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Biochimica et Biophysica Acta 1761 (2006) 725–735
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Arabidopsis thaliana expresses two functional isoforms of Arvp, a protein involved in the regulation of cellular lipid homeostasis☆

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Received 23 November 2005; received in revised form 17 March 2006; accepted 27 March 2006
Available online 19 April 2006

Abstract

Arv1p is involved in the regulation of cellular lipid homeostasis in the yeast *Saccharomyces cerevisiae*. Here, we report the characterization of the two *Arabidopsis thaliana ARV* genes and the encoded proteins, AtArv1p and AtArv2p. The functional identity of AtArv1p and AtArv2p was demonstrated by complementation of the thermosensitive phenotype of the *arv1*Δ yeast mutant strain YJN1756. Both *A. thaliana* proteins contain the bipartite Arv1 homology domain (AHD), which consists of an NH_2-terminal cysteine-rich subdomain with a putative zinc-binding motif followed by a C-terminal subdomain of 33 amino acids. Removal of the cysteine-rich subdomain has no effect on Arvp activity, whereas the presence of the C-terminal subdomain of the AHD is critical for Arvp function. Localization experiments of AtArv1p and AtArv2p tagged with green fluorescent protein (GFP) and expressed in onion epidermal cells demonstrated that both proteins are exclusively targeted to the endoplasmic reticulum. Analysis of β-glucuronidase (GUS) activity in transgenic *A. thaliana* plants carrying chimeric *ARV1*::*GUS* and *ARV2*::*GUS* genes showed that *ARV* gene promoters direct largely overlapping patterns of expression that are restricted to tissues in which cells are actively dividing or expanding. The results of this study support the notion that plants, yeast and mammals share common molecular mechanisms regulating intracellular lipid homeostasis.

Keywords: *Arabidopsis*; Lipid homeostasis; Sterol; Sphingolipid; Plant; Yeast

1. Introduction

The mechanisms regulating lipid homeostasis in eukaryotes and in particular those involved in intracellular lipid trafficking are still poorly understood at the molecular level. Nevertheless, a number of studies in animal and yeast cells support the existence of a coordinated regulation between sphingolipid biosynthesis and sterol metabolism [1–4]. Recently, the *Saccharomyces cerevisiae ARV1* gene was isolated in a genetic screen designed to identify genes that are essential for viability when yeast cells are impaired in sterol esterification [5]. The encoded Arv1p contains a novel NH_2-terminal domain, defined as the Arv1 homology domain (AHD), which includes a putative zinc-binding motif. Arv1p is predicted to have six transmembrane domains [5] and has been found to localize in the endoplasmic reticulum (ER) or the Golgi apparatus of yeast

Abbreviations: AHD, Arv1 homology domain; CaMV, cauliflower mosaic virus; DsRed, Discosoma red fluorescent protein; ER, endoplasmic reticulum; HA, peptide from human hemagglutinin; GPD, glyceraldehyde phosphate dehydrogenase; GFP, green fluorescent protein; GUS, β-glucuronidase; UBQ, polyubiquitin; IPC, inositolphosphorylceramide; MIPC, mannose inositolphosphorylceramide; MIP_2C, mannose diinositolphosphorylceramide; RT, reverse transcriptase; T3RE, ER-targeted variant of DsRed; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide; YNB, yeast nitrogen base

☆ The nucleotide sequence data for *A. thaliana* ARV1 and ARV2 cDNAs have been submitted to GenBank Database under the accession numbers AY758070 and AY758071, respectively.

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doi:10.1016/j.bbalip.2006.03.025

cells in a bud size- and/or growth phase-dependent manner, suggesting a possible cell-cycle regulated localization [6]. Deletion of *ARV1* leads to altered levels and distribution of intracellular sterols. The overall content of free sterols increases by about 50% and that of steryl esters by about 75%. Moreover, sterol concentration decreases in plasma membrane whereas elevated sterol levels are observed in ER and vacuolar membranes. Based on these results, a role for Arv1p in sterol trafficking has been proposed [5]. The finding that Arv1p interacts directly with Erg11p, the enzyme that demethylates lanosterol in the course of ergosterol biosynthesis, further substantiates the relationship between Arv1p and sterol metabolism in yeast [7]. Studies from Swain et al. [6] have shown that *arv1*Δ mutant yeast cells also display defects in their ability to properly synthesize and maintain sphingolipid levels. Mutant cells have reduced rates of biosynthesis and lower steady-state levels of complex sphingolipids while accumulating certain ceramide species. In line with these results, mutations in *ARV1* have also been isolated in a genetic screen designed to obtain yeast mutants with defects in sphingolipid metabolism [6]. These observations have led to propose that Arv1p either may primarily regulate sphingolipid metabolism, such that altered sterol levels and distribution might result from improper sphingolipid homeostasis, or act as a common dual regulator of both sphingolipid and sterol metabolism and transport [6]. A human Arv1p has also been cloned and shown to replace yeast Arv1p function completely in regulating yeast sterol and sphingolipid metabolism [5,6].

In regards to plants, far less attention has been paid to the mechanisms involved in the maintenance of cellular lipid homeostasis. In fact, one of the fundamental questions still to be addressed is how intracellular lipid homeostasis is maintained in plant cells and which mechanisms regulate cross-talk between the metabolism of different lipid species [8,9]. Some recent findings support the view that sterol and sphingolipid metabolism are also coordinately regulated in plants. Studies using leek seedlings have shown that inhibition of the sterol biosynthetic pathway also impairs phosphatidylserine and glucosylceramide synthesis [10]. More recently, plasma membranes of plant cells have been shown to contain lipid rafts [11,12], which are discrete microdomains of membranes enriched in sphingolipids and sterols previously identified and characterized in animal and yeast cells [13–15]. To gain insight into the mechanisms regulating lipid homeostasis in plant cells, we undertook the present study focusing on the functional characterization of the two *Arabidopsis thaliana ARV* genes and the encoded proteins.

2. Materials and methods

2.1. Plant materials and yeast strains

A. thaliana plants (var. Columbia 3) were grown either on soil irrigated with mineral nutrients [16] at 22 °C under a 8 h light/16 h dark illumination regime with 130 μmol photons m^{-2} s^{-1} "daylight" fluorescent illumination or axenically in Petri dishes containing solid (0.8% w/v agar) germination medium (GM; Murashige and Skoog medium (ICN Biomedicals) supplemented with 0.5 g l^{-1} MES pH 5.7) under the same light and temperature conditions. Suspension-cultured *A. thaliana* T87 cells were grown as described [17]. Yeast mutant strain YJN1756 (*MAT*α *arv1::KAN*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*) used in this study is derived from strain W303-1A [3]. The yeast strains were grown in either YPD [1% (w/v) yeast extract, 1% (w/v) bactopeptone, and 2% (w/v) glucose] or minimal medium [0.16% (w/v) YNB (yeast nitrogen base) without amino acids and without $(NH_4)_2SO_4$, 0.5% (w/v) $(NH_4)_2SO_4$, and 2% (w/v) glucose] supplemented with the appropriate amino acids and adenine.

2.2. Cloning of ARV1 and ARV2 cDNA sequences

A first-strand cDNA pool was synthesized by reverse transcription of 2.5 μg total RNA from axenically grown 10-day-old *A. thaliana* seedlings with an oligo (dT) primer (5′-GCGTCGACTGCAGGGCTTTTTTTTTTTTTTTT-3′). The cDNA mixture was used as template for independent PCR reactions using primer pairs ARV1-f1 and ARV1-r1 for the amplification of ARV1 cDNA, and ARV2-f1 and ARV2-r1 for the amplification of ARV2 cDNA. The *A. thaliana* genomic sequence AC007323 (GenBank) was used to design primers ARV1-f1 (5′-CGGGATTCAGACCCGGACTCTAATTGCT-3′; reverse complement of bp 8420 to 8439) and ARV1-r1 (5′-CGGTCGACCTGGAAGCTGATGGGATCATAC-3′, bp 6601 to 6622), and the *A. thaliana* genomic sequence AL161492 (GenBank) was used to design primers ARV2-f1 (5′-CGGGATCCCAACAGTCACAGACACAGAG-3′; bp 65298 to 65317) and ARV2-r1, (5′-CGGTCGACCACAAGTTCCTTGCAAGAGAAG-3′; reverse complement of bp 66757 to 66778). *Bam*HI and *Sal*I restriction sites (underlined) were added at the 5′ end of the forward and reverse primers, respectively. The amplified cDNAs were cloned into pGemT-Easy (Promega), yielding plasmids pGTARV1 and pGTARV2.

2.3. Construction of yeast expression vectors and functional complementation of a S. cerevisiae ARV1 null mutant

The ARV1 and ARV2 cDNA fragments were excised as *Bam*HI–*Sal*I fragments from pGTARV1 and pGTARV2 and cloned into the corresponding sites of the yeast expression vector pJR1133, which is identical to pJR1138 [18] except for containing the *URA3* marker instead of *LEU2*. The resulting plasmids, pJRAtArv1p and pJRAtArv2p, carried the cDNA fragments under the control of the yeast glyceraldehyde phosphate dehydrogenase (GPD) gene promoter. To construct plasmids pJRAtArv2p-HA, pJRAtArv2pΔ35-HA, pJRAtArv2pΔ46-HA, pJRAtArv2pΔ56-HA and pJRAtArv2pΔ68-HA, the corresponding cDNA fragments were amplified by PCR using as forward primer either ARV2-f1, ARV2Δ35 (5′-GGATCC**ATG**GAAGAAGTAGCAGAC-3′), ARV2Δ46 (5′-GGATCC**ATG**CTATTGATTATTTTATC-3′), ARV2Δ56 (5′-GGATCC**ATG**-CACAAAACAAAGGCT-3′) or ARV2Δ68 (5′-GGATCC**ATG**GTTGTTAATCAAGAA-3′), a common reverse primer ARV2-HA-r (5′-GTCGAC**TCA***CGCATAGTCAGGAACATCGTATGGGTA*TACGATTCTGAAAAAATAAGACATATA-3′), and pGTARV2 as template. *Bam*HI and *Sal*I restriction sites (underlined) were added at the 5′ end of the forward and reverse primers, respectively. Translational start and stop codons are shown in bold. The sequence coding for the hemagglutinin (HA) epitope (YPYDVPDYA) is shown in italics. The PCR products were cloned into pGemT-Easy, excised as *Bam*HI/*Sal*I fragments, and cloned into the corresponding sites of pJR1133. To create pAtArv2p4Ser-HA, the amino acid substitutions Cys^8Ser, Cys^{11}Ser, Cys^{32}Ser, and Cys^{35}Ser were introduced by a PCR-based overlap extension method [19]. To generate substitutions Cys^{32}Ser and Cys^{35}Ser, independent PCR reactions were carried out using pGTARV2 as template and either primer pair ARV2-f1 and ARV2-C32/35S-r (5′-TACTTCTTCGC-**T**ATTCTCGC**T**TTTCATGAG-3′) or primer pair ARV2-C32/35S-f (5′-CTCATGAAA**A**GCGAGAAT**A**GCGAAGAAGTA-3′) and ARV2-C-r (5′-GCAAGCTTCTATAAGTATCTAGAAG-3′). Nucleotide changes to introduce cysteine to serine substitutions are shown in bold. Primer ARV2-C-r contains a *Hin*dIII restriction site (underlined). About 200 ng of each PCR product were mixed and used as template in the subsequent PCR-based overlap extension reaction, which was carried out using primers ARV2-f1 and ARV2-C-r. The resulting fragment was cloned into pGemT-Easy yielding clone pGTC32/35S. Substitutions Cys^8Ser and Cys^{11}Ser were introduced using pGTC32/35S as template and either primer pair ARV2-f1 and ARV2-C8/11S-r (5′-CTTGTGCCCAC**T**CTCTACAC**T**CGTCTTCTT-3′) or primer pair ARV2-