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ARTICLE *in* FEBS LETTERS · JUNE 2008

Impact Factor: 3.17 · DOI: 10.1016/j.febslet.2008.04.042 · Source: PubMed

FEBS Letters 582 (2008) 1743–1748

The hydrophobic segment of *Arabidopsis thaliana* cluster I diacylglycerol kinases is sufficient to target the proteins to cell membranes

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Received 26 March 2008; revised 17 April 2008; accepted 22 April 2008

Available online 6 May 2008

Edited by Ulf-Ingo Flügge

Abstract Diacylglycerol kinases (DGKs) catalyze the phosphorylation of diacylglycerol into phosphatidic acid. To fulfill their role in many signalling processes, DGKs must be located at, or in, membranes. Most mammalian DGKs are cytosolic and are recruited to membranes upon stimulation, except for ε type DGKs that are permanently membrane-associated through a hydrophobic segment. Nothing is known about the mechanism(s) involved in the membrane localization of plant DGKs. By fusion to fluorescent proteins, we show that two DGKs from cluster I in *Arabidopsis thaliana* possess amino-terminal hydrophobic segments that are sufficient to address them to endoplasmic reticulum membranes.

Keywords: Diacylglycerol kinases; Integral protein; Hydrophobic domain; GFP; Membrane sequestering; *Arabidopsis thaliana*

1. Introduction

Diacylglycerol kinases (DGKs) catalyze the phosphorylation of diacylglycerol (DAG) into phosphatidic acid (PA). During signalling processes, DAG is produced by phospholipases C (PLC) or by phospholipases D coupled to PA-phosphatases [1]. Both DAG and PA are bioactive molecules. DAG is known to bind to protein kinase C homology-1 (C1) domains (or phorbol ester/diacylglycerol binding domains, first described in protein kinases C). DAG regulates, through this interaction, proteins such as protein kinases C, Unc-13, and chimaerins. On the other hand, PA can regulate signalling proteins such as phosphatidylinositol-4-phosphate 5-kinases and sphingosine kinases. Therefore, DGK is a DAG consumer as well as a PA generator and thus regulates the balance between DAG and PA. Furthermore, DGK-generated PA can produce cytidine diphosphate (CDP)-DAG, which can lead to phosphatidylinositol (PI) via the action of PI synthase. Then, DGK has a role in the recycling of phosphoinositides consumed by PLC. In higher vertebrates, DGK isozymes are involved in development, neural and immune responses, cytoskeleton reorganization and carcinogenesis [1]. In plants, DGK has recently been reported to be involved in the nitric oxide signalling cascade during plant defence [2], in root development [3] and in cold response [4].

To catalyze PA formation, DGKs must be located in membranes. So far, in mammalian cells, ten different DGK isoforms have been isolated. Most of them are cytosolic in non-stimulated cells, but they translocate to membranes upon stimulation [5,6]. The recruitment of DGKs to membranes depends on regulatory events such as PLC-mediated calcium increases or phosphorylation events [7]. However, it has been shown recently that DGKs from the ε subfamily are permanently associated to membranes via a hydrophobic segment [8]. In plants, nothing is known about the mechanisms underlying the membrane localization of DGKs. In the *Arabidopsis* genome, seven DGK isoforms have been identified and they form three clusters. AtDGK1 and AtDGK2 belong to cluster I. They possess two cysteine-rich domains related to C1 domains. These domains are absent in the other *Arabidopsis* DGKs [9]. Besides, in the amino-terminal regions of AtDGK1 and AtDGK2, a hydrophobic segment can be found that is absent from the other isoforms. We checked whether this hydrophobic segment has a role in membrane targeting. By fusion with fluorescent proteins, we show that this region is sufficient and necessary to sequester AtDGK1 and AtDGK2 to endoplasmic reticulum (ER) membranes.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana Columbia plants were grown under a 16 h light/8 h dark cycle at 22 °C. Onions (*Allium cepa* L.) were obtained from a local supermarket. Transformed *Arabidopsis* plants were grown on 50 μg mL^{-1} kanamycin in MS/2 agar. *A. thaliana* Columbia cells were cultivated as in [4].

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Abbreviations: ATPase, adenosine triphosphatase; DAG, diacylglycerol; DGK, diacylglycerol kinase; G-6-Pase, glucose-6-phosphatase; ER, endoplasmic reticulum; GFP, green fluorescent protein; PA, phosphatidic acid; PI, phosphatidylinositol; PLC, phospholipase C; UDPase, uridine diphosphatase; YFP, yellow fluorescent protein

0014-5793/$34.00
doi:10.1016/j.febslet.2008.04.042

2.2. Cell transformation

Cells from *A. thaliana* suspension cultures were transformed by co-cultivation with *Agrobacterium tumefaciens* (adapted from [10]).

2.3. Transient expression in A. thaliana protoplasts

Protoplasts from a 5-day-old cell suspension were transiently transformed by yellow fluorescent protein (YFP)- or green fluorescent protein (GFP)-fusion constructs using 20–40 μg of plasmid using the polyethylene glycol method [11]. Transformed cells were incubated at 22 °C for 24 h and analyzed by confocal laser scanning microscopy.

2.4. Membrane isolation from A. thaliana suspension cells

Cells were filtered, then ground in homogenization buffer (1 mL g^{-1} of fresh weight) containing 100 mM MOPS-KOH (pH 7.5), 600 mM sucrose, 4 mM EDTA, 0.1 mM PMSF, 5 mM DTT, 5 mM ascorbic acid, 0.05% (w/v) cysteine, 0.6% (w/v) PVP K25 and 0.2% (w/v) BSA. The homogenate was filtered and subjected to differential centrifugations at 3750×*g* for 10 min, 18 200×*g* for 35 min and 38 400×*g* for 90 min. The resulting microsomal pellet was gently resuspended in buffer containing 10 mM MOPS-KOH (pH 7.3), 1 mM EDTA, and 10 mM KCl. Plasma membranes were isolated as described in [12]. For ER, Golgi membrane and nuclear membrane isolation, the protocol was adapted from [13,14] (see supplemental data).

2.5. Enzymatic activities

Adenosine triphosphatase (ATPase), uridine diphosphatase (UDPase), and glucose-6-phosphatase (G-6-Pase) activities were assayed as described in [4]. In vitro DGK activity was measured as in [15] with modifications (see supplemental data).

2.6. Protein extraction from A. thaliana suspension cells and Western blot analysis

Proteins were extracted from the supernatant as described in supplemental data. Protein extracts were separated on SDS–PAGE and transferred onto nitrocellulose membranes. After blocking, membranes were incubated with rabbit polyclonal primary antibody raised against GFP (ab6556, Abcam, diluted 1/250). The secondary antibody was an anti-rabbit horseradish peroxidase conjugate, used at a 1/15 000 dilution. Detection was performed using an Amersham ECL detection kit.

2.7. Plasmid constructs

For transient expression experiments, full length or truncated fragments of *AtDGK1* and *AtDGK2* cDNAs were amplified and cloned in frame with the GFP of the pBI-GFP vector [16] using XbaI and BamHI restriction sites. Truncated fragments led to constructs missing the C-terminal part of AtDGK1 and AtDGK2 or both the C-terminal part and the hydrophobic segment of the proteins.

For stable expression, fragments of AtDGK1 and AtDGK2 were amplified and cloned downstream of the CaMV 35S promoter in the binary vector pCP-60, a pBIN19 derivative, using KpnI and BamHI restriction sites. This led to constructs containing the putative transmembrane domain of both proteins in frame with the YFP. All constructs (Fig. 1A) were verified by DNA sequencing.

2.8. Confocal microscopy and image processing

Images were collected with a Leica TCS SP2 upright laser scanning confocal microscope (Leica Microsystems). Excitation wavelengths were 488 nm for GFP, 514 nm for YFP and 561 nm for FM4-64. Emission was detected at 500–570 nm for GFP, 516–580 nm for YFP and 650–700 nm for FM4-64. All multi-labelling signals were acquired in sequential line scanning mode.

3. Results

3.1. Localization of bulk DGK activity

We first looked for DGK activity in different subcellular fractions of *A. thaliana* suspension cells. Plasma membranes, ER and Golgi membranes were isolated by cellular fractionation from microsomes. Nuclear membranes were purified from nuclei. For plasma membranes, ER and Golgi membranes, enrichment in DGK activity from microsomes paralleled the enrichment of the different fractions in ATPase activity specific of plasmalemma, in G-6-Pase and in UDPase, respectively (Table 1). DGK activity measured in the cytosolic fraction was 134 times lower than in the microsomes. An activity was also found in nuclear membranes. All activities were dependent on exogenous DAG supply and were inhibited by R59022, a DGK inhibitor. These results suggest the membrane localization for bulk DGK activity in *Arabidopsis*.

3.2. Predictions of AtDGK1 and AtDGK2 secondary structures

AtDGK1-7 sequences were analyzed at TMHMM (http://www.cbs.dtu.dk/services/TMHMM/, [17]) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/, [18]). Both software packages predicted the presence of an amino-terminal hydrophobic segment that could form a transmembrane helix in AtDGK1 and AtDGK2, while no transmembrane segment was predicted for AtDGK3-7. Depending on the software used, the helix in AtDGK1 began at residue 25 or 27 and it was 23-residues long, while in AtDGK2, it began at residue 21 and it was 21- to 23-residues long (Fig. 1B). We wanted to determine whether these putative helixes were a determinant of the membrane localization of these enzymes.

3.3. The absence of the N-terminal hydrophobic segment of AtDGK1 and AtDGK2 results in a non-membrane localization

We first tried to express full-length AtDGK1 and AtDGK2 sequences fused to a fluorescent protein under the control of the 35S CaMV promoter. These chimaeric proteins were not detected either in transiently transformed *Arabidopsis* protoplasts or onion cells, or in stably transformed *Arabidopsis* plants. Therefore, GFP was fused to truncated forms of AtDGK1 and AtDGK2, missing the catalytic and accessory domains. The fluorescence analysis of *Arabidopsis* protoplasts transformed with the $AtDGK1_{1\text{-}320}$-GFP and $AtDGK2_{1\text{-}310}$-GFP constructs (Fig. 1A) showed a membrane localization pattern typical of the ER (Fig. 2a and c), while in the control, GFP alone, fluorescence was diffuse through the cytosol and the nucleus (Fig. 2i). In protoplasts expressing the $AtDGK2_{1\text{-}310}$-GFP protein, the stippled fluorescence around the cell and the fluorescence design around nucleus are also clues for an ER localization (Fig. 2c). When chimaeric proteins were deleted of their putative transmembrane domain, the fluorescence patterns were totally different (Fig. 2e and g), i.e. comparable to GFP control ($AtDGK1_{54\text{-}320}$-GFP; Fig. 2e) or in nucleus ($AtDGK2_{47\text{-}310}$-GFP; Fig. 2g). In both cases, no fluorescence associated with membranes could be detected.

3.4. The N-terminal hydrophobic segment of AtDGK1 and AtDGK2 targets fluorescent proteins to reticulum membranes

The first 50 amino acid residues of AtDGK1 and the first 43 residues of AtDGK2 were fused to the YFP (Fig. 1A). These fragments contain the hydrophobic segment of the proteins. When transiently expressed in onion epidermal cells, both constructs led to a strong fluorescence associated with the ER network, while GFP alone fluoresced mainly in the cytosol and the nucleus (Suppl. Fig. S1). These constructs were used to stably transform *A. thaliana*. In roots and leaves of 8- and

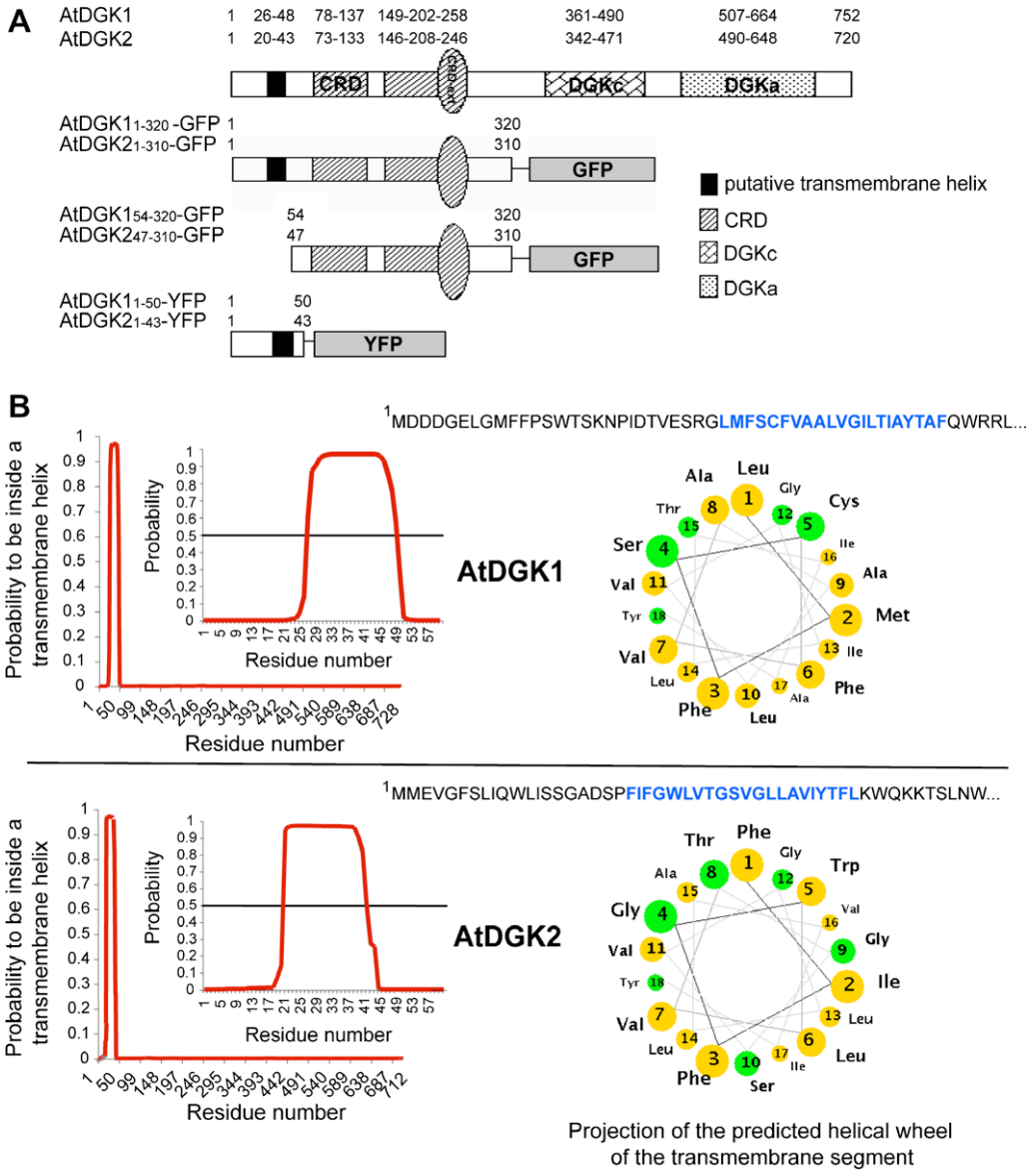

Fig. 1. (A) AtDGK1 and AtDGK2 proteins and the different fusion proteins. Numbers represent amino acids position along the protein sequence. DGKa: accessory domain; DGKc: catalytic domain; CRD: cystein-rich domain; CRD-ext: ext-CRD-like domain; GFP: green fluorescent protein; YFP: yellow fluorescent protein. (B) A putative N-terminal transmembrane helix was detected in AtDGK1 and AtDGK2. The sequences of the predicted transmembrane helices are in blue. (Left panel) In the diagrams, the red lines represent the probability for a residue to be inside a transmembrane helix as calculated with a hidden Markov model by http://www.cbs.dtu.dk/services/TMHMM/. (Right panel) Projections of the predicted helical wheel are represented as a cross sectional view of the axis. Non-polar residues are represented in yellow and polar residues in green. The wheel was drawn using a device available at http://cti.itc.virginia.edu/~cmg/Demo/**wheel/wheel**App.html.

Table 1
DGK specific activity measured in different membrane fractions

Membrane fractions	Microsome-derived membranes			Nuclear membranes
	Plasma membrane	Endoplasmic reticulum	Golgi apparatus	
Fraction enrichment from microsomes	×2.7	×2.25	×1.9	Not relevant
DGK specific activity ($\times 10^{-3}$ pmoles min^{-1} μg^{-1})	42	58	43.6	9.4
DGK activity enrichment compared to microsomes	×2.9 ± 0.1	×2.8 ± 0.2	×1.8 ± 0.4	Not relevant

Plasma membranes, endoplasmic reticulum membranes and Golgi were purified from microsomes, in separate experiments. For each membrane fraction, a purification factor was calculated as the enrichment in vanadate sensitive ATPase, glucose-6-phosphatase and uridine diphosphatase specific activities in plasma membranes, endoplasmic reticulum and Golgi apparatus fractions, respectively, compared to those activities in microsomes. DGK was calculated in each membrane fractions, including nuclear membranes. For microsome-derived membranes, DGK activity enrichment was calculated as a ratio of the DGK specific activity in the membrane fractions to the one in the corresponding initial microsomal fractions.

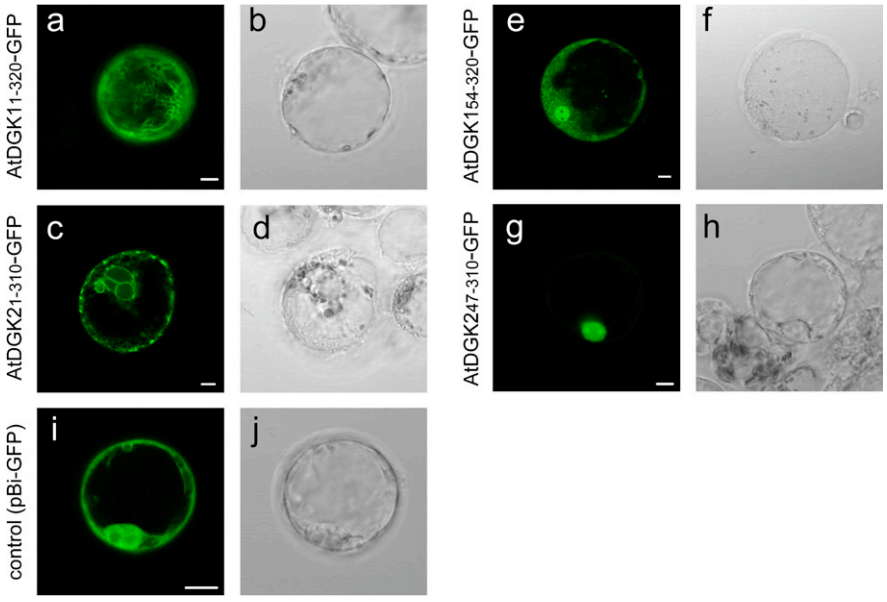

Fig. 2. Subcellular localization of chimeric proteins transiently expressed in protoplasts of *Arabidopsis thaliana* suspension cells. Protoplasts expressing AtDGK1$_{1-320}$-GFP (a), AtDGK2$_{1-310}$-GFP (c), AtDGK1$_{54-320}$-GFP (e), AtDGK2$_{47-310}$-GFP (g) or the control pBi-GFP (i). Note the ER localization (a, c) when the helix is expressed and the nuclear and cytoplasmic localization when the helix is not express. (b, d, f, h, j): Bright field images. Scale bar: 8 μm.

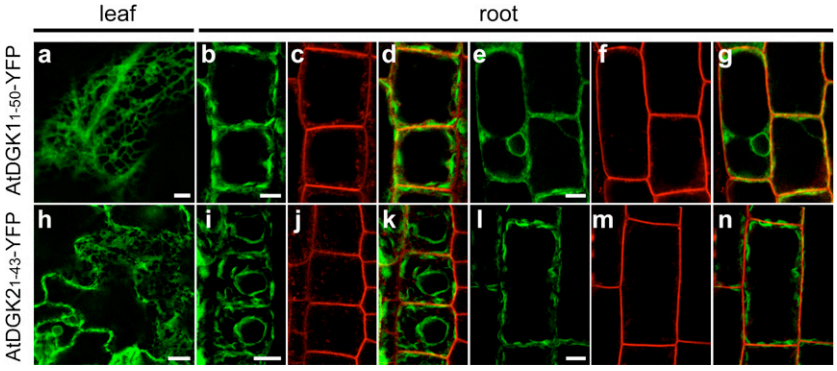

Fig. 3. Subcellular localization of chimeric peptides stably expressed in *Arabidopsis* plants. AtDGK1$_{1-50}$-YFP and AtDGK2$_{1-43}$-YFP expressed in the leave (a and h, repectively) or in the root (b–g and i–n, respectively). (d, g, k, n): merged pictures. Note that FM4-64 fluorescence (c, f, j, m) is distinct from the ER-localized YFP fluorescence. Scale bar: 7 μm (a and h: scale bar: 8 μm).

15-day-old T2 transformants, respectively (Fig. 3), the fluorescence patterns were similar for each DGK-fragment. In lower leaf epidermal cells (Fig. 3a and h), fluorescence was typical of the ER, showing sheet-like regions connected by tubular elements [19]. In root cells, fluorescence appeared as a sub-cortical and perinuclear network (Fig. 3b, e, i, and l). Furthermore, the fluorescence distribution was similar to that observed in roots of plantlets expressing GFP-HDEL, an ER marker (Suppl. Fig. S2). A dye specific marker of the plasma membrane, FM4-64 [20], was used to stain root cells. The FM4-64 intensity peak was clearly distinct from the AtDGK1$_{1-50}$-YFP or AtDGK2$_{1-43}$-YFP intensity peak indicating that those two peptide fusions were not localized to the plasma membrane (distance between FM4-64 peak and YFP peak was higher than the optical resolution limit: 380 nm versus 200 nm) (Suppl. Fig. S3). This was confirmed using *A. thaliana* suspension cells stably transformed with AtDGK1$_{1-50}$-YFP and AtDGK2$_{1-43}$-YFP constructs. The fluorescence patterns, similar for both constructs, exhibited an ER pattern and did not overlap with the fluorescence of FM4-64 (Fig. 4A). The transformed suspension cells were used to extract proteins for Western blotting with antibodies recognizing GFP/YFP. In the membrane fractions, the antibody reacted with a protein showing apparent molecular mass of 32.5 and 31.7 kDa, for AtDGK1$_{1-50}$-YFP and AtDGK2$_{1-43}$-YFP, respectively (Fig. 4B). These are the expected values for the fusion proteins. In the soluble fraction of AtDGK1$_{1-50}$-YFP transformed cells, a protein having a lower molecular mass of about 25 kDa was observed, likely corresponding to a cleaved YFP.

4. Discussion

AtDGK1 and AtDGK2 proteins have a hydrophobic segment in their amino-terminal region. In fusion with fluorescent proteins, the N-terminal portion of each protein, including the

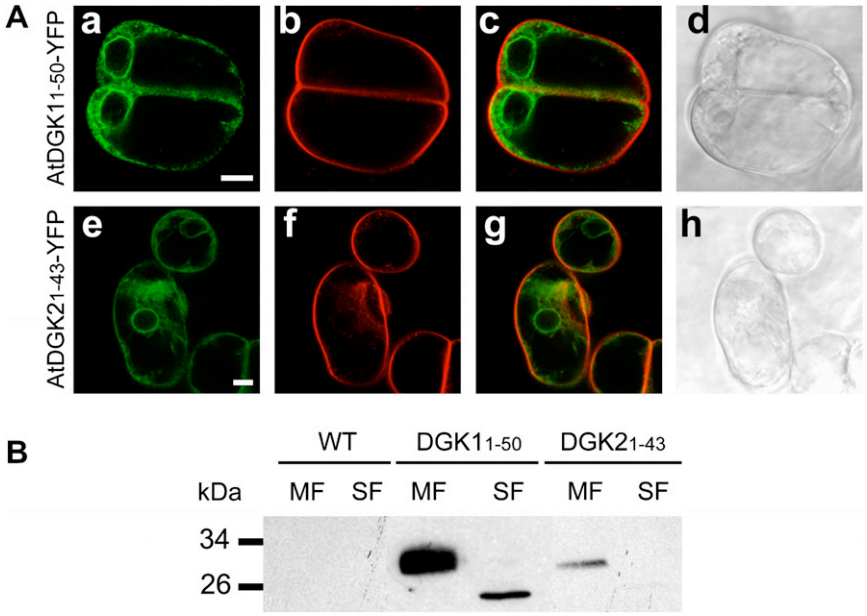

Fig. 4. Subcellular localization of chimeric peptides stably expressed in *Arabidopsis* suspension cells. (A) Confocal microscopy; (a) AtDGK1$_{1-50}$-YFP. (e) AtDGK2$_{1-43}$-YFP. (c, g) Merged pictures. Note that both YFP (a, e) and FM4-64 fluorescence (b, f) did not overlap. (d, h) Bright field. Scale bar: 8 μm. (B) Western blot of AtDGK1$_{1-50}$-YFP and AtDGK2$_{1-43}$-YFP proteins in soluble and membrane fractions (SF and MF, respectively) of transformed *Arabidopsis* cells.

hydrophobic domain, are shown to address the fusion to ER membranes. However, when the hydrophobic domain is missing, the fusion is soluble. The fact that for AtDGK2$_{47-310}$-GFP, the fluorescence is found in the nucleus may be due to the presence of very basic residues. When the hydrophobic segment is absent, the protein is not sequestered to membranes and the basic motif would act as a nuclear localization signal and the protein would be imported to the nucleoplasm. When the hydrophobic fragment alone is fused to the YFP, it is sufficient to target the fusion in ER membranes.

Therefore, DGKs of cluster I in *A. thaliana* appear to be transmembrane proteins. This is not a common mechanism for the membrane localization of DGKs. In Mammals, DGKθ translocates from the cytosol to the plasma membrane in response to noradrenaline [6] and DGKα translocates from the cytoplasm to the plasma membrane or to the Golgi network in response to arachidonic acid [5]. DGKε is the unique mammalian isoform with a predicted transmembrane helix between residues 20–40. It has been recently highlighted that this helix plays a role in permanently sequestering the enzyme to a membrane [8,21]. We show here that this mechanism concerns not only animal DGKε but also plant AtDGK1 and AtDGK2. Mammalian DGKs can be grouped into five types, depending on their structural features. All mammalian DGKs have at least two cysteine-rich domains related to C1 domains and a catalytic domain. Besides, most mammalian DGKs have other structural motifs, such as an EF hand (type I DGKs), a PH domain (types II and V), a MARCKS domain (type IV) or a proline rich domain and Ras binding domains (type V). DGKε, which constitutes the type III mammalian DGK, is the only isoform not to have extra structural motifs [7]. Interestingly, cluster I DGKs from *A. thaliana* have no structural motifs other than the cysteine-rich domains and the catalytic domain [7,9]. Therefore, *Arabidopsis* cluster I DGKs appear to be structurally related to DGKε and here we show that they are also related by their permanent sequestering to membranes.

While no DGK activity was found in the soluble fractions of *Arabidopsis* cells, it was detected in most membrane compartments. The highest specific activity was found in reticulum membranes. It is tempting to speculate that AtDGK1-2 contribute to this activity. In fusion with GFP, rat DGKε was targeted to ER membranes of COS7 cells [22]. However, our data do not allow us to unambiguously conclude on the final localization of cluster I DGKs. The eliminated C terminal half of the proteins in the fusion experiment may indeed affect the final localizations. For instance we cannot exclude that the activity detected in plasma membrane might in part be due to cluster I DGKs. But this plasmalemma activity could also be due to cluster II and III DGKs. These proteins have no hydrophobic segments, but they can be recruited to membranes.

In mammals, DGKε isoforms are important for phosphoinositide recycling. It would be interesting to see whether together with the structural and localization features, *Arabidopsis* cluster I DGKs and mammalian DGKε share conserved functions.

Acknowledgements: This work was supported by the Ministère de l'Enseignement Supérieur et de la Recherche, the Centre National pour la Recherche Scientifique and the Université Pierre et Marie Curie. We are grateful to Dr. Béatrice Satiat-Jeunemaître (Institut des Sciences du Végétal, CNRS) for helpful discussions. We acknowledge Dr. P. Ratet and Dr. S. Breaux for the kind gift of pCP60 and pBI-GFP, respectively. We thank Dr M. Hodges (IBP, UMR8618, Université Paris-Sud) for carefully reading the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.04.042.

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