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Cloning and expression of cDNAs encoding ADP-ribosylation factor in carrot seedling

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Abstract

A homology based strategy yielded two cDNA clones designated *arf-001* (1097 bp) and *arf-002* (897 bp) from carrot seedlings. These genes contained open reading frames which encode the proteins of 192 and 181 amino acid residues, respectively, with the significant homology to ADP-ribosylation factors of plants, animals and microbial sources. Genomic Southern blot hybridization analyses revealed that both genes showed one main signal, respectively; however, several related genes might be present in carrot genome. The products of these genes obtained by over-expression in *Escherichia coli* showed the specific binding activity toward GTP. The expression of *arf-001* was mainly observed in leaf and stem tissues while *arf-002* was appreciably expressed in roots as analyzed by RT-PCR. The transcriptional level of *arf-001* showed the transient increase by the exposure of carrot seedlings to ethylene. In contrast, *arf-002* appeared to be a house-keeping gene, and its expression level was maintained at constant level upon the treatment with various stimuli and under stress conditions. These results suggest that the *arf-001* and *002* play the distinct physiological roles in carrot cells.

Keywords: GTP-binding protein; ADP-ribosylation factor; Gene cloning; Gene expression; RT-PCR; *Daucus carota*

1. Introduction

Monomeric GTP-binding proteins are involved in regulating essential functions of eukaryotic cells, such as cell differentiation, intracellular vesicle transport and cytoskeleton organization [1–4]. Based on the amino acid homology and deduced functions, the low molecular weight GTP-binding proteins are generally classified into Ras superfamilty and several other subfamilies [1–3]. ADP-ribosylation factor (ARF), a member of Arf/sar subfamily of small G-proteins [3,4] were originally identified as the proteins required for cholera toxin-mediated ADP-ribosylation of the α-subunit (Gαs) of the trimeric GTP-binding protein complexes which results in the activation of adenyryl cyclase, in vitro [4,5]. However, ARFs were subsequently shown to be associated with Golgi membranes, and several lines of evidence suggested that these proteins function in vesicular transport from endoplasmic reticulum to plasma membrane via Golgi apparatus [4,6,7]. It has been also demonstrated [2–4] that the binding of ARF to Golgi membrane is controlled by GTP hydrolysis and *N*-myristoylation of a conserved glycine near *N*-terminal in the structure. On the other hand, it has been recently demonstrated [8,9] that ARF regulates the activity of phospholipase D in a GTP-dependent manner, and, in addition, this class of proteins has also been shown to activate phosphatidylinositol kinases, directly, in secretory neuroendocrine cells [10].

A number of genes encoding ARF proteins have been isolated from microorganisms, animals and plants. In higher plants, although several cDNAs presumably encoding ARFs have been isolated from rice, potato, *Arabidopsis*, etc. [11–13], only very litlle information is available about the transcriptional control and the biological functions of these ARFs. In order to examine the structures and physiological functions of plant ARFs, in the present study, we attempted to isolate cDNA clones encoding this protein and two genes, designated *arf-001* and *arf-002*, were obtained from carrot seedlings. The change in the transcriptional level of these genes upon the treatment of carrot with various stimuli and under stress conditions was examined to understand the physiological functions of the products of these genes.

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2. Materials and methods

2.1. Materials

Carrot (*Daucus carota* L.) seeds were germinated under sterile conditions on Murashige and Skoog's agar medium [14] in test tubes (25 mm in diameter) and grown at 26 °C under constant illumination for 3 weeks. Oligogalacturonides were prepared by the partial hydrolysis of the pectin fraction of carrot according to the previously reported method [15]. ATP, UTP, GTP, GMP and GTP-γ-S were purchased from Boehringer Mannheim while 2-chloroethylphosphonic acid (2-CEPA), an ethylene generating reagent, was from Sigma. Isopropyl-thio-β-D-galactopyranoside (IPTG) was obtained from Wako Pure Chemicals. [^{35}S]GTP-γ-S (specific activity 46.2 TBq/mmol) was from Perkin-Elmer. All other chemicals were of reagent grade.

2.2. Cloning of arf genes

Total RNA was isolated from carrot seedlings with RNeasy Plant Mini Kit (Qiagen) and the RNA obtained (approximately 5 μg) was subjected to the rapid amplification of cDNA end (RACE) methods using the GeneRacer Kit (Invitrogen) after the generation of cDNA template by reverse-transcription (RT) reaction with AMV-RT. 3′-RACE was performed with GeneRacer Oligo dT as the reverse primer, and with the appropriate gene specific forward primers for polymerase chain reaction (PCR) amplification of the DNA fragments. 5′-CAT GTC CTG TTT GTT TGC AAA CAC CAA-3′ was employed as the gene specific forward primer for cloning of *arf-001* and 5′-TTC ACA GTG TGG GAC GTC GGC GGG CAA GAC-3′ was for *arf-002*. 5′-RACE was carried out with GeneRacer RNA Oligo as the forward primer, and 5′-CAT GTC CTG TTT GTT TGC AAA CAC CAA-3′ and 5′-AGC AGC ATT CAT TGC ATT TGG AAG AT-3′ were employed for *arf-001* and *arf-002* as the reverse primers, respectively. The DNA fragments obtained were subcloned into the pCR2.1-TOPO vector (Invitrogen), and their nucleotide sequences were determined on both strands using the dye-terminator method with M13-20 and RV-P (Takara) as the sequencing primers on a PRISM 3100 Genetic Analyzer (Applied Biosystems).

2.3. Southern blot hybridization of arf genes

Genomic DNA from carrot seedlings was prepared using Nucleocon Phytopure (Amersham Biosciences) according to the instruction manuals. Three different restriction digests of carrot genomic DNA were prepared using *Eco*RI, *Eco*RV and *Hin*dIII, and were electrophoresed on a 0.8% agarose gel, then, transferred onto a Immobilon-NY+ (Millipore). The DNA fragments of 605 and 381 bp, which contain the translatable regions and 3′ untranslatable regions of *arf-001* and *002*, were amplified by PCR and were directly labeled with AlkPhos Direct Labeling and Detection System (Amersham Biosciences) to be used as the probes, respectively. The membrane was hybridized with the probes for 24 h at 55 °C in a solution containing 6×SSC, and, after appropriate washings, the filters were dried and exposed to an X-ray film for 3 h at room temperature.

2.4. Preparation of recombinant proteins of ARF

Over-expression of *arf-001* and *002* genes in *Escherichia coli* was performed using *E. coli* Expression System with Gateway Technology (Invitrogen). The translatable regions of *arf-001* and *002* were amplified by PCR and were firstly subcloned into Directional TOPO entry vector (pENTR, Invitrogen), and then, the DNA segments were transferred into the expression vector, pDEST14, according to the instruction manuals. The constructed expression vectors harboring the translatable regions of *arf* genes were introduced into *E. coli* BL21/DE3. The transformed cultures were grown at 37 °C overnight, and then supplemented with 500 ml of fresh medium. IPTG was added to the culture, when it reached an optical density of 0.6 at 590 nm, to obtain a final concentration of 0.4 mM, and the incubation was further continued for 3 h at 37 °C. The bacterial cells were harvested by centrifugation at 8000 × *g* for 10 min, and, after sonic oscillation, the proteins were precipitated by centrifugation at 13,000 × *g* for 10 min at 4 °C. As analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), small amounts of contaminants were found in the cell homogenates, and therefore, the samples were further purified by an ion exchange column chromatography. The expressed ARF proteins were re-dissolved in approximately 1 ml of 10 mM K-phosphate buffer (pH 7.8) containing 1 mM EDTA and 1 mM dithiothreitol, and the samples were applied onto a DEAE-Toyopearl 650 column (Tosoh, 1.5 cm × 1.6 cm diameter) previously equilibrated with the same buffer. After washing with the phosphate buffer, the column was eluted with the buffer containing increased concentrations of KCl (stepwise gradient from 0 to 500 mM). The fractions were desalted by dialysis and the proteins in the samples were analyzed by SDS-PAGE.

2.5. Assay of GTP-binding activity of recombinant proteins

GTP-binding activity of recombinant proteins was determined by the incubation of the purified ARF proteins with [^{35}S]GTP-γ-S, essentially, according to the method described previously [16]. The assay mixture consisted of, in a total volume of 200 μl, 20 mM Tris–HCl (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol, 25 mM $MgSO_4$, 25 mM KCl, 0.3 mM dimyristoylphosphatidylcholine, 0.3% Tween 80 (by volume) and 10 μg ARF proteins. The binding reaction was initiated by the addition of 50 nM of [^{35}S]GTP-γ-S (7.4 kBq). In some experiments, 1 μM of non-radiolabeled GTP, GMP, AMP or UTP was added to the assay mixture 10 min prior to the start of the reaction. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 1 ml ice cold binding buffer free from GTP-γ-S, and the mixtures were transferred to a micro-filtration apparatus (Bio-Dot, Bio-Rad Laboratories), respectively. The samples were filtered through a nitrocellulose membrane (0.22 μm) by rapid suction, and the proteins