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

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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.

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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

Abbreviations: BSA, bovine serum albumin; EEA, *Euonymus europaeus* agglutinin; EUL, *Euonymus* lectin; EGFP, enhanced green fluorescent protein.

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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 polyvinylidene 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′ CCCGCTTTCTAGAATGAAAGGAAAGATCTTCCAGAGCTG 3′). Amplification of the EUL domain alone was achieved with forward primer evd 494 (5′ GGCGGAGAATTCACCATGGCCGGAAGAGCAACGGTGAAGG 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

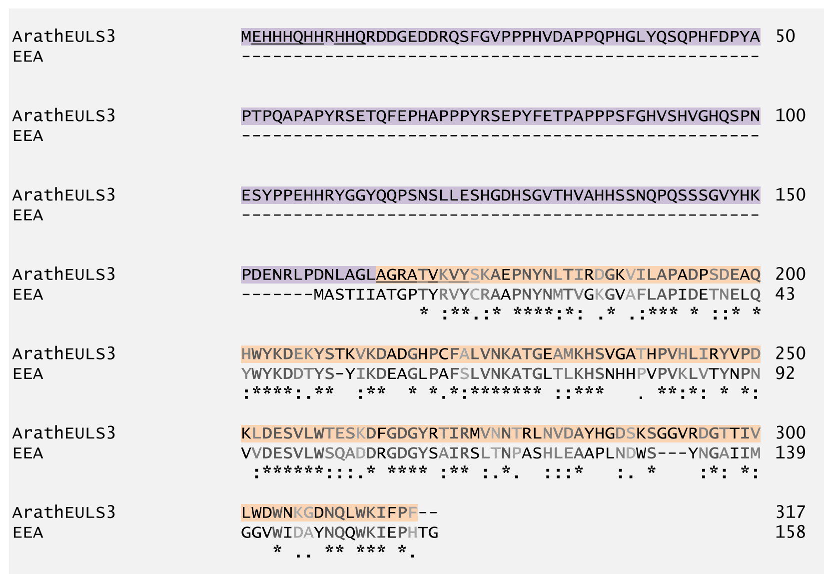

Fig. 1. Sequence comparison between the *Euomymus* lectin (EEA) and the *Arabidopsis* EUL protein (ArathEULS3) using ClustalW. The ArathEULS3 sequence consists of an N-terminal domain absent from EEA (shown in purple) and a C-terminal domain homologous to EEA (shown in orange). Identical residues are 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.)

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