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Molecular characterization of Helja, an extracellular jacalin-related protein from *Helianthus annuus*: Insights into the relationship of this protein with unconventionally secreted lectins

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ABSTRACT

Jacalin-related lectins (JRLs) encompass cytosolic, nuclear and vacuolar members displaying the jacalin domain in one or more copies or in combination with unrelated domains. *Helianthus annuus* jacalin (Helja) is a mannose-specific JRL previously identified in the apoplast of *Helianthus annuus* seedlings, and this protein has been proposed to follow unconventional secretion. Here, we describe the full-length Helja cDNA sequence, which presents a unique jacalin domain (merolectin) and the absence of a signal peptide, confirming that the protein cannot follow the classical ER-dependent secretory pathway. Helja mRNA is present in seeds, cotyledons, roots and hypocotyls, but no transcripts were detected in the leaves. Searches for sequence similarity showed that Helja is barely similar to other JRLs present in *H. annuus* databases and less than 45% identical to other monocot or dicot JRLs. Strikingly, most of the merolectins recovered through data mining using Helja as a query were predicted as apoplastic, although most of these proteins lack the signal peptide required for classical secretion. Thus, Helja is the first bait identified to recover putative unconventionally secreted lectins. Because the recovered JRLs are widely distributed among the plant kingdom, an as yet unknown role for jacalin lectins in the apoplast is emerging.

Introduction

Lectins are carbohydrate-binding proteins, highly specific for a wide range of sugar motifs, with the ability to agglutinate cells. These proteins are ubiquitous in nature, occurring in viruses, bacteria, animals and plants (Lis and Sharon, 1986; Ingale and Hivrale, 2013). Different criteria have been used for the classification of lectins, including carbohydrate specificity, evolutionary relationships and sequence or tri-dimensional structural similarities (Jiang et al., 2010; Lannoo and Van Damme, 2010). Moreover, the lectin superfamily includes proteins that display a unique or variable number of domains for carbohydrate binding, and these regions might also be associated with unrelated domains. This criterion defines the major lectin groups known as merolectins (a single domain), hololectins (two or more identical domains), superlectins (two or more different domains recognizing structurally unrelated sugars) and chemerolectins (combination with unrelated domains). Additionally, plant lectins have been organized into 12 families based on a genome-wide analysis of phylogeny and protein domain structure comparisons. These families include B-lectin, Lectin legB, Jacalin, Chitin-bind 1, Ricin B Lectin, EEA, LysM, Phloem, Calreticulin, Gal-binding Lectin, Gal Lectin and Lectin C (Jiang et al., 2010).

Although previous studies have summarized lectin functions, this subject has only recently been unraveled (Ingale and Hivrale, 2013; Singh and Zimmerli, 2013). In plants, a significant number of these proteins are involved in biotic or abiotic stress responses, while others have been identified as storage proteins (Xiang et al., 2011; Lannoo and Van Damme, 2014). These proteins are encoded through constitutive and inducible genes (Peumans and Van Damme, 1995; Van Damme et al., 2004a,b; Xiang et al., 2011) that, in turn, can be controlled via regulatory elements that respond

Abbreviations: CDS, coding sequence; EST, expressed sequence tag; Helja, *Helianthus annuus* jacalin; JRL, Jacalin-related lectin; ORF, open reading frame; RT-PCR, reverse transcription polymerase chain reaction.

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to hormones, such as jasmonates or ABA (Wang and Ma, 2005; Jia and Rock, 2013).

The jacalin family comprises members with galactose or mannose affinity (Peumans et al., 2000), but only a few proteins, out of the more than 2800 sequences deposited in the UniProt database, have been thoroughly characterized. The galactose-specific jacalin of *Artocarpus integriflolia* has been localized to storage vacuoles, while the merolectin Calsepa, a 15 kDa mannose-specific jacalin of *Calystegia sepiuym*, was inmmunolocated in the cytoplasm rhizomes (Peumans et al., 2000). Additionally, proteins with multiple domains, such as VER2 have been identified, and this regulator of wheat vernalization was shown to have a C-terminal jacalin domain and a disease response domain in the N-terminal of the protein. VER2 is regulated through phosphorylation to control its cytoplasmic or nuclear location (Xing et al., 2009). Other proteins displaying several jacalin domains include the so called "Myrosinase binding proteins", which are associated with the vacuole and protein-body-like structures and plant defense mechanisms during herbivore and insect attack (Geshi and Brandt, 1998). Based on these data, members of the jacalin family have primarily been identified as intracellular proteins (Lannoo and Van Damme, 2010; Van Damme et al., 2002). Despite this information, we have previously detected in *Helianthus annuus* extracellular fluids a 16 kDa jacalin-related lectin (JRL), called *Helianthus annuus* jacalin (Helja), with binding affinity for mannose (Pinedo et al., 2012). Several lines of evidence, notably immunolocalization experiments through confocal microscopy, have demonstrated that Helja is located in the apoplast of sunflower seeds. Nevertheless, the N-terminal signal peptide that drives the proteins to the secretory pathway is absent in the genomic sequence of this protein (Pinedo et al., 2012; Regente et al., 2012). This feature, together with the fact that the protein is not glycosylated and its secretion is insensitive to brefeldin A (Regente et al., 2012), suggests that Helja might be an unconventionally secreted lectin.

To gain insights into apoplastic JRLs, we analyzed the Helja coding sequence (CDS) to establish phylogenetic relationships with other members of this protein family. We showed that Helja is clearly distant from other sunflower JRLs. Interestingly, the plant JRLs most similar to Helja, are devoid of signal peptides and predicted as extracellular, based on bioinformatics tools developed to evaluate subcellular localization. These data enhance the current understanding of the complexity of the jacalin family and might provide further information for identifying the as yet unknown functions of JRL in the apoplast.

Material and methods

Biological materials

Escherichia coli strain GigaSingles (genotype: *endA1 hsdR17* ($r_{K12}^{-}m_{K12}^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac*F′ (*proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Z*Δ*M15*::Tn*10*) (Tet^{R})] was acquired from Novagen and grown at 37 °C in liquid Luria Bertani (LB) medium at 200 rpm or on solid LB medium supplemented with 1.5% (w/v) agar.

Helianthus annuus L. seeds, line 10347, were obtained from Advanta Semillas S.A.I.C, Argentina. The seeds were surface-sterilized in 20 g/L sodium hypochlorite for 30 min, extensively rinsed and imbibed overnight in sterile distilled water. To produce seedlings, the imbibed seeds were peeled and germinated in moistened filter paper for 4 days in a chamber at 25 ± 1 °C and a 14 h:10 h (L:D) photoperiod, as previously described (Corti Monzón et al., 2014). The seedlings were subsequently transferred to nutrient solution for ten additional days of hydroponic culture under the same conditions indicated above. The solution was maintained at a constant volume through daily additions. At the end of the period, roots, first leaves, cotyledons and hypocotyls were harvested and immediately processed for RNA extraction.

Freshly harvested Jerusalem artichoke (*Helianthus tuberosus*) tubers were used to isolate extracellular washing fluids and total protein extracts.

Isolation of Extracellular Fluids and Total Extracts for the detection of Helianthus annuus *jacalin (Helja)-related proteins*

Jerusalem artichoke (*H. tuberosus*) tubers (20 g) were washed with tap water to eliminate soil and then were sliced to obtain cubes with a side length of approximately 1.5 cm. Extracellular washing fluids (EF) were prepared by immersion of the washed cubes in infiltration buffer followed by the infiltration–centrifugation procedure described for the isolation of Helja (Pinedo et al., 2012). Yields were close to 40–50 μL g^{-1} FW^{-1} of EF and its proteins were concentrated by addition of 3 volumes of cold acetone and centrifugation. The pellet was solubilized in 90 μL of sample buffer prepared according to Laemmli (1970).

To obtain artichoke total protein extracts, two g of tubers were homogenized in 6 mL of infiltration buffer, filtered through a nylon cloth and centrifuged for 30 min at 26,000 × *g*. The supernatant was named total extract (TE).

Protein electrophoretic separation and protein blot were performed as previously described (Pinedo et al., 2012) by loading EF and TE in 12% SDS-PAGE. Anti-Helja antibodies were used at a 1:4000 dilution and alkaline phosphatase conjugated anti rabbit secondary antibodies at 1:7000.

RNA isolation, cDNA synthesis, PCR amplification and vector ligation

Total RNA was extracted from 1 g fresh weight (FW) of *H. annuus* seeds. Frozen and ground seed tissue was mixed with Trizol® reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA). The precipitate obtained after the addition of isopropanol was washed with ethanol 70% before dissolution and digestion with DNase I. The sample of total RNA (1 μg) was used as template for first-strand synthesis in a reaction mix containing oligo-dT and M-MLV reverse transcriptase according to the manufacturer's instructions (Invitrogen). The obtained RT reaction mixture was diluted 5 times and used as a template in subsequent PCR. The oligonucleotides employed as primers were manually designed on the basis of the sequence of HA412-HO_S039609_scaffold_annotation recovered from data mining in the genomic dataset of scaffoldings generated from the *H. annuus* HA412-OH line (Genomics of Sunflower-University of British Columbia-Canada) using the BAB18761.1 sequence (jacalin from *H. tuberosus*) as a query (Nakagawa et al., 2000). The primer parameters were assessed using Oligo Calc (Kibbe, 2007). Thus, the Helja forward and reverse primer sequences were 5′-ATG GCT AAC AAC TAC GTT GAG G-3′ and 5′-CTA GGG ACT AAG TAC GAC G C-3′, respectively. The PCR reaction (25 μL) contained 1 μL of RT reaction mixture, 250 μM of each deoxynucleotide triphosphate, 2 mM $MgCl_2$, 10 μM of each primer, 2.5 μL 10× reaction buffer and 1 U of Taq DNA polymerase (Invitrogen 11615-036). The reactions were incubated at 95 °C for 3 min, followed by 35 cycles at 95 °C for 45 s, 58 °C for 45 s and 72 °C for 90 s, with a final extension step of 72 °C for 2 min.

The resulting PCR amplification product of the expected size was recovered from a 1% agarose gel and purified using the Wizard Plus Gel and PCR Clean-Up System (Promega). The O'Gene ruler ladder mix was purchased from Fermentas (Glen Burnie, MD). The purified cDNA was ligated into the pJET1.2/blunt vector according to the manufacturer's instructions (CloneJET™ PCR Cloning Kit, Thermo Scientific).

cDNA cloning and sequencing

The *Escherichia coli* strain GigaSingles was grown at 37 °C in liquid LB medium. When necessary, 100 μg mL^{-1} ampicillin was added. Competent cells were obtained through standard procedures using $CaCl_2$ (Inoue et al., 1990) and transformed with the pJET1.2/blunt vector carrying the amplified cDNA. Positive colony screening was performed through plasmid extraction (isolated using Highway ADN PuriPrep kit from INBIO (Argentina) or Wizard-Plus SV Miniprep DNA purification System (Promega) and analysis with the *Bgl* II restriction enzyme.

Two independent randomly selected positive transformants were sequenced in both directions (Instituto de Biotecnología-Unidad de Genómica-INTA Castelar, Argentina) using the Helja forward and reverse primers described above. Reverse sequences were converted to the antiparallel strand, and the four readings were aligned using Clustal Omega at http://www.ebi.ac.uk/Tools/msa/clustalo/ (Sievers et al., 2014). The open reading frame (ORF) was identified to obtain the coding sequence (CDS) sequence.

Expression analysis

Total RNA was extracted from 1 g FW of organs harvested *from H. annuus* seedlings and used in RT and PCR reactions as described above. The Helja forward and reverse primers were designed as previously detailed. Actin (GenBank FJ487620.1) was used as a housekeeping gene. The actin forward and reverse primer sequences were 5′-AGG GCG GTC TTT CCA AGT AT-3′ and 5′-ACA TAC ATG GCG GGA ACA TT-3′ according to Moreno-Pérez et al. (2010). The PCR products were loaded onto 1% agarose gels and subsequently stained with SYBR® Safe DNA Gel Stain (Life Technologies). The images were captured using the G:BOX F3 image system (Syngene).

Data mining

Both, jacalin as a key word and the Helja CDS as a query were used to challenge Sunflower Unigene Repository (SUR v1.0) containing 41.013 unique sequences obtained through the assembly of expression sequence tags (ESTs) (Fernandez et al., 2012). The FASTA alignment software available at http://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml (Pearson and Lipman, 1988) was used with Helja sequence as a query, selecting Nucleotide–Nucleotide (DNA/RNA fasta) with default settings. The recovered accessions were manually filtered with the following cut off: 55% identity, 50% query cover, scores higher than 140 and E value lower than 10^{-11}.

Helja was also used as a query in a blastp search restricted to the non-redundant collection of viridiplantae organisms at the NCBI database (http://blast.be-md.ncbi.nlm.nih.gov/Blast.cgi) using default parameters. The sequences recovered from the data mining analysis were manually filtered for merolectins, retaining the first 22 sequences with the following cut off: 40% identity, 90% query cover, maximum score higher than 90 and E value lower than 10^{-20}.

Phylogenetic analysis

The *H. annuus* sequences retrieved after data mining were translated into the six potential reading frames using http://web.expasy.org/translate, and the results were filtered through comparison with the Helja amino acid sequence. The sequences were aligned with Mafft 7.0 (Katoh and Standley, 2013) at http://mafft.cbrc.jp/alignment/server/and Genedoc (http://www.nrbsc.org/gfx/genedoc/gdfeedb.htm) (Nicholas et al., 1997) after reduction to a group of non-redundant representative sequences with CD-Hit (Huang et al., 2010; Li and Godzik, 2006) set at 95% identity. Maximum likelihood phylogenies were built using PhyML software (Phyml 3.0 at http://www.atgc-montpellier.fr/phyml/) with the default parameters (Guindon et al., 2010). Bootstraps branch support measures were performed for over 100 iterations. The resulting phylogenetic trees were edited using Dendroscope (Huson, 2012) at http://ab.inf.uni-tuebingen.de/software/dendroscope. The same procedure was performed using the viridiplantae sequences similar to Helja.

Additional biocomputational analyses

In silico prediction of donor and acceptor splice sites were performed at http://www.cbs.dtu.dk/services/NetGene2/ (Hebsgaard et al., 1996). InterProScan 5 was used to analyze protein function and classification at http://www.ebi.ac.uk/Tools/pfa/iprscan5/ (Zdobnov and Apweiler, 2001; Goujon et al., 2010; Jones et al., 2014), while the occurrence of plant lectin domains was tested through PlecDom at http://www.nipgr.res.in/plecdom.html (Shridhar et al., 2009). Non-classical secretion signals were analyzed using the SecretomeP server trained with mammalian sequences at http://www.cbs.dtu.dk/services/SecretomeP/, which was last accessed October 15, 2014 (Bendtsen et al., 2004). The prediction of cellular localization was performed with wolfPSORT and LocTree3 software at http://www.genscript.com/psort/wolf_psort.html and https://rostlab.org/services/loctree2/, respectively (Horton et al., 2007; Goldberg et al., 2014).

The occurrence of signal peptide was assessed using the SignalP 4.0 algorithm (Petersen et al., 2008) and UniProt, the resource of the protein sequence and functional information, was used to obtain experimental evidence of the jacalin location at http://www.uniprot.org/ (UniProt Consortium, 2008).

Results

Cloning and expression analysis of Helja

We have previously obtained the partial sequence for Helja through mass spectrometric analysis (Pinedo et al., 2012) according to similarity with the jacalin BAB18761.1 from *H. tuberosus* (Nakagawa et al., 2000). *In silico* domain identity searches revealed that Helja is a JRL, although the complete sequence of this protein remained unknown because it was not available in public databases. To obtain the full-length Helja CDS, we performed data mining using the genomic dataset of scaffoldings generated from the *H. annuus* HA412-OH line (Sunflower Genome Project, University of British Columbia) using the BAB18761.1 sequence as a query. This search recovered the HA412-HO_S039609_scaffold_annotation with an open reading frame (ORF) putatively encoding Helja (Fig. 1A). Thus, the primers designed to anneal with the deduced ORF from HA412-HO_S039609_scaffold_annotation facilitated the amplification of a unique product of approximately 450 bp through reverse transcription polymerase chain reaction (RT-PCR) performed using cDNA obtained from the mRNA of mature *H. annuus* seeds. The cDNA was further cloned and sequenced showing a complete CDS of 438 nt (GenBank KJ681498). The cloned cDNA is consistent with both the scaffold annotation and the *in silico* donor and acceptor splice site predictions. From ATG to the first TAG, it presents 1444 nt, but the nucleotide subtraction corresponding to the two predicted introns at positions 24–112 (89 nt) and 314–1230 (917 nt) generates a fragment of 438 nt (shadowed in Fig. 1A), which precisely matches the cloned cDNA size and sequence. The full-length cDNA encoded a predicted protein of 145 amino acids (Fig. 1B), with a theoretical pI of approximately 4.5 and molecular weight of 15.3 kDa. Significantly, no N-terminal hydrophobic peptide could be detected using the SignalP *in silico* tool. Thus, these data suggest that the primary translation product encoding Helja lacks the signal peptide, consistent with the hypothesis regarding the unconventional sorting of this protein (Pinedo et al., 2012; Regente et al., 2012). Additionally, the Helja protein sequence lacks the KKKK stretch characteristic of nuclear

Fig. 1. Helja nucleotide and amino acid sequences. (A) Nucleotide sequence of the scaffold HA412-HO_S039609 recovered from the database of the Sunflower Genome Project at British Columbia University using the jacalin-like sequence BAB18761.1 from *Helianthus tuberosus* as a query. Start and stop codons are highlighted in italics and underlined. The boxes indicate the nucleotide sequence from which the forward and reverse primers were designed and annealed for cloning and sequencing Helja cDNA. The gray shadowed nucleotides belong to the exons and the cDNA sequence experimentally obtained. (B) Deduced amino acid (aa) sequence from the cDNA.

localization, a feature associated with some members of the jacalin family (Lannoo and Van Damme, 2010).

To analyze the expression of Helja, we investigated the mRNA transcript levels in seeds and in different organs of young plants obtained from 15-day-old seedlings. RT-PCR revealed that the mRNA encoding Helja was expressed in the seeds, cotyledons, roots and hypocotyls, but no expression was detected in the leaves (Fig. 2).

Jacalin-related lectins in H. annuus

To identify other JRLs in *H. annuus*, a tblastn search using Helja sequence against non-redundant (nr) database, restricted to *H. annuus*, was performed using the NCBI site. However, no matches to the sequences available in this database were identified. Therefore, the Sunflower Unigene Repository (SUR v1.0), containing 41.013 unique sequences obtained through the assembly of expression sequence tags (ESTs) (Fernandez et al., 2012), was explored. The Helja CDS was used as a query to challenge SUR v1.0, and several sequences were recovered. The results were manually filtered according to scores higher than 140 and E values lower than 10^{-11} as cut off, and 17 different accessions putatively encoding jacalins (4 contigs and 13 singletons) were selected (Supplementary Fig. 1). Because the sequences were annotated as “lectin”, “agglutinin” or “unknown protein”, these sequences were translated into six potential reading frames and examined for the presence of specific

Table 1
Jacalin domain identification in *Helianthus annuus* sequences.

H. annuus accessions in SUR 1.0	Annotation in SUR 1.0	Plecdom	Interproscan
C_11167	Jacalin-like	Jacalin	Jacalin
S_38559	–	Jacalin	–
S_18319	Lectin 3	–	Jacalin
C_10625	Jacalin-like	Jacalin	Jacalin
S_18731	1tuber agglutinin	Jacalin	Jacalin
S_38848	1tuber agglutinin	Jacalin	Jacalin
S_18730	Jasmonate-induced protein	Ricing-B	Jacalin
S_18318	Jacalin-like	Jacalin	Jacalin
C_8578	1tuber agglutinin	Jacalin	Jacalin
S_32984	1tuber agglutinin	Jacalin	Jacalin
S_33030	Mannose-specific recombinant lectin	Jacalin	Jacalin
S_14237	Jacalin-lectin family	Jacalin	Jacalin
S_33751	Jasmonate-induced protein	Jacalin	Jacalin
S_34168	Lectin [*Helianthus tuberosus*]	Jacalin	Jacalin
S_33830	–	Jacalin	Jacalin
C_9314	–	Jacalin	–
S_32274	–	Jacalin	–

The sequences were recovered from the Sunflower Unigene Repository (SUR v1.0) using Helja cDNA sequence as query in the FASTA alignment software (Pearson and Lipman, 1988) with default setting and cut off scores higher than 140 and E value lower than 10^{-11}. They were checked *in silico* for jacalin domain using InterproScan and PlecDom algorithms. (–) Indicates unknown annotation.

domains using well-established methods in Interproscan and Plecdom, the software specifically designed for the identification of lectin families (Shridhar et al., 2009). The results, summarized in Table 1, confirmed through both algorithms, that 12 of these proteins presented jacalin domains. Among these sequences, the *H. annuus* sequence S_33830, previously devoid of gene ontology annotation, was identified. Interestingly, the Plecdom algorithm identified three additional non-annotated sequences (S_38559, C_9314 and S_32274) as members of the JRL. This result suggests that PlecDom has a higher potency for jacalin domain identification than Interproscan.

A multiple alignment was performed using the 12 double positive jacalin sequences from *H. annuus*, and the phylogenetic relationships of these sequences were analyzed using PhyML, a Maximum Likelihood method (Guindon et al., 2010). Two groups of accessions were easily identified from this alignment: one group encompassed Helja, with a set of primarily short sequences (C_8578, S_33751, S_33030, S_33830 and S_32984), and the other comprised JRLs, possessing additional amino acid stretches in the N- and C-terminal regions (C_11167, C_10625, S_ 38848, S_18731 and S_18318) (Supplementary Fig. 2). Interestingly, these

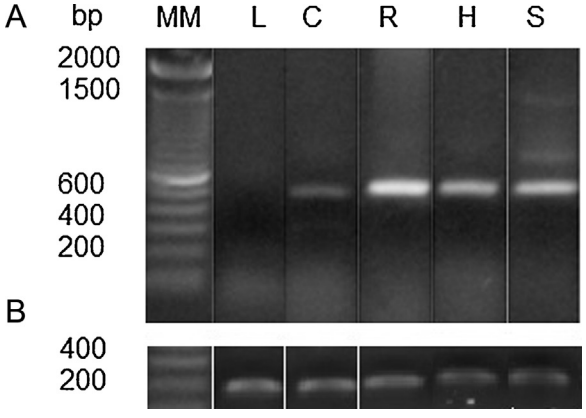

Fig. 2. Helja expression in organs obtained from *Helianthus annuus* seeds and 15-day-old seedlings. Agarose gels (1%) were used for the electrophoretic visualization of the RT-PCR products, obtained using first-strand cDNA from the leaves (L), cotyledons (C), roots (R), hypocotyls (H) and seeds (S) as the template and the primers for Helja (A) or actin (B). MM, molecular mass in bp. The gels were stained with Syber safe.

groups appeared distantly separated in the resulting unrooted phylogenetic tree performed using the lectin from *Artocarpus integrifolia* Jacalin as the outgroup (Fig. 3). Helja emerged as a unique member separate from these clusters (Fig. 3). Despite the fact that the tree showed moderate well-supported clades with bootstraps values lower than 75 over 100 iterations, the bulk of the results showed that Helja is barely similar to the other JRLs present in *H. annuus*.

The relationship of Helja with other plant jacalins

To obtain a deeper understanding of the sequence relationship of Helja with other members of the jacalin family, a blastp was performed over the nr database limited to Viridiplantae organisms using Helja as a query. Data mining identified accessions displaying either the jacalin domain alone or in association with other domains, such as protein kinase, dirigent, ATPase or leucine-rich repeats. Therefore, to obtain a functional comparison, unique jacalin domain sequences (merojacalins) were selected (Table 2). As expected, the blastp analysis showed that jacalin BAB18761.1 from *H. tuberosus* displays the highest score (253) and identity (95%) compared with Helja. Notably, a sharp decrease in the percentage identity was observed for the remaining matches, although some of these sequences corresponded to closely related species, such as *H. tuberosus* (Table 2). A multiple alignment was then performed using these sequences filtered with the CD-Hit program (Li and Godzik, 2006) to remove sequences above a 95% identity threshold. The alignment clearly showed the occurrence of conserved and invariant residues associated with the β prism domain characteristic of the jacalin family (Fig. 4). Interestingly, the 12 C-terminal amino acids from Helja were completely different from the C-terminal stream of the protein predicted from BAB18761.1, the closest related *H. tuberosus* JRL. A subsequent comparison of the selected accessions using the maximum likelihood method PhyML rendered an unrooted phylogenetic tree split into 3 unit; remarkably, one of these units showed Helja and the *H. tuberosus* lectin BAB18761.1 separated from the other *H. tuberosus* jacalins with a well-supported bootstrap value (Supplementary Fig. 3). The second cluster contains only jacalins from monocots (*Hordeum vulgare*, *Zea mays* and *Ananas comosus*) and the third branch displays accessions from both monocots (*Phoenix dactylifera* and *Musa acuminate)* and dicots (*Populus trichocarpa*). The low bootstrap support revealed high variability, although these sequences displayed the consensus

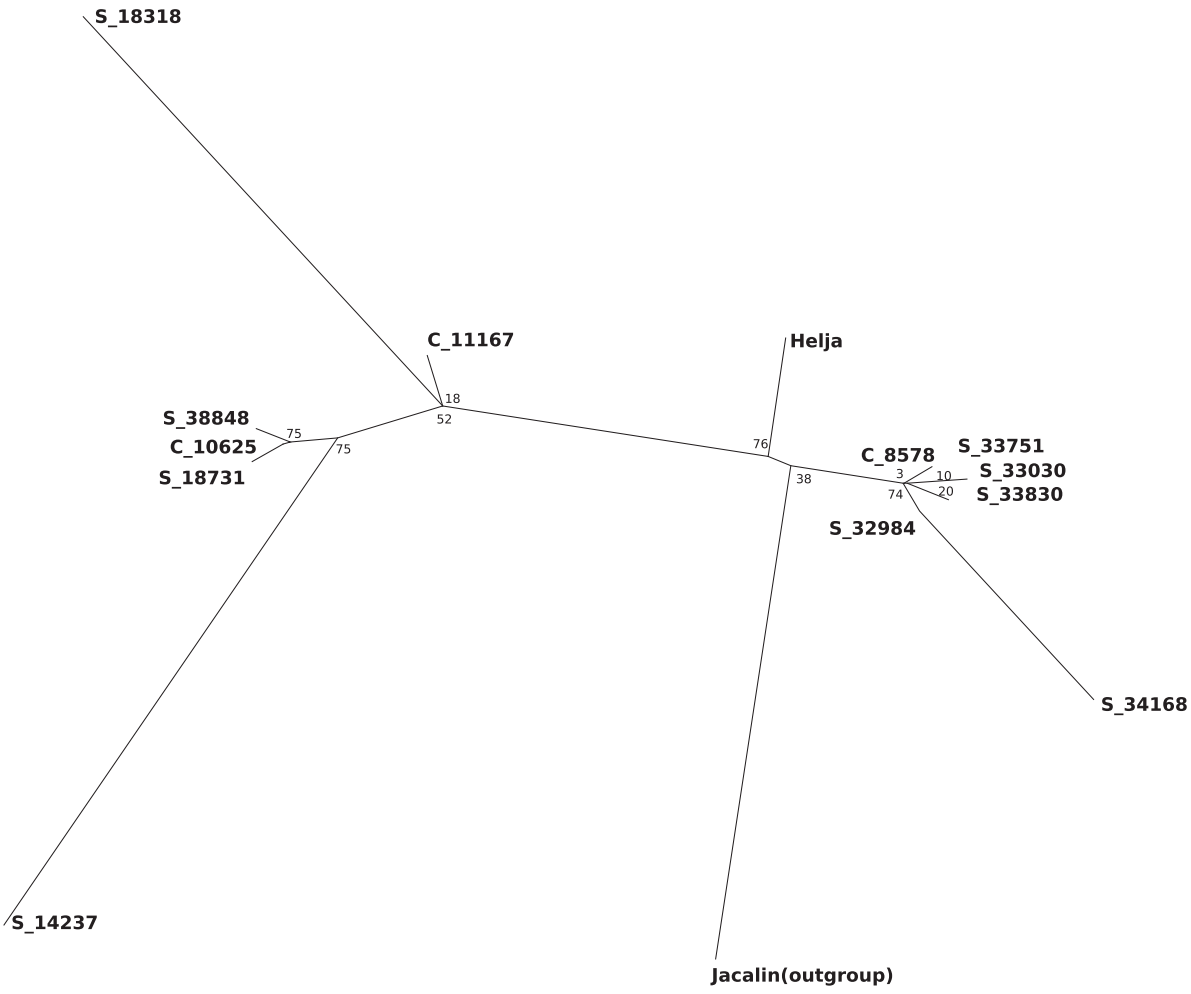

Fig. 3. Phylogenetic relationships of *Helianthus annuus* JRLs. The tree was built up with the sequences recovered from the Sunflower Unigene Repository (SUR v1.0) database using Helja CDS as a query and filtered for jacalin domains using Interproscan and Plecdom algorithms (Table 1). Jacalin, the *Artocarpus integrifolia* (*jack* fruit) lectin, was used as the outgroup.

Table 2
Viridiplantae JRLs producing significant identity with Helja.

Accession	Annotation/*species*	Score	*E*	Cov %	Id %
BAB18761.1	Lectin/*Helianthus tuberosus*	253	1e – 83	92	95
XP_008804539	Mannose/glucose-specific lectin-like/*Phoenix dactylifera*	112	2e – 28	96	43
AAL84814.1	Tuber agglutinin/*Helianthus tuberosus*	112	4e – 28	96	43
XP_008780478	Mannose/glucose-specific lectin-l/*Phoenix dactylifera*	112	5e – 28	96	41
AAD11578.1	Lectin 2/*Helianthus tuberosus*	111	6e – 28	96	43
AAM12553.1	Tuber agglutinin/*Helianthus tuberosus*	111	7e – 28	96	42
AAL84816	Tuber agglutinin/*Helianthus tuberosus*	110	1e – 27	96	43
AAL84817.1	Tuber agglutinin/*Helianthus tuberosus*	110	2e – 27	96	43
AAD11576.1	Lectin 3/*Helianthus tuberosus*	110	2e – 27	96	43
AAD11577.1	Lectin HE17/*Helianthus tuberosus*	108	5e – 27	95	43
1C3K_A	Chain A, H. Tuberosus Lectin/*Helianthus tuberosus*	108	6e – 27	96	42
AAL84815.1	Tuber agglutinin/*Helianthus tuberosus*	107	2e – 26	96	42
XP_006372713.1	POPTR_0017s043602g hypothet protein/*Populus trichocarpa*	106	1e – 25	96	45
AAQ07258.1	*Jacalin-like lectin/Ananas comosus*	104	2e – 25	96	40
1X1V_A	Lectin–methyl–alpha-mannose complex/Chain A/*Musa acuminata*	102	9e – 25	98	42
2BMY_A	Chain A, Banana Lectin/*Musa acuminata*	102	2e – 24	98	42
ADW77219	lectin AAA Group/*Musa acuminata*	101	3e – 24	98	41
ABS86034	Mannose-binding lectin AAA Group/*Musa acuminata*	97.4	1e – 22	93	42
XP_008648490	Salt stress-induced protein-like/*Zea mays*	95.9	6e – 22	100	40
AFW74546	ZEAMMB73_667809/*Zea mays*	93.6	4e – 21	100	39
Q5U9T2	Horcolin or mannose specific lectin/*Hordeum vulgare*	91.7	2e – 20	96	40
AFW82809	ZEAMMB73_630227/*Zea mays*	91.3	7e – 20	94	42

The sequences were recovered from NCBI database using Helja amino-acidic sequence as query in a blast p search performed over non-redundant database sequences and cut off of maximum score higher than 90 and E value lower than 10^{-20}. They were manually filtered for merolectins. Accession numbers follow the nomenclature indicated at NCBI. Cov % and Id% indicate coverture and identity, respectively, over the Helja sequence.

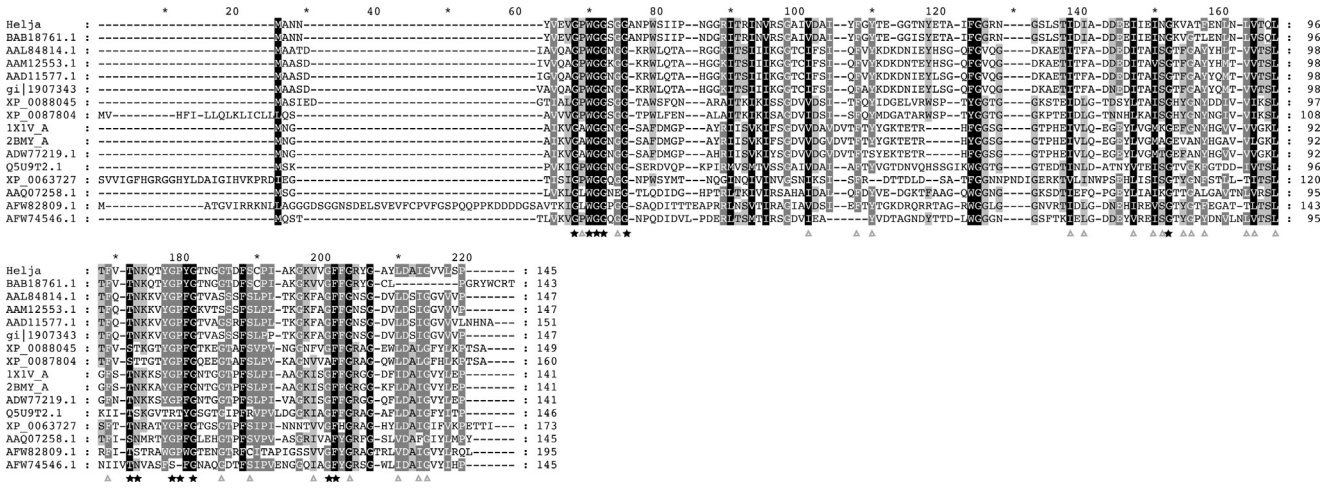

Fig. 4. Multiple alignment of Helja with viridiplantae JRLs sequences. The selected viridiplantae sequences from Table 2 were aligned with MAFFT 70 and filtered using CD-Hit. The residues were shadowed to distinguish 100% (black), 80% (gray) and 60% (light gray) similitude. The accession numbers are indicated according to the NCBI accession number. Conserved and invariant residues of the β prism domain characteristic of the JRL family are indicated using triangles and asterisks, respectively.

amino acids of the jacalin domain. *In silico* analyses to predict protein localization, were performed using the viridiplantae JRLs recovered in Table 2. Additionally, the UniProt information related to the subcellular localization of these proteins was assessed. Only *H. vulgare* Q5U9T2 was annotated as an apoplastic protein in UniProt (Table 3). This gene encodes Horcolin, an extracellular jacalin devoid of signal peptide isolated from coleoptiles (Grunwald et al., 2007). Helja and Horcolin are 40% identical and display 96% coverage along the unique jacalin domain. LocTree3, SignalP and SecretomeP were used as predictors of cellular location. SecretomeP identified 18 out of the 23 accessions with scores higher than 0.50 (Table 3), suggesting that these 18 sequences could be associated with disordered structures, a characteristic of unconventionally secreted proteins in mammals (Bendtsen et al., 2004). Interestingly, LocTree3, the program used to identify sequence patterns for 18 different locations in eukaryotes (Goldberg et al., 2014), predicted that 22 out of the 23 accessions are secreted into apoplast, with accuracies higher than 80%. However, only XP_008780478 carries a signal peptide detectable through the SignalP algorithm (Table 3). As a control, LocTree3 was also applied to identify known cytosolic and nuclear jacalins (Ver2, Jacalin and Nictaba), and all of these proteins were predicted to be intracellular. Thus, Helja is a useful bait to recover unconventionally secreted lectins in plants.

Experimental evidence was obtained as a proof of concept regarding the occurrence in the apoplast of at least one of the predicted extracellular JRLs reported in Table 3. Since the sequence of the JRL BAB18761.1 from tubers of *Helianthus tuberosus* displays significant identity to Helja we have used Helja antibodies

Table 3
Subcellular localization survey of viridiplantae JRLs producing significant identity with Helja.

Acession	LocTree 3				SignalP	SecP	Experim. Localization
	Score	Accuracy %	Class	Localization			
Helja	26	84	Secreted	Apoplast	No	0.447	Apoplast [17]
BAB18761.1	22	84	Secreted	Apoplast	No	0.399	No
XP_008804539	27	84	Secreted	Apoplast	No	0.400	–
AAL84814.1	21	83	Secreted	Apoplast	No	0.554	No
XP_008780478.	17	82	Secreted	Apoplast	Yes	0.554	–
AAD11578.1	22	84	Secreted	Apoplast	No	0.554	No
AAM12553.1	20	83	Secreted	Apoplast	No	0.487	No
AAL84816.	23	84	Secreted	Apoplast	No	0.532	No
AAL84817.1	21	83	Secreted	Apoplast	No	0.532	No
AAD11576.1	22	84	Secreted	Apoplast	No	0.526	No
AAD11577.1	23	84	Secreted	Apoplast	No	0.582	No
1C3K_A	22	84	Secreted	Apoplast	No	0.499	-
AAL84815.1	20	83	Secreted	Apoplast	No	0.553	No
XP_006372713.1	17	82	Secreted	Apoplast	No	0.568	No
AAQ07258.1	25	84	Secreted	Apoplast	No	0.550	No
1X1V_A	28	85	Secreted	Apoplast	No	0.506	No
2BMY_A	27	84	Secreted	Apoplast	No	0.523	No
ADW77219	23	84	Secreted	Apoplast	No	0.564	No
ABS86034	29	85	Secreted	Apoplast	No	0.594	No
XP_008648490	32	86	Secreted	Apoplast	No	0.729	No
AFW74546	32	86	Secreted	Apoplast	No	0.732	No
Q5U9T2	100	99	Secreted	Apoplast	No	0.627	Apoplast [43]
AFW82809	–	–	–	–	No	0.768	–

In silico and experimental evidence was obtained. The score and % of accuracy in the prediction, as well as the class and localization were obtained using LocTree3 algorithm. The presence of signal peptide or disordered structures characteristic from unclassically secreted proteins was tested using SignalP or SecretomeP (SecP), respectively. Experimental localization information was obtained from UniProt. Accession numbers follow the nomenclature indicated at NCBI.

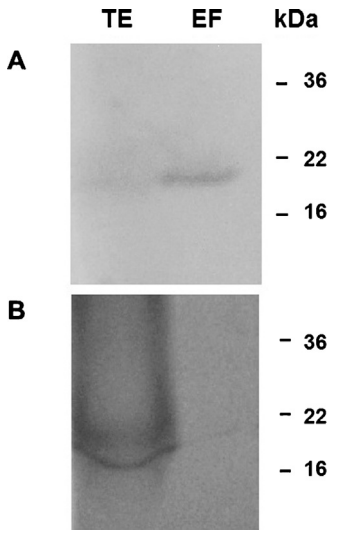

Fig. 5. Immunodetection of a JRL in the extracellular fluids from *H. tuberosus* tubers. Total soluble (TE) and extracellular (EF) proteins were isolated and loaded on a 12% SDS–PAGE, transferred to nitrocellulose, blocked and incubated sequentially with 1:4.000 anti Helja polyclonal antibodies and alkaline phosphatase-conjugated anti-rabbit IgG 1:7000 (A). The same samples were loaded in gel and stained with Coomassie B. Blue (B). Molecular weight markers are indicated on the right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to analyze whether a similar extracellular JRL could be detected. A band of the expected MW (Nakagawa et al., 2000) was observed through inmunoanalysis in the apoplastic fluids (EF) isolated from *H. tuberosus* tubers (Fig. 5A). No similar band could be detected in the intracellular fraction (TE) even though much more protein occurs in TE according to Coomassie Brillant Blue staining (Fig. 5B). In summary, the band inmunorecognized in EF, confirms the existence of an extracellular JRL in this species as well as the high sensitivity of the cross reactivity of the anti-Helja antibodies supporting the occurrence of a widespread mechanism for unconventional secretion.

Discussion

The complete cDNA sequence of Helja is presented here, and the results revealed that this extracellular JRL is synthesized devoid of signal peptide, despite its demonstrated apoplastic location. The extracellular localization of Helja was previously verified using a biochemical approach that includes MALDI–TOF/TOF mass spectrometric detection of the protein in intercellular fluids. The fraction was isolated and double-checked for the absence of cytosolic contaminants. Consistently, immunolocalization assays using confocal microscopy validated the apoplastic accumulation of this protein (Pinedo et al., 2012). Thus, the lack of signal peptide in the Helja CDS confirms the hypothesis that this protein is unconventionally secreted (Pinedo et al., 2012; Regente et al., 2012) and refines the picture of the merojacalins in plants, as only a few members have been characterized concerning their subcellular localization. Hence, single domain jacalins with mannose-binding specificity, such as Calsepa and Morniga M, are encoded by sequences devoid of signal peptides and are located in the cytoplasm and nucleus, respectively (Peumans et al., 2000; Van Damme et al., 2002). However, the galactose-specific lectins Jacalin and Morniga G, are encoded as preproproteins with a signal peptide that directs these proteins to the vacuolar compartment (Peumans et al., 2000; Van Damme et al., 2002). Helja transcripts were detected in *H. annuus* seeds, roots, flowers and hypocotyls, but not in the leaves. Although no information is available on the putative function Helja in the apoplast, the wide distribution of this protein in sunflower seedlings suggests a non-negligible role.

Asteraceae is one of the largest plant families, however, only a small number of JRLs have been identified in this family. The proteins are primarily restricted to approximately 10 JRLs in *H. tuberosus*, a species of unknown genome. Thus, the accessions identified in this work provide a significant contribution to this group. Species with fully sequenced genomes show a highly variable number of JRLs. More than 20 and 40 JRLs have been identified in *Z. mays* and *Arabidopsis*, respectively, and only 6 accessions have been identified in the *Glycine max* genome (Jiang et al., 2010; Chandra et al., 2006). The 12 sunflower unigenes recovered in this study through data mining follow the trend of sequence variability reported for JRLs in other species (Jiang et al., 2010), and points out to the functional importance of these proteins.

Interestingly, when Helja was used as a template to detect similar proteins among the whole sequences from plants deposited in public databases, only the *H. tuberosus* merolectins BAB18761.1 was highly similar, although the last 10 amino acids were clearly different. Consistent with this observation, no Helja tryptic fragments matched the C-t region of BAB18761.1 in previous proteomic data mining (Pinedo et al., 2012). The remaining sequences recovered belong to both mono and dicotyledons and displayed coverage higher than 94%, but were only 39 to 45% identical. These features were reflected in the topology of the phylogenetic tree, where Helja was branched with the accession BAB18761.1 and separated from other *H. tuberosus* sequences. The relatively low level of sequence conservation between these JRLs and the other jacalins from Asteraceae suggest that variations have evolved independently from an ancestor of *Helianthus* genus. More importantly, while the JRLs have primarily been localized to the cytoplasm, nucleus or vacuole, data mining using Helja as a query recovered an extracellular jacalin, Horcolin (Q5U9T2) among a large group of accessions. This protein, devoid of signal peptide, was purified from extracellular fluids isolated from *H. vulgare* coleoptiles. Helja and Horcolin are present in distantly related groups in *H. annuus*, an Asteraceae dicot, and *H. vulgare*, a Triticeae monocot, although these sequences are 40% identical. Remarkably, the algorithms inferring the subcellular localization of eukaryotic proteins recognized a pattern for secretion to the apoplast in all the accessions of the merolectins similar to Helja (Table 3), despite the fact that only one of the sequences presents a signal peptide in the N-terminus to drive the protein through the classic secretion pathway. Amino acid modifications and structural divergence in the JRL could be requisite for non-classical secretion and might justify the *in silico* clustering of putatively extracellular JRL. Therefore, the Helja sequence emerged as a good bait to defy databases and recover putative unconventional secreted lectins. These predictions were strongly supported by the fact that, among the proteins listed in Table 3, experimental evidence confirmed the presence of a 16 kDa band recognized by antibodies raised against Helja in the intercellular washing fluids isolated from *H. tuberosus* tubers. Together, these data sustain the fact that the unconventional secretion of jacalins emerges as a widespread mechanism.

A number of proteomic approaches, primarily using *Arabidopsis thaliana*, have recurrently shown the occurrence of JRLs (with and without the signal peptide in their sequences) in the apoplast (Ding et al., 2012; 2014), supporting the idea that these proteins play a significant biological role in this location. Strikingly, no sequences from this dicotyledonous model species appeared among the sequences recovered in the data mining experiments performed in the present study, despite the relative enrichment of *Arabidopsis* accessions in the databases.

In summary, the phylogeny of *H. annuus* JRL is consistent with the hypothesis that significant differences in the Helja sequence are accumulated to facilitate new functions in the apoplast. The

comparison of the evolution of different families of lectins in *Arabidopsis*, *Oryza sativa* and *Glycine max* showed a species-specific expansion rate and high variation in the number of members in each group (Jiang et al., 2010). Moreover, the authors identified JRLs as the group displaying the highest rates of birth to death evolution. The variable number of JRLs in different species likely reflects a high expansion rate, suggesting that jacalins are prone to acquire mutations in duplicated genes that, in turn, might lead to neofunctionalization. The finding of putatively unconventionally secreted JRLs in diverse taxonomic groups suggests the occurrence of conserved sorting mechanisms among plants. These leaderless proteins might be secreted through at least one of the four pathways for non-classical secretion previously recognized in yeast or mammals (Nickel, 2005) and more recently recognized in plants (Ding et al., 2014; Drakakaki and Dandekar, 2013). Moreover, previous evidence obtained by our group suggests that Helja is more likely associated with multivesicular bodies (MVB), as this protein was detected in apoplastic vesicles that resembled the vesicles released after the fusion of MVB with the plasma membrane (Regente et al., 2009). A novel type of JRL with apoplastic localization is emerging and awaiting functional characterization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.06.004

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