Lectin activity of the nucleocytoplasmic EUL protein from *Arabidopsis thaliana*

Jonas Van Hove [a,1], Elke Fouquaert [a,1], David F. Smith [b], Paul Proost [c], Els J.M. Van Damme [a,*]

[a] *Laboratory of Biochemistry and Glycobiology, Department of Molecular Biotechnology, Ghent University, Coupure Links 653, 9000 Ghent, Belgium*
[b] *Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, USA*
[c] *Laboratory of Molecular Immunology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium*

ARTICLE INFO

ABSTRACT

The *Euonymus* lectin (EUL) domain was recognized as the structural motif for a novel class of putative carbohydrate binding proteins. Confocal microscopy demonstrated that the lectin from *Euonymus europaeus* (EEA) as well as the EUL protein from *Arabidopsis thaliana* (ArathEULS3) are located in the nucleocytoplasmic compartment of the plant cell. ArathEULS3 as well as its EUL domain were successfully expressed in *Pichia pastoris* and purified. The EUL domain from *Arabidopsis* interacts with glycan structures containing Lewis Y, Lewis X and lactosamine, indicating that it can be considered a true lectin domain. Despite the high sequence identity between the EUL domains in EEA and ArathEULS3, both domains recognize different carbohydrate structures.

© 2011 Elsevier Inc. All rights reserved.

1. Introduction

In recent years evidence is accumulating that plants subjected to specific abiotic or biotic stimuli respond by the expression of low amounts of a new class of plant lectins, now referred to as the group of inducible plant lectins [1,2]. In 2008 the molecular cloning of the *Euonymus europaeus* agglutinin (EEA) from spindle tree led to the discovery of a new family of lectins [3]. Since then the *Euonymus* lectin domain (EUL) is considered as the structural unit of a novel family of putative carbohydrate-binding proteins.

An extensive screening of publicly accessible genome databases revealed that the EUL domain is widespread among green plants [3]. Furthermore some sequences consist of a single EUL domain linked to an unrelated N-terminal domain whereas others comprise two in tandem arrayed EUL domains. Based on the overall domain architecture of EUL proteins a classification system for this protein family was proposed [4]. Only EEA was shown to exhibit carbohydrate binding activity but until now there are no reports that prove the lectin activity of the other EUL proteins. Transcriptome analyses revealed that the production of EUL proteins is upregulated under stress conditions [4–7]. The S3 type of EUL proteins containing an N-terminal domain (>100 amino acids) linked to an EUL domain was shown to be expressed in most if not all Viridiplantae, suggesting an important role for this type of proteins.

Unfortunately the identification and characterization of the stress inducible EUL lectins is hampered by their very low expression levels. In an attempt to study the biological properties of the S3 type of EUL protein from *Arabidopsis thaliana* (ArathEULS3) the localization of the protein was studied in plant cells. Furthermore the recombinant protein was expressed in *Pichia pastoris* and purified. Analyzes were done both with the full length protein as well as with the EUL domain only. Our results show for the first time that ArathEULS3 and its EUL domain possess carbohydrate-binding activity and hence can be considered as lectins.

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana cell suspension cultures ecotype Landsberg *erecta* (Plant System Biology-Dark type culture: PSB-D) were maintained on a 7 day culture cycle by adding 10 ml of the cell culture to 90 ml of medium containing 4.43 g/liter Murashige and Skoog Basal salts with minimal organics (Sigma–Aldrich), 30 g/liter sucrose, 0.5 mg/liter α-naphthaleneacetic acid, 0.05 mg/liter kinetin, pH 5.7. The cells were grown on a rotary shaker (150 rpm) at 25 °C in the dark.

Tobacco BY-2 suspension cells were cultured as described in Fouquaert et al. [8].

2.2. Construction of expression vectors

A full length cDNA clone corresponding to At2g39050 (EULS3 of *A. thaliana*) was ordered from the Experimental Plant Division group

within the Department of Biological Systems of the BioResource Center of the RIKEN Tsukuba Institute (Ibaraki, Japan) [9,10].

Vectors for expression of EEA and ArathEULS3 linked to EGFP were constructed using the Gateway™ technology of Invitrogen (Carlsbad, CA, USA). The coding sequence of EEA was amplified as an attB PCR product using the cDNA clone *LECEEA* as a template (Accession number EF990656, [3]). The entire coding sequence of ArathEULS3 was amplified using the forward primer EUL$_{Ar}$-f (5′ AAAAAGCAGGCTTCACCATGGAGCAC CACCACCAGCAT 3′) and the reverse primer EUL$_{Ar}$-r (5′ AGAAAGCTGGGTGTCAGAAAGGAAAGA TCTTCCAGAG 3′) with or without stop codon in case of C-terminal or N-terminal fusion to EGFP, respectively. To obtain attB PCR products a nested PCR was performed using *ArathEULS3* gene-specific primers for the first PCR reactions and primers EVD 2 (5′ GGGGACAAGTTTGTACAAAAAAGCAGGCT 3′) and EVD 4 (5′ GGGGACCACTTTGTACAAGAAAGCTGGGT 3′) for the second PCR reactions. Cycling parameters for the first PCR reaction were as follows: 5 min 94 °C, 25 cycles (15 s 94 °C, 30 s 50 °C, 1 min 72 °C), 5 min 72 °C. The amplified fragments were cloned in the pDONR221 vector (Invitrogen) by a BP clonase reaction. After sequencing of the entry clones, subsequent LR reactions were performed with the pK7WGF2 and the pK7FWG2 destination vectors [11] to fuse the EUL sequence C-terminally or N-terminally to EGFP, respectively.

2.3. Expression analyses

Tobacco BY-2 cells were transiently transformed with EGFP-fusion constructs using particle bombardment as described by Fouquaert et al. [8].

For stable transformation a two-day old *Arabidopsis* cell culture was cocultivated with *A. tumefaciens* cells harboring the expression vector [12] as described by Van Leene et al. [13]. Seven days after subculturing plant cells were harvested and proteins extracted. The protein content was estimated using the Bio-Rad Protein Assay, based on the Bradford [14] dye-binding procedure using bovine serum albumin (BSA) as a standard. SDS–PAGE on 15% polyacrylamide gels was performed under reducing conditions as described by Laemmli [15]. For Western blot analysis, samples separated by SDS–PAGE were electrotransferred to 0.45 µm polyvinyl-idene fluoride (PVDF) membranes (Biotrace™ PVDF, PALL, Gelman Laboratory, USA). After blocking the membranes in Tris-Buffered Saline (TBS: 10 mM Tris, 150 mM NaCl and 0.1% (v/v) Triton X-100, pH 7.6) containing 5% (w/v) BSA, blots were incubated for 1 h with a rabbit polyclonal anti-EUL antibody (raised by Thermo Scientific (Rockford, IL, USA) against the EUL domain of ArathEULS3), diluted 1/500 in TBS. The secondary antibody was a 1/1000 diluted goat anti-rabbit IgG labeled with horseradish peroxidase (Dako Cytomation, Glostrup, Denmark). Immunodetection was achieved by a colorimetric assay using 3,3′-diaminobenzidine tetrahydrochloride (Sigma–Aldrich, St. Louis, MI, USA) as a substrate.

2.4. Confocal microscopy

Image analysis was carried out with a Nikon C1 confocal microscope mounted on an inverted TE2000 Eclipse epifluorescence body (Nikon Instruments, Badhoevedorp, The Netherlands) using a 60× Plan Apo objective lens (NA of 0.95) coupled with a standard Nikon CCD camera. EGFP was excited with a 488 nm line of an argon ion laser and emission light was selected with a HQ 515/30 nm filter. Images were analyzed with ImageJ (http://www.rsb.info.-nih.gov/ij).

2.5. Construction of the polyhistidine fusion vector and expression in P. pastoris

The EasySelect *Pichia* Expression Kit from Invitrogen was used to clone and express ArathEULS3 in the *P. pastoris* strain KM71H (Invitrogen, Carlsbad, CA, USA). The full ArathEULS3 sequence was amplified by PCR using the forward primer evd 307 (5′ GGCGGAGAATTCACCATGGAGCACCACCACCAGCATCACCG 3′) and reverse primer evd 308 (5′ CCCGCTTTCTAGAATGAAAGGAAA-GATCTTCCAGAGCTG 3′). Amplification of the EUL domain alone was achieved with forward primer evd 494 (5′ GGCGGAGAATT-CACCATGGCCGGAAGAGCAACGGTGAAGG 3′) and reverse primer evd 308 (5′ CCCGCTTTCTAGAATGAAAGGAAAGATCTTCCAGAGCTG 3′). Amplification conditions were as follows: 2 min at 94 °C, 25 cycles (15 s 94 °C, 30 s 55 °C, 1 min 72 °C), 5 min 72 °C. The amplification was carried out in a 25 µl reaction volume, containing
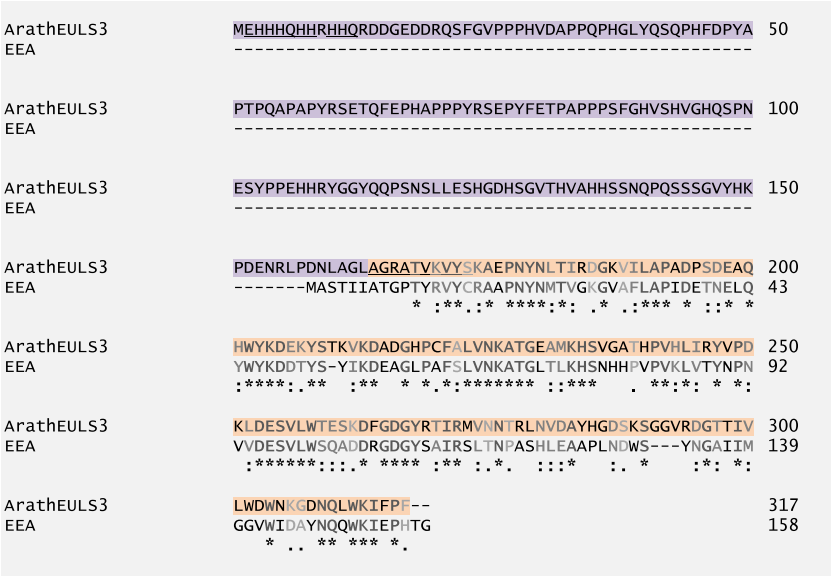
```
ArathEULS3    MEHHHQHHRHHQRDDGEDDRQSFGVPPPHVDAPPQPHGLYQSQPHFDPYA  50
EEA           --------------------------------------------------

ArathEULS3    PTPQAPAPYRSETQFEPHAPPPYRSEPYFETPAPPPSFGHVSHVGHQSPN  100
EEA           --------------------------------------------------

ArathEULS3    ESYPPEHHRYGGYQQPSNSLLESHGDHSGVTHVAHHSSNQPQSSSGVYHK  150
EEA           --------------------------------------------------

ArathEULS3    PDENRLPDNLAGLAGRATVKVYSKAEPNYNLTIRDGKVILAPADPSDEAQ  200
EEA           -------MASTIIATGPTYRVYCRAAPNYNMTVGKGVAFLAPIDETNELQ  43
                     * :**.:* ****:*: .* .:*** * ::* *

ArathEULS3    HWYKDEKYSTKVKDADGHPCFALVNKATGEAMKHSVGATHPVHLIRYVPD  250
EEA           YWYKDDTYS-YIKDEAGLPAFSLVNKATGLTLKHSNHHPVPVKLVTYNPN  92
              :****.**  :** * *.*:*******  ::***   . **:*: * *:

ArathEULS3    KLDESVLWTESKDFGDGYRTIRMVNNTRLNVDAYHGDSKSGGVRDGTTIV  300
EEA           VVDESVLWSQADDRGDGYSAIRSLTNPASHLEAAPLNDWS---YNGAIIM  139
              :******::.* **** :** :.*. :::*  :. *    :*: *:

ArathEULS3    LWDWNKGDNQLWKIFPF--                                 317
EEA           GGVWIDAYNQQWKIEPHTG                                 158
              * .. ** *** *.
```

Fig. 1. Sequence comparison between the *Euomymus* lectin (EEA) and the *Arabidopsis* EUL protein (ArathEULS3) using ClustalW. The ArathEULS3 sequence consists of an N-terminal domain absent from EEA (shown in purple) and a C-terminal domain homologous to EEA (shown in orange). Identical residues are indicated by asterisks whereas homologous residues are shown by dashes. The N-terminal amino acid sequences determined for the recombinant ArathEULS3 and EUL domain purified from *Pichia* are underlined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
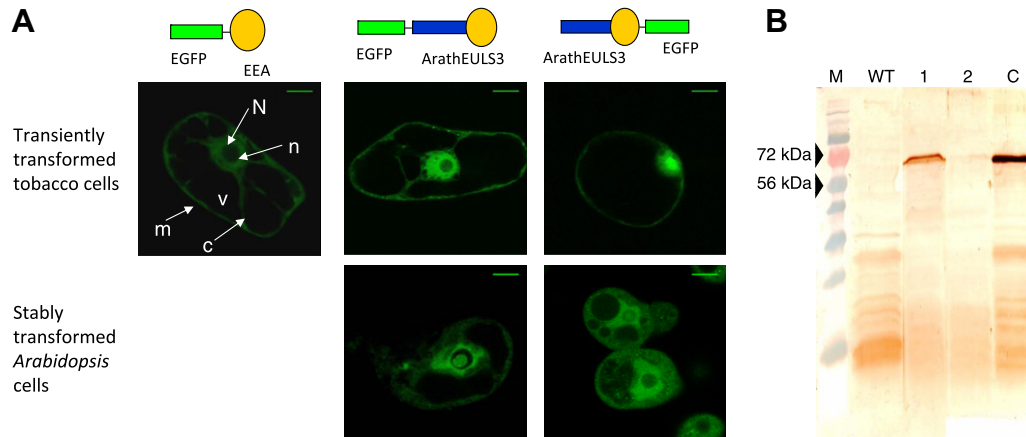
Fig. 2. (A) Confocal images of transiently transformed tobacco BY-2 cells and stably transformed *Arabidopsis* PSB-D cells expressing EGFP–EEA, EGFP–ArathEULS3 and ArathEULS3–EGFP. Scale bars represent 25 nm. Cell compartments: n, nucleolus; N, nucleus; m, cell membrane; c, cytoplasm; v, vacuole. (B) Western blot analysis of crude protein extracts from *Arabidopsis thaliana* cells expressing ArathEULS3–EGFP (lane 1) and EGFP–ArathEULS3 (lane 2). A rabbit polyclonal antibody raised against the EUL domain was used as primary antibody. In each lane approximately 50 μg total protein was loaded. Lane M, Pageruler™ prestained protein ladder (Fermentas, St Leon-Rot, Germany). Lane WT, crude protein extract from non-transformed cells. Lane C was loaded with a TAP-tagged ArathEULS3 protein (65 kDa) as a positive control.

40 ng DNA template using *Pfx* DNA polymerase. The PCR products were digested with *EcoRI* and *XbaI*, and cloned into the *Escherichia coli/P. pastoris* shuttle vector pPICZB, containing a polyhistidine tag downstream from the multiple cloning site. The shuttle vectors were transformed into *E. coli* TOP 10 cells using heat shock transformation and transformants were selected on LB agar plates containing 25 μg/ml zeocin. Plasmids were purified using the E.Z.N.A. Plasmid Mini kit I (Omega Bio-Tek, GA, USA) according to the manufacturer's instructions. The complete coding sequence of the insert was verified by nucleotide sequence analysis using 5′ AOX1 and 3′ AOX1 vector-specific forward primer evd 21, 5′ GAC-TGGTTCCAATTGACAAGC 3′ and reverse primer evd 22, 5′ GCAAATGGCATTCTGACATCC 3′, respectively (LGC Genomics GmbH, Berlin, Germany). Plasmid DNA from transformed *E. coli* was linearized using the *SacI* restriction enzyme (Fermentas, Sankt Leon-Rot, Germany) overnight at 37 °C. Approximately 10 μg of the linearized DNA was transformed into *Pichia* strain KM-71H cells by electroporation (Bio-Rad, Hercules, CA, USA) with following pulse settings: 25 μF, 1.5 kV, 200 Ω. Transformants were selected for zeocin resistance on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, 100 μg/ml zeocin) plates.

2.6. Production and purification of the recombinant ArathEULS3 lectin and the EULS3 domain in P. pastoris

Expression of the full length ArathEULS3 and the EUL domain in *P. pastoris* was performed as described by Al Atalah et al. [16]. The recombinant proteins were purified from pelleted cells extracted in 20 mM 1,3 diaminopropane with addition of acid washed glass beads (ø 250–500 μm). All supernatants were collected and the pH was set at 10 before loading on the Q Fast Flow column equilibrated in 20 mM 1,3 diaminopropane. After washing the column was eluted with 0.1 M Tris–HCl pH 7.6 containing 0.5 M NaCl. The eluted samples were pooled and imidazole (IZ) was added to a final concentration of 25 mM before loading on a Nickel Sepharose column. After washing with washing buffer (0.1 M Tris–HCl pH 7.6 containing 0.5 M NaCl and 25 mM IZ) a stepwise elution of the column was performed using 0.1 M Tris–HCl pH 7.6 and 0.5 M NaCl, with increasing IZ concentrations ranging from 50 to 250 mM IZ. The eluted fractions were analyzed with SDS–PAGE and Western blot. Fractions considered to be pure were pooled and concentrated on a Q Fast Flow column using the same methodology as described above.
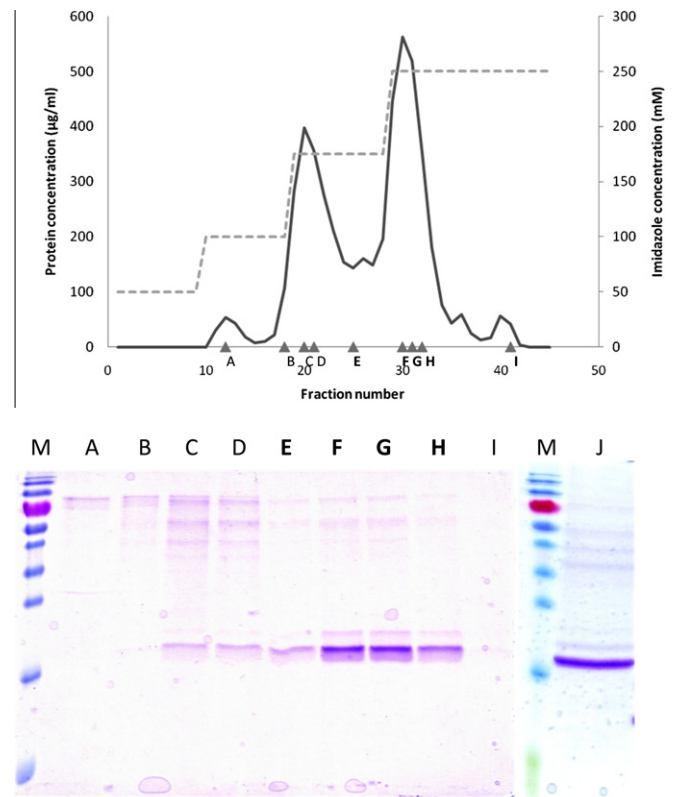

Fig. 3. A: elution pattern of EUL domain from ArathEULS3 from Nickel-Sepharose. Full line: protein absorbtion measured at 280 nm. Dashed line: stepwise elution with increasing concentrations of IZ (50–100–175–250 mM) in Tris buffer. Triangles: number of analyzed fractions. B: SDS–PAGE of eluted fractions from Nickel-Sepharose. M: prestained protein marker (Fermentas). A–I refer to fractions indicated in Fig. 3A. Lane J shows the purified EUL domain from ArathEULS3.

2.7. Molecular mass determination

The molecular mass of the recombinant proteins was experimentally determined with a gel filtration using a Superose 20 column (attached to an AKTA FPLC system, Amersham) in PBS containing 0.1 M galactose at a flow rate of 0.5 ml/min, and compared to a set of molecular mass standards ranging from 12.6 to 240 kDa.

2.8. Glycan array screening

The microarrays are printed as described previously [17] and version 4.2 with 511 glycan targets was used for the analyzes reported here (http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtml). Analyzes were performed as described by Al Atalah et al. [16].

3. Results and discussion

3.1. The EUL domain within ArathEULS3 shows high sequence similarity to EEA

Sequence alignment on the cDNA sequences encoding ArathEULS3 and EEA revealed that ArathEULS3 is a chimeric protein containing a C-terminal 154 amino acid EUL domain with 45% sequence identity and 74% sequence similarity to the EUL domain of EEA (Fig. 1). BlastP analyses with the sequence of the N-terminal domain of ArathEULS3 did not show homology to any known protein.

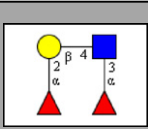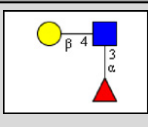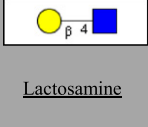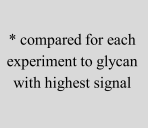### 3.2. The E. europaeus agglutinin and ArathEULS3 are nucleocytoplasmic proteins

Analysis of the amino acid sequences of EEA and ArathEULS3 using the SignalP tool did not show the presence of a signal peptide, indicating that these EUL proteins are synthesized on free ribosomes. Using the PSORT database, no NLS or other targeting sequences could be retrieved.

To check the location of the proteins the sequences were fused to EGFP and expressed in plant cells. Analysis by confocal microscopy demonstrated that the EGFP–EEA fusion protein is located in the nucleus and cytoplasm of tobacco BY2 cells at different time points after transformation (Fig. 2A). Very similar results were obtained for fusion constructs of ArathEULS3 transiently expressed in BY2 cells or stably transformed in *Arabidopsis* cells. Fig. 2A shows that both the N-terminal and the C-terminal EGFP fusions for ArathEULS3 are located in the nucleus and in the cytoplasmic strands transversing the vacuole.

Western blot analysis was performed on crude protein extracts of the transformed *Arabidopsis* cell lines as well as on the wild type cells to check the integrity of the fusion protein. An immunoreactive band of approximately 70 kDa was clearly detected in the cells transformed with ArathEULS3–EGFP using polyclonal anti-EUL antibodies. However no or only a very weak immunoreactive band was detected for the N-terminal fusion construct, which is in agreement with the lower fluorescence signals observed in confocal microscopy for cells expressing these constructs. No signal was detected for non-transformed cells (Fig. 2B). Taking into account that the calculated molecular mass of the ArathEULS3 protein is approximately 36 kDa and EGFP has a size of 27 kDa it can be concluded that the ArathEULS3–GFP construct expressed in the plant cell is expressed as an intact fusion protein.

Table 1
Overview of the top 30 glycan structures with highest reactivity on the glycan array. The most recurring structures are highlighted: Lewis Y structures are shown in bold, Lewis X motifs are shown in italic and lactosamine motifs are underlined. The percentage relative fluorescence units (% RFU) is shown for the top 30 glycans in the glycan array for ArathEULS3 and EEA.

Glycan Structure	% RFU*	
	ArathEUL	EEA
Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3GalNAcα-Sp14	100,0	0,03
GalNAcα1-3(**Fucα1-2)Galβ1-4(Fucα1-3)GlcNAc**-3GalNAc-Sp14	73,6	0,05
<u>Galβ1-4GlcNAc</u>β1-4(<u>Galβ1-4GlcNAc</u>β1-2)Manα1-3(GlcNAcβ1-4)(<u>Galβ1-4GlcNAc</u>β1-6(<u>Galβ1-4GlcNAc</u>β1-2)Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-Sp21	67,5	0,06
Neu5Acα-3*Galβ1-4(Fucα1-3)GlcNAc*β-Sp0	46,6	0,04
Galα1-3Galβ1-3GlcNAcβ1-2Manα1-3(Galα1-3Galβ1-3GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-Sp19	46,6	0,13
GlcNAcα1-4<u>Galβ1-4GlcNAc</u>β1-3*Galβ1-4(Fucα1-3)GlcNAc*β1-3*Galβ1-4(Fucα1-3)GlcNAc*β-Sp0	37,2	0,06
GlcNAcβ1-3GalNAcα-Sp8	37,0	0,09
*Galβ1-4(Fucα1-3)GlcNAc*β1-4*Galβ1-4(Fucα1-3)GlcNAc*β1-4*Galβ1-4(Fucα1-3)GlcNAc*β-Sp0	22,7	0,02
GlcNAcβ1-3Galβ-Sp8	21,2	0,06
Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα1-6(**Fucα1-2Galβ1-4(Fucα1-3)GlcNAc**β1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-6AA	18,9	0,08
Galβ1-4Galβ-Sp10	17,0	0,05
Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα-Sp14	15,8	0,04
<u>Galβ1-4GlcNAc</u>β-(OCH2CH2)6NH2	15,6	0,03
Neu5Acα2-3-Galβ1-3(*Galβ1-4(Fucα1-3)GlcNAc*β1-6)GalNAc-Sp14	14,8	0,09
Galα1-3(Fucα1-2)Galβ1-3GalNAcβ-Sp8	14,5	21,47
GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	13,2	0,05
[6OSO3](Fucα1-2)Galβ1-3[6OSO3]GlcNAcβ-Sp0	12,6	0,04
Fucα1-2Galβ1-3(Fucα1-4)GlcNAc-Sp8	11,8	0,08
Galα1-3(Fucα1-2)*Galβ1-4(Fucα1-3)GlcNAc*β-Sp8	10,7	0,06
Neu5Acα2-3*Galβ1-4(Fucα1-3)GlcNAc*β1-3GalNAcα-Sp14	10,1	0,11
Neu5Acα2-3(GalNAcβ1-4)<u>Galβ1-4GlcNAc</u>β1-3GalNAcα-Sp14	9,7	0,02
<u>Galβ1-4GlcNAc</u>β1-2Manα1-3(<u>Galβ1-4GlcNAc</u>β1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	9,1	0,09
Galα1-3Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-3(Galα1-3Galβ1-3(Fucα1-4)GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-Sp19	9,1	0,01
Galβ1-3(Neu5Acα2-3*Galβ1-4(Fucα1-3)GlcNAc*β1-6)GalNAcα-Sp14	9,0	0,03
*Galβ1-4(Fucα1-3)GlcNAc*β1-6GalNAc-Sp14	8,4	0,07
Fucα1-2Galβ-Sp8	8,3	0,08
[3OSO3]Galβ1-4(Fucα1-3)[6OSO3]GlcNAc-Sp8	8,0	0,03
Neu5Acα2-6<u>Galβ1-4GlcNAc</u>β1-2Manα1-3(Neu5Acα2-6<u>Galβ1-4GlcNAc</u>β1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	7,7	0,07
Fucα1-2<u>Galβ1-4GlcNAc</u>β1-2Manα1-3(Fucα1-2<u>Galβ1-4GlcNAc</u>β1-2Manα1-6)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAcβ-Sp22	7,2	0,03
Fucα1-2<u>Galβ1-4GlcNAc</u>β1-2(Fucα1-2<u>Galβ1-4GlcNAc</u>β1-4)Manα1-3(Fucα1-2Galβ1-4 GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp12	7,2	0,27

Lewis Y

Lewis X

Lactosamine

* compared for each experiment to glycan with highest signal

In contrast to EEA that is expressed at relatively high levels in the arilli of the spindle tree, the *Arabidopsis* protein is a non-abundant protein which production is upregulated under stress conditions [4]. Further elucidation of the physiological role of the EUL lectins inside the plant cell will require a more detailed functional study of the protein and its interacting partners. Therefore the protein was produced in *Pichia*, purified and characterized.

3.3. Purification and characterization of the ArathEULS3 full length protein and the EULS3 domain

Both the full ArathEULS3 protein and the EUL domain were successfully cloned into the *E. coli/P. pastoris* pPICZB shuttle vector and expressed into *P. pastoris* strain KM71H. Intracellular expression was detected using Western blot analysis with an antibody directed towards the His-tag present in the recombinant proteins. The recombinant His-tagged proteins for ArathEULS3 and the EUL domain were purified using a combination of anion exchange chromatography and metal affinity chromatography (Fig. 3A and B). On average 2 mg recombinant protein per liter culture volume was obtained.

The calculated molecular mass of the recombinant ArathEULS3 protein including a c-myc epitope and the (His)6 tag is 37.8 kDa. Upon gel filtration the ArathEULS3 protein eluted in two peaks corresponding to a calculated molecular mass of 77.6 and 35.8 kDa, respectively, suggesting that the recombinant protein exists partly as a monomer but also forms dimers.

N-terminal protein sequence analysis for the full recombinant ArathEULS3 protein and the EUL domain yielded the peptide sequences EHHHQHHVHHQD and AGRATVKVYS, respectively, corresponding to the N-terminus of ArathEULS3 (83% AA sequence identity) and the EUL domain (100% AA sequence identity).

3.4. The ArathEULS3 protein is a functional lectin

The carbohydrate-binding capacity of the full length ArathEULS3 lectin and its putative lectin domain were analyzed using the glycan array technology, and compared to the carbohydrate binding properties of EEA. Table 1 gives an overview of the top 30 glycan structures that reacted best upon analysis of ArathEULS3. It is shown that ArathEULS3 reacts with glycans containing one or more Lewis X (Galβ1–4(Fucα1–3)GlcNAc-), Lewis Y (Fucα1–2Galβ1–4(Fucα1–3)GlcNAc-) or lactosamine (Galβ1–4GlcNAc-) motifs. These glycan motifs are generally accepted to be typical for higher animals, and unlike Lewis A motifs, they have not yet been found in plants [18–20]. Our results prove for the first time that the ArathEULS3 protein is a functional carbohydrate binding protein. It remains to be shown how the recognition of these glycans is important for the plant. Similar results were obtained for the recombinant protein containing only the EUL domain.

In contrast, the glycan binding results for EEA showed affinity of EEA towards Lewis D (Galα1–3(Fucα1–2)Galβ1–3GlcNAc-) motifs and blood type B (Galα1–3(Fucα1–2)Galβ1–4GlcNAc-) glycan structures [3]. The array results support the concept that both EEA and ArathEULS3 are carbohydrate binding proteins, though their binding sites apparently have evolved into recognition of slightly different glycan structures.

Acknowledgments

References

[1] E.J.M. Van Damme, A. Barre, P. Rougé, W.J. Peumans, Cytoplasmic/nuclear plant lectins: a new story, Trends Plant Sci. 9 (2004) 484–489.

[2] N. Lannoo, E.J.M. Van Damme, Nucleocytoplasmic plant lectins, Biochim. Biophys. Acta. 1800 (2010) 190–201.

[3] E. Fouquaert, W.J. Peumans, D.F. Smith, P. Proost, S. Savvides, E.J.M. Van Damme, The old *Euonymus europaeus* agglutinin represents a novel family of ubiquitous plant proteins, Plant Physiol. 147 (2008) 1316–1324.

[4] E. Fouquaert, W.J. Peumans, T.T.M. Vandekerckhove, M. Ongenaert, E.J.M. Van Damme, Proteins with an *Euonymus* lectin-like domain are ubiquitous in Embryophyta, BMC Plant Biol. 9 (2009) 136.

[5] A. Moons, J. Gielen, J. Vandekerckhove, D. Van, G. der Straeten, M. Gheysen, Van. Montagu, An abscisic-acid- and salt-stress-responsive rice cDNA from a novel plant gene family, Planta 202 (1997) 443–454.

[6] F. Riccardi, P. Gazeau, M.P. Jacquemot, D. Vincent, M. Zivy, Deciphering genetic variations of proteome responses to water deficit in maize leaves, Plant Physiol. Biochem. 42 (2004) 1003–1011.

[7] S.C. Carpentier, E. Witters, K. Laukens, H. Van Onckelen, R. Swennen, B. Panis, Banana (*Musa* spp.) as a model to study the meristem proteome: acclimation to osmotic stress, Proteomics 7 (2007) 92–105.

[8] E. Fouquaert, S.L. Hanton, F. Brandizzi, W.J. Peumans, E.J.M. Van Damme, Localization and topogenesis studies of cytoplasmic and vacuolar homologs of the *Galanthus nivalis* agglutinin, Plant Cell Physiol. 48 (2007) 1010–1021.

[9] M. Seki, P. Carninci, Y. Nishiyama, Y. Hayashizaki, K. Shinozaki, High-efficiency cloning of *Arabidopsis* full-length cDNA by biotinylated CAP trapper, Plant J. 15 (1998) 707–720.

[10] M. Seki, M. Narusaka, A. Kamiya, J. Ishida, M. Satou, T. Sakurai, M. Nakajima, A. Enju, K. Akiyama, Y. Oono, M. Muramatsu, Y. Hayashizaki, J. Kawai, P. Carninci, M. Itoh, Y. Ishii, T. Arakawa, K. Shibata, A. Shinagawa, K. Shinozaki, Functional annotation of a full-length *Arabidopsis* cDNA collection, Science 296 (2002) 141–145.

[11] M. Karimi, D. Inzé, A. Depicker, GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation, Trends Plant Sci. 7 (2002) 193–195.

[12] G. Ditta, S. Stanfield, D. Corbin, D.R. Helinski, Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*, Proc. Natl. Acad. Sci. USA 77 (1980) 7347–7351.

[13] J. Van Leene, H. Stals, D. Eeckhout, G. Persiau, E. Van De Slijke, G. Van Isterdael, A. De Clercq, E. Bonnet, K. Laukens, N. Remmerie, K. Henderickx, T. De Vijlder, A. Abdelkrim, A. Pharazyn, H. Van Onckelen, D. Inzé, E. Witters, G. De Jaeger, A tandem affinity purification-based technology platform to study the cell cycle interactome in *Arabidopsis thaliana*, Mol. Cell. Proteomics 6 (2007) 1226–1238.

[14] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[15] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[16] B. Al Atalah, E. Fouquaert, D. Vanderschaeghe, P. Proost, J. Balzarini, D.F. Smith, P. Rougé, Y. Lasanajak, N. Callewaert, E.J.M. Van Damme, Expression analysis of the nucleocytoplasmic lectin 'Orysata' from rice in *Pichia pastoris*, FEBS J. 278 (2011) 2064–2079.

[17] O. Blixt, S. Head, T. Mondala, C. Scanlan, M.E. Huflejt, R. Alvarez, M.C. Bryan, F. Fazio, D. Calarese, J. Stevens, N. Razi, D.J. Stevens, J.J. Skehel, I. van Die, D.R. Burton, I.A. Wilson, R. Cummings, N. Bovin, C.H. Wong, J.C. Paulson, Printed covalent glycan array for ligand profiling of diverse glycan binding proteins, Proc. Natl. Acad. Sci. USA 101 (2004) 17033–17038.

[18] R. Leonard, The presence of LewisA epitopes in *Arabidopsis thaliana* glycoconjugates depends on an active alpha4-fucosyltransferase gene, Glycobiology 12 (2002) 299–306.

[19] N. Melo, Identification of the human LewisA carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (*Vaccinium myrtillus* L.), FEBS Lett. 415 (1997) 186–191.

[20] T.K. Dam, Fine specificities of two lectins from *Cymbosema roseum* seeds: a lectin specific for high-mannose oligosaccharides and a lectin specific for blood group H type II trisaccharide, Glycobiology 21 (2011) 925–933.