESULTS AND DISCUSSION

Proteomic Analysis of Sunflower Extracellular Fluids

A protocol based on infiltration-centrifugation was used for the isolation of extracellular fluids (EF) from 7-days-old sunflower seedlings and the EF fraction was then tested for the absence of significant intracellular components, as previously described [19]. Denaturing 12% PAGE showed that the protein pattern of apoplastic fluids was clearly different from the profile observed in total soluble extracts (TE) from seedlings Fig. (**1A**). In addition, a western blot analysis using antibodies for the cytosolic marker glyceraldehyde-3-P dehydrogenase (GAPDH) Fig. (**1B**) was able to detect this protein only in the TE fraction. Altogether, these results demonstrate that the obtained EF fraction was devoid of detectable levels of cytosolic proteins. So, all the bands seen on EF samples were automatically recovered, digested with trypsin and submitted to a MALDI-TOF spectrometric analysis in order to identify the proteins. Sequence coverage of 20 %, a Mascott score of 65 and a peptide number of 4 were established as cut off for tentative identification. Using this strategy 15 proteins were putatively identified, some of them generated from the same band in the gel. The information obtained is summarized in Table **1** where it can be seen that most of the matched proteins belong to plant genera different from *Helianthus* and represent still unknown sunflower proteins. The functional classification of these proteins showed that they are involved in plant defense as well as cell wall and nucleic acid or protein metabolism and belong to families already described in the extracellular matrix of different species Table **1**.

The proteins putatively identified were classified according to the following criterion. Class I includes classical secretory proteins containing a signal peptide detectable by the Signal P 3.0 software. Class II comprises proteins without signal peptide but presenting secretion features of certain mammalian secreted proteins, identified with the Secretome P software. Finally, we have included in Class III those proteins that resulted Signal P and secretome P negative.

Only a minor part of the matched proteins presented a signal peptide in their amino-terminus (Class I in Table **1**). Class I includes a hypothetical *A. thaliana* protein, a cinnamate 4-hydroxylase, a pentatricopeptide repeat-containing protein, a splicing factor sc35 and an amidase. The fact that contamination with intracellular components was discarded and that new functions are being associated to the apoplast [2], suggest putative novel localizations of proteins previously known to be associated to other cell compartments. A deeper individual analysis is needed to determine the meaning of these findings.

The rest of the proteins putatively identified by MALDI-TOF were tested with Secretome P, an algorithm designed to detect characteristic disordered structures of non-classical and leaderless secreted proteins of mammals [14]. Intriguingly, five proteins were Secretome P positive (score above 0.5) and are shown as class II in Table **1**. Among these are a maturase, a mutator like-transposase, an agglutinin, a LEA protein and a 26S proteasome subunit. Finally, five of the matched proteins were Signal P and Secretome P negative (class III in Table **1**).

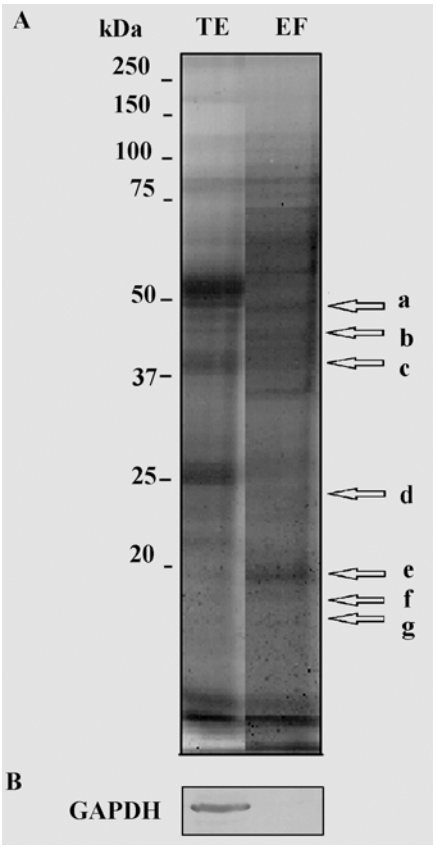

Fig. (1). Differential profiles of total soluble and extracellular proteins from sunflower seedlings. Total soluble (TE) and extracellular (EF) proteins (40 µg) were loaded on a 12% SDS-PAGE and stained with Colloidal Coomassie Blue (A) or transferred to nitrocellulose and immunodetected with anti-GAPDH serum and alkaline phosphatase-conjugated anti-rabbit IgG (B). Molecular weight markers are indicated on the left. The arrows and letters on the right indicate bands identified by MALDI-TOF peptide fingerprint.

In summary, two thirds of the proteins putatively identified lacked the signal peptide known to be responsible for protein secretion. The possibility of contamination with intracellular components was minimized on the basis of the immunodetection of GAPDH in the total soluble protein fraction but not in TE, and the fact that the abundant intracellular Rubisco subunits were not detected in the EF by any of the highly sensitive spectrometric analysis performed. Hence, our results point out the occurrence of leaderless extracellular proteins in sunflower apoplast.

Table 1. Proteins Identified in the Extracellular Fluids of Sunflower Seedlings by MALDI/TOF Peptide Mass Fingerprinting

Class	Protein name	Band	ID	Source	Score	MW kDa exp/theor	PN	Cov. %	Signal P	Secretome P	Ref
I	Hypothetical Protein	a	Q9SZB1	*Arabidopsis thaliana*	75	44/32.7	11	39	Yes	na	na
I	Cinnamate 4-Hydroxylase	a	Q9AXP9	*Populus balsamifera*	68	44/58.2	13	22	Yes	na	na
I	Pentatricopeptide Motif	b	Q9FME4	*Arabidopsis thaliana*	104	40/59.7	17	39	Yes	na	na
I	Splicing Factor Sc35	c	Q9FYB1	*Arabidopsis thaliana*	62	39/35.1	10	31	Yes	na	na
I	Amidase	d	Q7XMY1	*Oryza sativa*	69	24/20.4	8	54	Yes	na	na
II	Maturase fragment	a	Q8M7I2	*Prosopanche americana*	92	44/61.5	16	24	No	Yes	---
II	Mutator Like-Transposase	a	Q9ZQL0	*Arabidopsis thaliana*	84	44/62.6	15	26	No	Yes	**26**
II	Jacalin-Artichocke Agglutinin	e	Q9FS32	*Helianthus tuberosus*	208	16/15.5	6	74	No	Yes	**6, 10, 26,28,29**
II	26S Proteasome Sub 7 AAA ATPase	f	Q9FXT9	*Oryza sativa*	65	15/48.2	12	32	No	Yes	**9**
II	Lea-Emb15 fragment	f	Q40842	*Picea Glauca*	77	15/11.3	8	58	No	Yes	**26**
III	Heat Shock Protein	a	Q9SRD5	*Arabidopsis thaliana*	91	44/217	27	29	No	No	**6,9**
III	Hypothetical Defense Protein	b	Q9SIT3	*Arabidopsis thaliana*	93	40/44.9	14	31	No	No	na
III	Embryonic Protein DC8	b	S04909	*Daucus carota*	81	40/60.2	15	27	No	No	**12**
III	Hypothetical Protein	c	Q9FWW5	*Arabidopsis thaliana*	91	39/62.8	16	28	No	No	na
III	Protein Kinase-Like fragment	g	Q8RWN3	*Arabidopsis thaliana*	87	14/108.3	19	30	No	No	**9,12,32**

Band: Band identified on the gel in Fig. (**1**); PN : Peptide number; cov : % coverage; exp/theor: experimental/theoretical; Ref: References of proteins belonging to the same families already detected in the apoplast of other species; na: not analyzed.
The analysis with Signal P software was used to detect the N-terminal signal peptides signature (Class I), and Secretome P for pathway- independent features of mammalian secreted proteins (Class II). Class III includes proteins resulting Signal P and Secretome P negative.

Characterization of a Leaderless Jacalin-like Lectin

In order to validate the proteomic data obtained we have selected a protein to deepen the analysis. A closer examination of the leaderless proteins grouped in class II and III Table **1** reveals that the band *e*, matching an agglutinin from *Helianthus tuberosus* [22] (Uniprot Q9FS32), displays the highest Mascott score (208) and coverage as well as a good agreement between the theoretical and experimental molecular mass (16 kDa). Bioinformatic tools predicted that the agglutinin is a mannose binding lectin (InterproScan IPR 001229) with a jacalin domain (Pfam PF01419). However, jacalins are mostly recognized as intracellular cytoplasmic/nuclear proteins [23-25]. Nevertheless, some putative members of this family with and without signal peptide sequences were repeatedly identified in the apoplast but not validated by additional experimental procedures [6,10,26-29]. Interestingly, some of these reports avoided intracellular contamination by using non destructive methods for protein recovery such as media of hydroponically grown seedlings [28] or cell culture [6]. Thus, the sunflower putative jacalin like lectin with no classical signal-motif directing the protein to the apoplast was selected to confirm both, the preliminary identification as well as the extracellular localization. We took advantage of the potential binding properties of this protein and submitted EF to D-Mannose affinity chromatography. The fraction of proteins retained in the matrix and eluted with 0.2 M mannose displayed a major polypeptide of 16 kDa and additional bands of around 25, 33, 40, 45 and 60 kDa in SDS-PAGE Fig. (**2**). To confirm the identity of the 16 kDa band a subsequent MALDI-TOF/TOF MS analysis was performed and confirmed the previous identification as

an agglutinin, with a Mascot score of 235. Additional support to the identification was given by an ion score of 140 obtained by the tryptic fragment of 2133.95 Da (**Supplementary** Fig. (**1**)). When the tryptic peptides obtained in all the spectrometric analysis performed for the 16 kDa putative jacalin were overlapped with the matched *H. tuberosus* sequence they showed a coverage of 71 % including 9 out of 11 invariant residues that determine the β prism pattern of jacalins Fig. (**3**). So, according to its mannose affinity and MALDI-TOF/TOF identification the 16 kDa lectin is a *H. annuus* jacalin, from now on named Helja.

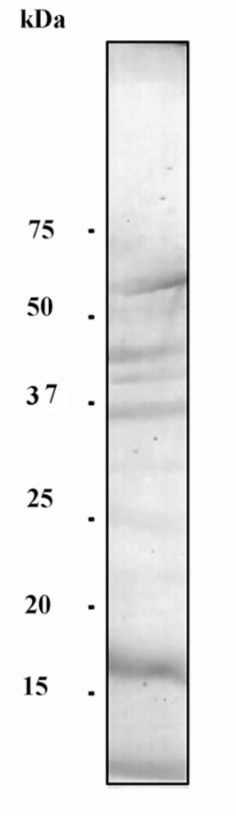

Fig. (2). Extracellular proteins with mannose-binding affinity. The EF (4 ml) was loaded on a D-mannose-agarose matrix and the retained proteins were eluted with 0.2 M mannose and concentrated 6 fold before the analysis in a 12% SDS-PAGE. Gels were stained with Colloidal Coomassie Blue. Molecular weight markers are indicated on the left.

mmunolocalization Confirms that Helja is an Apoplastic Protein

An aliquot of Helja was used as antigen in a standard immunization protocol to produce polyclonal antibodies, which in turn were used in western blot analysis. Preliminary experiments indicated that Helja was enriched in seeds, so both, seeds total soluble (STE) and extracellular fluids (SEF) were isolated from imbibed seeds and submitted to 12% PAGE denaturing gels and immunodetection. The antibodies detected a unique band of around 16 kDa in the apoplastic fraction and were not able to detect any protein in STE showing that Helja was specifically recognized in apoplastic fluids Fig. (**4**). Therefore, seed sections were obtained, incubated with the anti-Helja antibodies and revealed with fluorescent secondary antibodies. These assays showed Helja fluorescence restrained to the walls of cotyledon's parenchymatic cells Fig. (**5A** and **C**). The absence of signal after incubations with preimmune serum supports the specificity of the recognition Fig. (**5E**).

Hence, using biochemical and cellular approaches we confirmed the putative identification and location of the agglutinin Helja. It is effectively an extracellular Jacalin-Related Lectin (JRL) since it was able to make binding to mannose, a typical feature of the family, and immunolocation assays revealed its association to the cell walls. Therefore, this leaderless jacalin is a *bona fide* apoplastic sunflower protein. Since Helja was predicted to be extracellular by Secretome P, the program trained to detect non classically secreted mammalian proteins, the verified extracellular location sustains the usefulness of this predictor in plants and envisages the identification of new unconventionally secreted proteins. However, the fact that proteins in group III were negative for this algorithm could reflect the important differences between the plant and mammalian extracellular matrix and emphasizes the necessity of training the program with plant proteins.

To our knowledge, Helja is the first protein predicted to undergo non classical secretion experimentally confirmed in plants as well as the first JRL identified in sunflower. A blastx search in the Swiss-Prot database using the *Helianthus tuberosus* agglutinin (Q9FS32) as query revealed Q5U9T2.1, a mannose binding-lectin, as the best hit (38% identity). This protein, named Horcolin, is a jacalin immunolocated in the cell walls of *Hordeum vulgare* coleoptiles despite that the

```
MANNYVEVGP WGGSGGANPW SIIPNDGRIT RINVRSGAIV DAIYFGYTEG  50
          *Δ  Δ*   Δ*                          Δ       Δ

GISYETAIFG GRNGSLSTID IADDEEIIEI NGKVGTLENL NLVSQLTFVT 100
                     Δ   Δ         Δ   *  Δ Δ      Δ  Δ Δ *

NKQTYGPYGT NGGTDFSCPI AKGKVVGFFG RYGCLPGRYW CRT        143
*     **   *    Δ    Δ         ΔΔ**  Δ
```

Fig. (3). Sequence of the *Helianthus tuberosus* Jacalin-like lectin similar to Helja. The tryptic peptides from the 16 kDa sunflower protein obtained in 7 independent MALDI/TOF experiments are represented in gray on the sequence of the *H. tuberosus* agglutinin (Uniprot Q9FS32). The conserved and invariant residues which define the characteristic β prism domain are indicated by triangles and asterisks respectively.

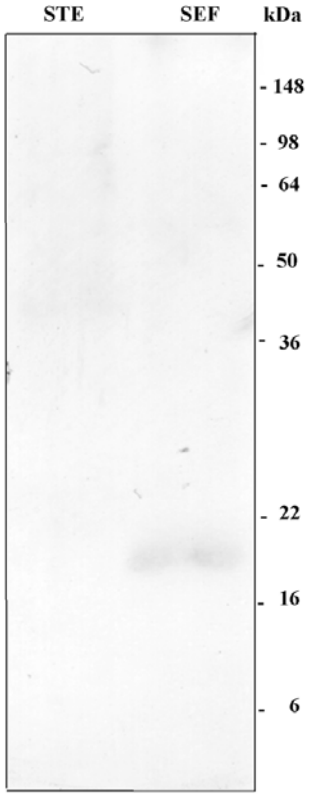

Fig. (4). Immunodetection of Helja, the *Helianthus annuus* jacalin-like lectin. Total soluble (STE) and extracellular (SEF) seed proteins (50 μg) were loaded on a 12% SDS-PAGE, transferred to nitrocellulose, blocked and incubated sequentially with 1:1.000 anti Helja Igs and alkaline phosphatase-conjugated anti-rabbit IgG (B). Molecular weight markers are indicated on the right.

sequence evaluation with different bioinformatic tools revealed neither signal peptide nor other targeting consensus [30]. Q9FS32 was also aligned with translated nucleotide sequences of the jacalin like lectins from both *Helianthus tuberosus,* and *Helianthus annuus* obtained from NCBI. The identity values obtained were lower than 53% showing that Q9FS32 and Helja are clearly different from the other putative jacalin-like lectins of *Helianthus*. These bioinformatic data, altogether with the fact that the majority of plant lectins are synthesized with a signal peptide and targeted to the vacuolar and extracellular compartment [23] could reflect neofunctionalization during evolution acquired for some members of the JRL family, mainly with nuclear and cytoplasmic location, to let the adaptation to the extracellular media.

A new paradigm is clearly emerging around protein secretion and the molecular machinery involved is starting to be unraveled in mammals and yeasts [11]. At least 4 different mechanisms of alternative secretion for a reduced number of *bonafide* non- classically secreted proteins were suggested; they involve multivesicular bodies, secretory lysosomes, microvesicle shedding and direct translocation across the plasma membrane [11]. The animal exosomes are secreted from intracellular multivesicular bodies that are able

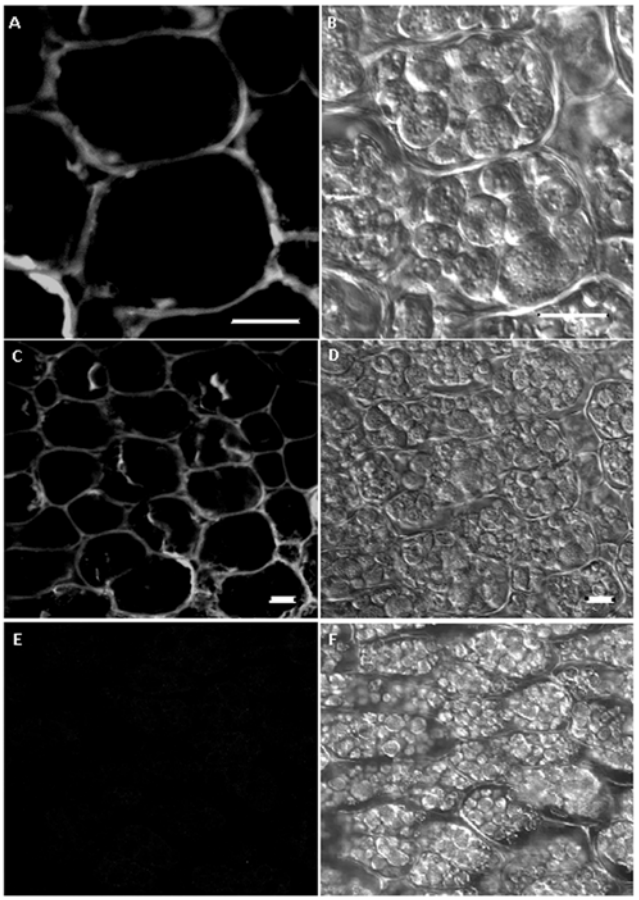

Fig. (5). Immunolocation of Helja. Confocal laser scanning microscopy showing immunofluorescent labeling (white) of seed sections. **Panel A – D:** Incubations with anti Helja antibodies 1/100 Panel **E-F :** Incubations with preimmune serum 1/100. The secondary antibody was Alexa Fluor 488-conjugated goat anti-mouse IgG. Bright field images are shown in the right side. Bar scale: 10 μm

to fuse with the plasma membrane resulting in the extracellular release of their luminal vesicles. The occurrence of leaderless proteins in extracellular microvesicles has also been reported in *Trypanosoma* secretome [13]. In plants, the complexity in the cell wall proteome and the trafficking of the secreted proteins has been recently updated [31]. In this context, the description of exosome-like vesicles in the apoplastic fluids of sunflower seeds must be highlighted. Moreover, these vesicles were shown to contain a 16 kDa protein matching *H. tuberosus* agglutinin [16], so it is tempting to speculate that multivesicular bodies might also be implicated in alternative secretion in plants. Our results support the hypothesis that plants might possess, as other eukaryotes, an alternative route to the endoplasmic reticulum/Golgi dependent secretory pathway.

ACKNOWLEDGEMENTS

This work was supported by grants from ANPCyT and the University of Mar del Plata (Argentina), the Agencia Española de Cooperacion Internacional (Project A/014378/07) and Asociación Universitaria Iberoamericana

de Postgrado (AUIP). LdlC and MR are members of the research career from the National Research Council (CONICET). ME, IQ and LP are fellows from UNMDP, CIC and CONICET respectively.

REFERENCES

[1] Jamet, E.; Albenne, C.; Boudart, G.; Irshad, M.; Canut, H.; Pont-Lezica, R. Recent advances in plant cell wall proteomics. *Proteomics*, **2008**, *8*, 893-908.

[2] Slabas, A.R.; Ndimba, B.; Simon, W.J.; Chivasa, S. Proteomic analysis of the *Arabidopsis* cell wall reveals unexpected proteins with new cellular locations. *Biochemical Society Transactions*, **2004**, *32*, 524-528.

[3] Boller, T. Peptide signalling in plant development and self/non-self perception. *Curr. Opinion Cell Biol.*, **2005**, *1*, 116–122.

[4] Maldonado, A.; Doerner, P.; Dixon, R.; Lamb, C.; Cameron, R. A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature*, **2002**, *419*, 399-340.

[5] Guillaumie, S.; San-Clemente, H.; Deswarte, C.; Martinez, Y.; Lapierre, C.; Murigneux, A.; Barrière, Y.; Pichon, M.; Goffner, D. MAIZEWALL. Database and developmental gene expression profiling of cell wall biosynthesis and assembly in maize. *Plant Physiol.* **2007**, *143*, 339-363.

[6] Oh, I.S.; Park, A.R.; Bae, M.S.; Kwon, S.J.; Kim, Y.S.; Lee, J.E.; Kang, N.Y.; Lee, S.; Cheong, H.; Park, O.K. Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola*. *Plant Cell*, **2005**, *17*, 2832–2847.

[7] Boudart, G.; Jamet, E.; Rossignol, M.; Lafitte, C.; Borderies, G.; Jauneau, A.; Esquerré-Tugayé, M.T.; Pont-Lezica, R. 2005. Cell wall proteins in apoplastic fluids of *Arabidopsis thaliana* rosettes: identification by mass spectrometry and bioinformatics. *Proteomics*, **2005**, *5*, 212–221.

[8] Bayer, E.M.; Bottrill, A.R.; Walshaw, J.; Vigouroux, M.; Naldrett, M.J.; Thomas, C.L.; Maule, A.J. *Arabidopsis* cell wall proteome defined using multidimensional protein identification technology. *Proteomics*, **2006**, *6*, 301–311.

[9] Jung, Y.H.; Jeong, S.H.; Kim, S.H.; Singh, R.; Lee, J.; Cho, Y.S.; Agrawal, G.K.; Rakwal, R.; Jwa, N.S. Systematic secretome analyses of rice leaf and seed callus suspension-cultured cells: workflow development and establishment of high-density two-dimensional gel reference maps. *J. Proteome Res.*, **2008**, *7*, 5187–5210.

[10] Cheng, F.; Blackburn, K.; Lin, Y.; Goshe, M.B.; Williamson, J.D. Absolute Protein Quantification by LC/MSE for Global Analysis of Salicylic Acid-Induced Plant Protein Secretion Responses. *J. Proteome Res.*, **2009**, *8*, 82–93.

[11] Nickel, W.; Rabouille, C. Mechanisms of regulated unconventional protein secretion. *Nature*, **2009**, *10*, 148-155.

[12] Chen, X.Y.; Kim, S.T.; Cho, W.K.; Rim, Y.; Kim, S.; Kim, S.W.; Kang, K.Y.; Park, Z.Y.; Kim, J.Y. Proteomics of weakly bound cell wall proteins in rice calli. *J. Plant Physiol.*, **2009**, *166*, 675-685.

[13] Geiger, A.; Hirtz, C.; Bécue, T.; Bellard, E.; Centeno, D.; Gargani, D.; Rossignol, M.; Cuny, G.; Peltier, J.B. Exocytosis and protein secretion in Trypanosoma BMC. *Microbiology*, **2010**, *10*, 20.

[14] Bendtsen, J.D.; Jensen, L.J.; Blom, N.; von Heijne, G.; Brunak, S. Feature-based prediction of non-classical and leaderless protein secretion. *Protein Engineering, Desing and Selection*, **2004,** *4*, 349-356.

[15] Cheng, F.; Zamski, E.; Guo, W.; Pharr, D.M.; Williamson, J.D. Salicylic acid stimulates secretion of the normally symplastic enzyme mannitol dehydrogenase: a possible defense against mannitol-secreting fungal pathogens. *Planta*, **2009**, *230*, 1093–1103.

[16] Regente, M.; Corti-Monzón, G.; Maldonado, A.; Pinedo, M.; Jorrín, J.; de la Canal, L. Vesicular fractions of sunflower apoplastic fluids are associated with potential exosome marker proteins. *FEBS Lett.*, **2009,** *583*, 3363-3366.

[17] Pinedo, M.L.; Segarra, C.I.; Conde, R.D. Occurrence of two endoproteinases in wheat leaf intercellular washing fuid. *Physiol. Plant*., **1993**, *88*, 287-293.

[18] Lohaus, G.; Pennewiss, K.; Satelmacher, B.; Hussmann, M.; Muehling, K.H. Is the infiltration-centrifugation technique appropriate for isolation of apoplastic fluid? A critical evaluation with different plan species. *Physiol. Plant*., 2001, *111*, 457-465.

[19] Regente, M.; Corti Monzón, G.; de la Canal, L. Phospholipids are present in extracellular fluids of imbibing sunflower seeds and are modulated by hormonal treatments. *J. Exp. Bot.*, **2008**, *59*, 553-562.

[20] Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis*, **1988**, *9*, 255-262.

[21] Baluska, F.; Parke, J.S.; Barlow, P.W. Specific patterns of cortical and endoplasmic microtubules associated with cell growth and tissue differentiation in roots of maize (*Zea mays* L.). *J. Cell Sci*., **1992,** *103*, 191-200.

[22] Nakagawa, R.; Yasokawa, D.; Okumura, Y.; Nagashima, K. Cloning and sequence analysis of cDNA coding for a lectin from *Helianthus tuberosus* callus and its jasmonate-induced expression. *Bioscience, Biotechnology, and Biochemistry*, **2000,** *64*, 1247-1254.

[23] Lannoo, N.; Van Damme, E.J.M. Nucleocytoplasmic plant lectins. *Biochim. Biophys. Acta*, **2010**, *1800*, 190-201.

[24] Van Damme, E.J.M.; Hause, B.; Hu, J.; Barre, A.; Rougé, P.; Proost, P.; Peumans, W. J. Two Distinct Jacalin-Related Lectins with a Different Specificity and Subcellular Location Are Major Vegetative Storage Proteins in the Bark of the Black Mulberry Tree. *Plant Physiol.*, **2002**, *130*, 757–769.

[25] Yang, H.; Czapla, T. Isolation and characterization of cDNA clones encoding jacalin lectins. *J. Biol. Chem.*, **1993**, *268*, 5905-5910.

[26] Basu, U.; Francis, J.L.; Whittal, R.M.; Stephens, J.L.; Wang, Y.; Zaiane, O.R.; Goebel, R.; Muench, D.G.; Good, A.G.; Taylor, G.J. Extracellular proteomes of *Arabidopsis thaliana* and *Brassica napus* roots: analysis and comparison by MudPIT and LC-MS/MS. *Plant Soil*, **2006**, *286*, 357–376.

[27] Jamet, E.; Canut, H.; Boudart, G.; Pont Lezica, R. Cell wall proteins: a new insight through proteomics. *Trends Plant Sci.*, **2006**, *11*, 33-39.

[28] Charmont, S.; Jamet, E.; Pont-Lezica, R.; Canut, H. Proteomic analysis of secreted proteins from Arabidopsis thaliana seedlings: improved recovery following removal of phenolic compounds. *Phytochemistry*, **2005**, *66*, 453–461.

[29] Kwon, H.; Yokoyama, R.; Nishitani, K. A proteomic approach to apoplastic proteins involved in cell wall regeneration in protoplasts of Arabidopsis suspension cultured cells. *Plant Cell Physiol*., **2005**, *46*, 843-857.

[30] Grunwald, I.; Heinig, I.; Thole, H.; Neumann, D.; Kahmann, U.; Kloppstech, K.; Gau, A. Purification and characterization o a jacalin-related, coleoptile specific lectin from *Hordeum vulgare*. *Planta*, **2007,** *226*, 225-234.

[31] Rose, J.C.K.; Lee, S. Straying off the Highway: Trafficking of secreted Plant Proteins and complexity in the plant cell wall Proteome. *Plant Physiol*., **2010**, *153*, 433-436.

[32] Cho, W.K.; Chen, X.Y.; Chua, H.; Rim, Y.; Kim, S.; Kim, S.T.; Kim, S.W.; Park, Z.Y.; Kim, J.Y. Proteomic analysis of the secretome of rice calli. *Physiol. Plant.*, **2009**, *135*, 331–341.

Received: April 27, 2011 **Revised: July 15, 2011** **Accepted: August 1, 2011**