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Evolutionary plasticity of plasma membrane interaction in DREPP family proteins

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ABSTRACT

The plant-specific DREPP protein family comprises proteins that were shown to regulate the actin and microtubular cytoskeleton in a calcium-dependent manner. Our phylogenetic analysis showed that DREPPs first appeared in ferns and that DREPPs have a rapid and plastic evolutionary history in plants. *Arabidopsis* DREPP paralogues called AtMDP25/PCaP1 and AtMAP18/PCaP2 are N-myristoylated, which has been reported as a key factor in plasma membrane localization. Here we show that N-myristoylation is neither conserved nor ancestral for the DREPP family. Instead, by using confocal microscopy and a new method for quantitative evaluation of protein membrane localization, we show that DREPPs rely on two mechanisms ensuring their plasma membrane localization. These include N-myristoylation and electrostatic interaction of a polybasic amino acid cluster. We propose that various plasma membrane association mechanisms resulting from the evolutionary plasticity of DREPPs are important for refining plasma membrane interaction of these signalling proteins under various conditions and in various cells.

1. Introduction

DREPP (Developmentally-Regulated Plasma Membrane Polypeptide) proteins comprise a family of plant-specific proteins that interact peripherally with the plasma membrane [1]. DREPP protein levels showed characteristic changes during the plant life cycle [2]. In *Arabidopsis*, DREPP proteins were studied in detail, especially with respect to binding to the plasma membrane and the cytoskeleton [3–5]. DREPP proteins have intrinsically disordered structures and coordinate Ca^{2+} and Cu^{2+} cations in the protein structure [6]. The *Arabidopsis* DREPP family contains PCaP1 (Plasma-Membrane Associated Cation-Binding Protein) protein, also named MDP25 (Microtubule-Destabilizing Protein) [3,7], and a divergent paralogue PCaP2, first described as a Microtubule-Associated Protein 18 (MAP18) [4,5,8]. AtPCaP1/MDP25 protein has been shown to bind to the plasma membrane by its N-terminally-located N-myristoylation, which serves as an anchor in the plasma membrane, and to an adjacent polybasic amino acid region that shows rather non-specific interaction with phosphatidylinositol phosphates (PtdInsPs) [9]. Li and co-workers [7] demonstrated that AtPCaP1/MDP25 protein bound to, and destabilized microtubules in *Arabidopsis*. Published data show that AtPCaP1/MDP25 shuttles between the cytoplasm and the plasma membrane, where it destabilizes microtubules. Qin and co-workers [10] demonstrated that AtPCaP1/MDP25 protein has also actin-severing activity and is involved in pollen tube growth regulation.

Cytoplasmic Ca^{2+} levels are key regulators of protein dissociation from the plasma membrane. AtPCaP1/MDP25 and AtPCaP2/MAP18 membrane-binding domains can competitively interact with Ca^{2+}/calmodulin and this interaction regulates plasma membrane association [3,7,8]. Rice paralogue OsDREPP2, a plasma membrane-binding protein, shows affinity to microtubules *in vitro*. Conserved Trp4 and Phe16 are important for binding microtubules and Ca^{2+}/CaM; calmodulin inhibits the binding of OsDREPP2 to microtubules [11].

A highly divergent paralogue of AtPCaP1/MDP25 from *Arabidopsis* called MAP18 was originally identified based on its microtubule-binding properties [5]. Keech and co-workers [12] identified MAP18 as a key regulator in a microtubule destabilization pathway during leaf senescence. Similarly to AtPCaP1/MDP25, AtPCaP2/MAP18 was also shown to modulate actin filaments in a Ca^{2+}-dependent way [13].

In a proteomic screen for MT-binding proteins in tobacco, we identified a tobacco DREPP protein among proteins interacting with MTs in the MT-binding assay. Detailed analysis of localization of tobacco DREPPs expressed in tobacco BY-2 cells was performed to understand the nature of plasma membrane interaction. Here we show that tobacco DREPP membrane localization depends on different mechanisms than *Arabidopsis* DREPPs. Diversity in plasma membrane association mechanisms in various plants was also proposed by our large scale phylogenetic analysis of DREPP protein family. To analyse plasma membrane-binding properties of the DREPP proteins, we introduced a new approach to evaluate plasma membrane affinity of GFP-tagged proteins.

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Our method is based on the decomposition of fluorescence signal from the cell cortical layer to the membrane and cytoplasmic components. By this approach, we demonstrated that DREPP-plasma membrane association depends on various mechanisms across plant species. These include combinations of N-myristoylation and protein-lipid electrostatic interactions. Our results suggest that mechanisms of plasma membrane interaction of peripherally associated membrane proteins can substantially vary at relatively low taxonomic levels of plant families.

2. Material and methods

2.1. Bioinformatic analysis

Multiple alignment of *DREPP* genes was performed manually in BioEdit [14] in relation to conserved amino acid residues after "toggle translation". Using *Arabidopsis* AtPCaP1/MDP25 protein as a query, NCBI tblastn program [15,16] was used to search for translated nucleotide sequences. In situations where taxa had only partially sequenced genomes, sequences were downloaded from EST database and joined to contigs using CAP3 program [17]. Phylogenetic analysis of nucleotide sequences was performed in MEGA6 software [18]. Only highly conserved central domains of genes were included in the analysis (with omitted unique insertions).

2.2. Plant material

The tobacco cell line BY-2 (*Nicotiana tabacum* L. cv. "Bright Yellow-2" [19]), was cultured in MS medium containing 4.3 $g \cdot L^{-1}$ of Murashige-Skoog salts (Sigma, St. Louis, Mo, USA), 1 $mg \cdot L^{-1}$ thiamine, 200 $mg \cdot L^{-1}$ KH_2PO_4, 100 $mg \cdot L^{-1}$ *myo*-inositol, 30 $g \cdot L^{-1}$ sucrose, and 0.9 μM 2.4-dichlorophenoxyacetic acid (2.4-D), pH 5.8. The transgenic BY-2 cell lines *DREPP-GFP* were maintained on the same medium supplemented with 20 $\mu g \cdot L^{-1}$ hygromycine. Every 7 days, 1 mL of cell suspension was transferred to 30 mL of fresh liquid medium and cultured in darkness at 25 °C on an orbital shaker (IKA KS501; IKA Labortechnik, Staufen, Germany; 120 rpm; orbital diameter, 30 mm).

2.3. Preparation of cDNA libraries

Pure mRNA was isolated from 3-week-old *Nicotiana tabacum* "Bright Yellow-2" plants or 2-week-old *Arabidopsis thaliana* plants "Columbia-0" cultivated on half-strength MS medium, using RNeasy Plant Mini Kit (#74904, Qiagen, Hilden, Germany). The cDNA was prepared using RT-PCR with oligo-dT_{23} primer and RevertAidTM M-MuLV Reverse Transcriptase (EP0442, Fermentas, Burlington, Canada) according to the manufacturer's instructions.

2.4. Cloning of DREPP genes and site-directed mutagenesis

In all cases, *Bam*HI-*Hin*dIII fragments of *DREPP* genes were amplified and cloned into the pDrive cloning vector (Qiagen PCR Cloning kit; #231124, Qiagen, Hilden, Germany). Next, fragments were inserted into the pGreenII 0129 vector containing *Aph IV* gene conferring hygromycine resistance in plants [20], then modified by the insertion of the *CaMV 35S* promotor, *EGFP* gene and nopalin synthase (*NOS*) terminator into a multiple cloning site. Prepared *DREPP* constructs were subjected to point mutagenesis using QuikChange Site-Directed Mutagenesis Kit (#200518; Stratagene, La Jolla, California, USA). Primers used for amplification of *DREPP* fragments are listed in Table S1 in supplementary material.

2.5. Transformation of plant material

Leaves of 1-month-old *Nicotiana benthamiana* were infiltrated with the *Agrobacterium tumefaciens* suspension. Briefly, overnight grown cultures of *A. tumefaciens* were washed twice in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH = 5.6), resuspended in the same buffer supplemented with 20 μM acetosyringone and infiltrated into leaves on the abaxial side [21]. After 2 days, infiltrated areas were subjected to microscopic analysis. Stable transformation of BY-2 cells was performed according to [22] with modifications described in [23]. Transgenic cell suspension cultures were maintained as described above, with the addition of 20 $\mu g \cdot L^{-1}$ hygromycine to the cultivation medium.

2.6. Confocal microscopy and FM4-64 staining

Confocal microscopy was performed under a Leica TCS SP2 confocal microscope (objective 63×, water immersion, ArKr laser). GFP was excited at 488 nm and FM4-64 at 514 nm. Emission was scanned at 500–550 nm and 750–850 nm for GFP and FM4-64, respectively. FM4-64 dye [24] (10 mM stock solution in dimethylsulfoxide) was added to 0.3 mL of BY-2 cells to a final concentration 10 μM. Cells were observed immediately. Equatorial optical sections of cells were captured. Images were analysed by Fiji software [25] using original macros based on a Plot Profile function. The resulting dataset was processed in R [26] as described in the next section. For each variant, more than 20 cells were evaluated and 50–300 cortical region profiles were collected in total. All lines were examined in at least two biological replicates; most lines were analysed in three independent biological replicates.

2.7. Image analysis

In order to identify a ratio of membrane-bound GFP-tagged peripheral membrane protein to the cytoplasmic fraction of GFP-tagged protein, we subtracted the membrane and cytoplasmic fluorescence signals from a profile data of cell cortical layer cross-sections. Measured fluorescence profiles were fitted by a two component model. The first fitted component was a normalised cortical cytoplasm layer profile of free-GFP signal obtained from GFP-expressing BY-2 cells. This component defined a profile of a cytoplasmic compound signal. The second component defined a profile of membrane fraction and was calculated from FM4-64 distribution for each individual measured profile (each sample was dually labelled with FM4-64 and GFP). FM4-64 distribution across the cortical cytoplasm layer profile was fitted by Gaussian form of Point-spread function for confocal microscopy including excitation and emission parameters. From this fit, estimated Point-spread function of membrane-bound GFP signal was re-calculated using different excitation and emission wavelengths when comparing FM4-64 (λ_{ex} = 514 nm, λ_{em} = 800 nm) and GFP (λ_{ex} = 488 nm, λ_{em} = 520 nm). A unitless value representing the membrane/cytoplasm signal distribution was obtained as a ratio of the integrated membrane component and the fitted intensity of the cytoplasmic component, followed by logarithmic transformation to compensate a heteroscedasticity in the dataset. Transformed data were used for statistical evaluation and the linear mixed-effects model with biological repetition as a source of random variability was applied (function lme from package nlme for R-project) [26]. Variants differing at a significance of 0.05 in Tukey-HSD post-hoc test (glht function from package multcomp) are denoted by different letters in graphs. Details of the image analysis are listed in Appendix A, source codes for ImageJ macros and R package 'peripheral' implementing our analysis can be downloaded from https://web.natur.cuni.cz/~vosolsob/peripheral.html.

3. Results

3.1. Protein DREPP is an apomorphy of Euphyllophyta group

A large set of EST sequences was collected from GeneBank and full-length contigs of *DREPP* genes from more than 100 species from all major plant families were generated. Because the most distant *DREPP* homologue was discovered in the fern *Adiantum capillus-veneris* and in contrast, *DREPP* was not detected in the genome of the spike moss