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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

adsorbed on the membrane were successively washed with the GTP-γ-S-omitted binding buffer (300 μl each, three times). The appropriate areas of the membrane filter were punched out with a cork bore, and the radioactivities associated with the proteins were determined.

2.6. RT-PCR analysis of arf genes

The expression level of *arf* genes was analyzed by RT-PCR. RNA was prepared from the roots, leaves and stems of carrot seedlings and from un-germinated carrot seeds. In the separate experiments, the seedlings were incubated with oligogalacturonides, or 2-CEPA according to the method described previously [17]. They were also incubated at low or high temperature (4 or 42 °C), or in the presence of 400 mM NaCl. At regular intervals, 100 mg of the seedlings were harvested, and total RNA was isolated from the respective samples as described above. Purity of RNA preparations was checked by electrophoresis and it was confirmed that contaminant DNA was negligible. Aliquots of RNA solutions (approximately 0.5 μg RNA equivalent) were added to the RT-PCR mixture prepared from the OneStep RT-PCR Kit (Qiagen), and after the RT reaction, PCR was carried out with the combination of appropriate primer pairs employing 5′-GAC CAT GCT CAA GGT GCG CAT TCA-3′ as the forward primer and 5′-GAC CAT GCT CAA GGT GCG CAT TCA-3′ as the reverse primer for *arf-001* (181 mer as the product), and 5′-GCT AAC AAA CAA GAT CTT CCG AAT GC-3′ as the forward and 5′-GCA TTC TTT CCG ACC ACT TGA AGG TCA GGT-3′ as the reverse for *arf-002* (396 mer as the product), respectively.

3. Results

3.1. Cloning of arf genes

We isolated two cDNA clones from carrot seedlings, which were assumed to encode ARF proteins, by means of RACE method based on the reported nucleotide sequences of the proteins from plant, animal and microorganism sources in the data bases. After 3′ RACE, the comparative analysis of the partial nucleotide sequences was carried out by BLAST, and the matched fragments were subjected to 5′RACE for further characterization of cDNAs encoding ARF or ARF-like small GTP binding proteins. The cDNA clone, designated as *arf-001* (GeneBank accession no. AY874441, 1097 bp) encodes a protein of 198 amino acid residues with the estimated molecular mass of 21,475 Da. On the other hand, *arf-002* (GeneBank accession no. DQ222228, 897 bp) encodes 181 amino acids with the molecular mass of 20,664 Da.

The primary sequences of the putative amino acids of *arf-001* and *002* products showed the significant homology with those of ARF proteins from various biological sources (Fig. 1).

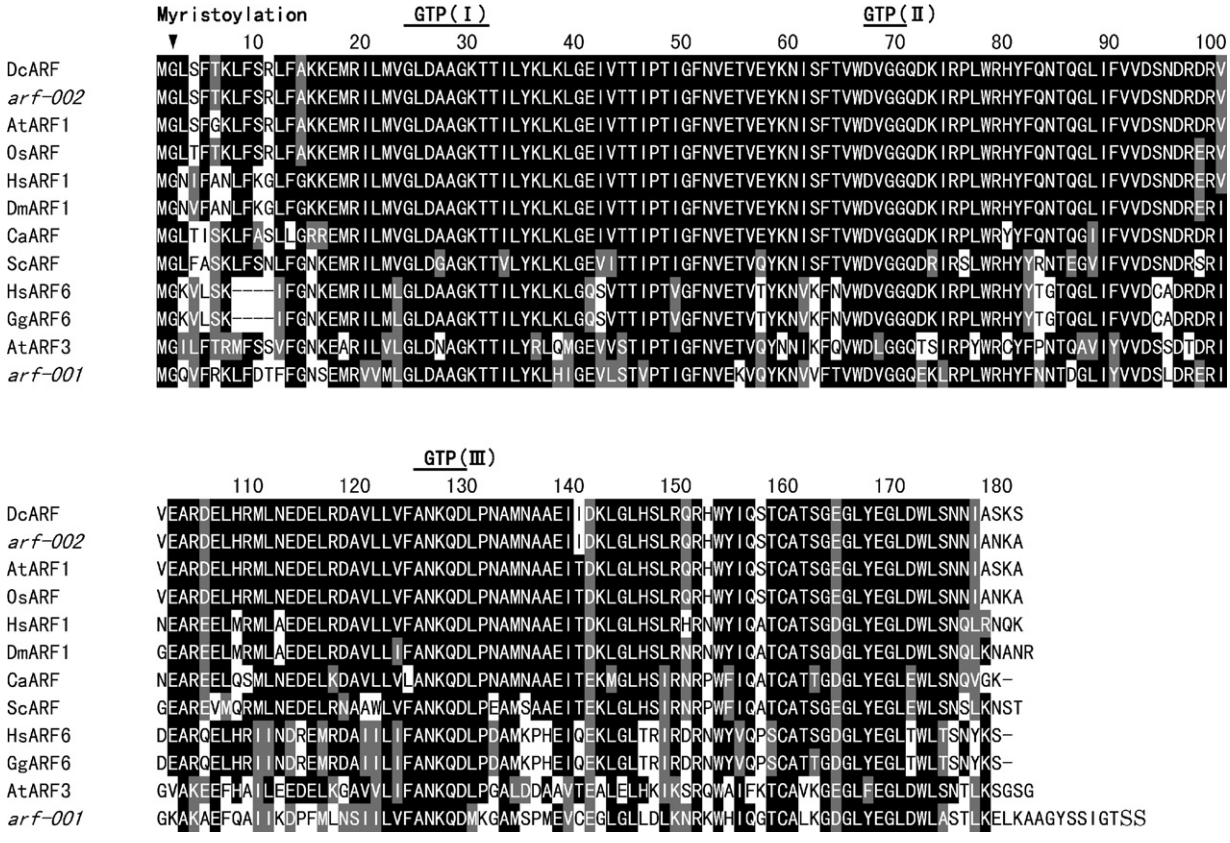

Fig. 1. Alignment of the deduced amino acid sequences of ARF proteins from various sources. The amino acid sequences from various biological sources were aligned by CLUSTALW, and their similarity was compared and analyzed by BOXSHADE. Three conserved structures for GTP-binding were presented as GTP (I)–GTP (III), and possible *N*-myristoylation site, glycine at 2 position, was indicated by an arrow head. The background of the identical amino acid residues was black and that of similar residues was gray. Abbreviations and GeneBank accession numbers are shown in the legend for Fig. 2.

The three characteristic motifs unique to the GTP-binding protein superfamily involved in GTP-binding and/or hydrolysis [11–13], GLDAAGKT (Fig. 1), DVGGQ and ANKQD, are well conserved. In addition, glycine at 2 position, the site of myristoylation after co-translational removal of methionine at *N*-terminal, which is characteristic to ARF proteins, is also involved in the translated products of these two *arf* genes. The putative amino acid sequences of the two *arf* gene products showed the significant homology to ARF proteins from various biological sources, and 63.7% amino acids of *arf-001* and 96.6% of *arf-002* were identical to those of ARFs from *Arabidopsis thaliana*, respectively.

The primary amino acid sequences of *arf-001* and *002* transcripts were further compared with ARF proteins from other biological sources by molecular phylogenetic tree analysis As shown in Fig. 2, it appeared that *arf-002* is close to a carrot *arf* gene product previously reported by Kiyosue and Shinozaki [18] and ARFs from *A. thaliana* [13], *Zea mays* [14] and *Oryza sativa* [15], while *arf-001* resembled to ARF3 of *A. thaliana,* and thus, this would be classified into another group of ARF proteins. It was proposed [2,18] that an ARF protein from *A. thaliana* with low homology to some other plant ARFs should be classified as 'ARF-related protein (ARL)', and therefore, the product of *arf-001* might be an ARL of carrot.

3.2. Southern blot analysis of arf genes

In order to characterize the genomic organization of the isolated two *arf* genes in carrot, Southern hybridization analysis was carried out using appropriate probes containing translatable and 3′ untranslatable regions of the respective genes. As shown in Fig. 3, the labeled probes for *arf-001* hybridized to carrot genomic DNA, and one major band was observed in the DNA fragments digested by either *Eco*RI, *Eco*RV or *Hin*dIII. Several weakly hybridized fragments were also detected in these samples. A similar set of the results was also obtained when the probe prepared for *arf-002* gene was employed for the hybridization experiments, and, as was in *arf-001*, one main band accompanying several weak signals was detected (Fig. 3).

From these results, we deduced that both of the two *arf* genes isolated in the present study, *arf-001* and *002*, might occur as a *arf* small or multi-gene family in the carrot genome, as in *A. thaliana* [19], *Pisum sativum* [20], cotton [21], etc. However, it is not clear whether the weakly hybridized bands represent additional *arf* genes or genes encoding closely related proteins in carrot.

3.3. GTP-binding activities of recombinant ARF proteins

To demonstrate the GTP-binding capability of the proteins encoded by carrot *arf* genes, translatable regions of the cDNAs

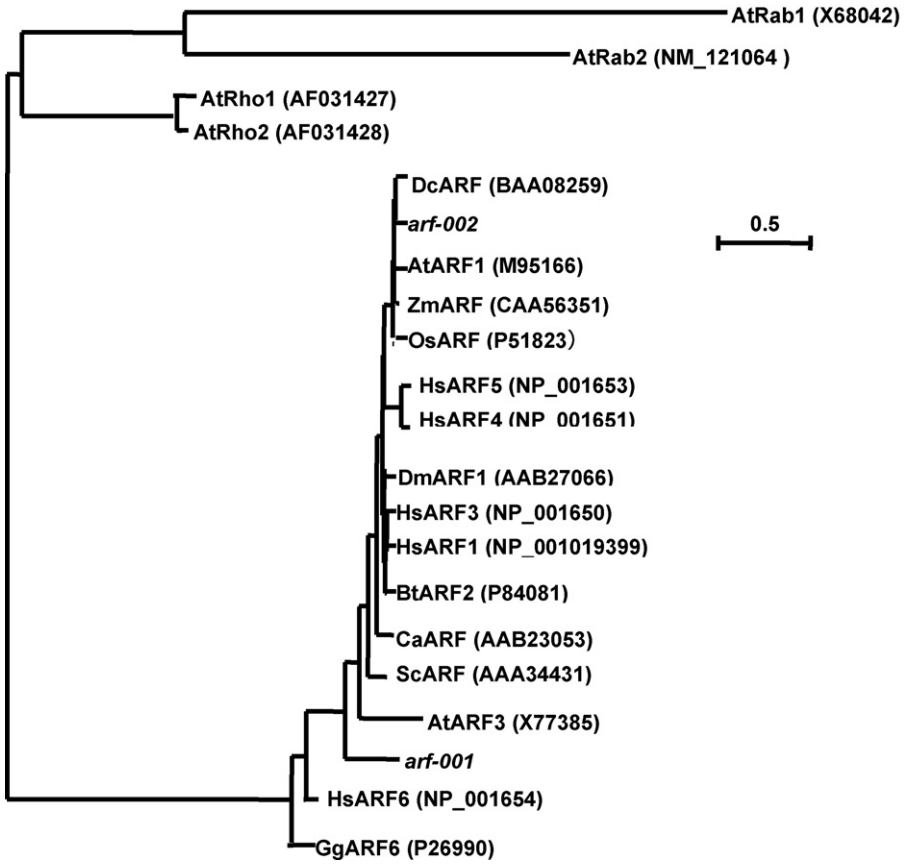

Fig. 2. Phylogenetic tree analysis of small GTP-binding proteins from various biological sources. Phylogenetic tree for the amino acid sequences of ARFs and some other monomeric G-proteins from various biological sources were constructed by NJplot. GeneBank accession numbers of the corresponding genes were shown in the parentheses, respectively, and abbreviations are as follows: At, *Arabidopsis thaliana*; Bt, *Bos taurus*; Ca, *Candida albicans*; Dc, *Daucus carota*; Dm, *Drosophila melanogaster*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*; Zm, *Zea mays*.

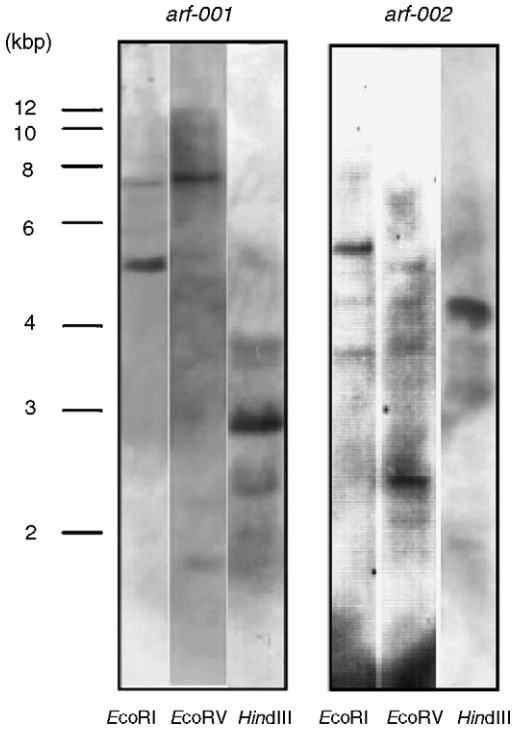

Fig. 3. Genomic Southern blot analyses of *arf-001* and *002* from carrot seedlings. Carrot genomic DNA was digested with *Eco*RI, *Eco*RV and *Hin*dIII, and, after the separation by electrophoresis, the DNA fragments were probed with the labeled fragments of *arf-001* or *002*, respectively.

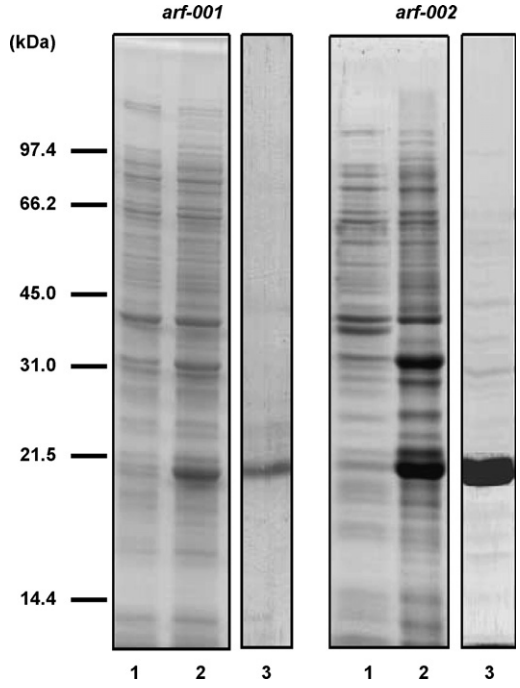

Fig. 4. SDS-PAGE analysis of recombinant ARF proteins produced by *E. coli*. *E. coli* cells were transformed with pDEST14 expression vector harboring translatable regions of *arf-001* or *arf-002*, and, after induction by IPTG, cells were harvested and the recombinant proteins were purified by an ion exchange chromatography (1) immediately before the addition of IPTG; (2) IPTG-treated cell homogenates; (3) purified recombinant ARF proteins).

were ligated into pDEST14 expression vector via pENTR. The proteins expressed in *E. coli* cells transformed with these recombinant plasmids showed the expected molecular masses of approximately 21 kDa on SDS–PAGE by Coomassie Brilliant Blue staining (Fig. 4), and the majority of the proteins were found in the cytosolic fraction of *E. coli*.

The ARF proteins obtained from the transformed *E. coli* cells were transferred onto a nitrocellulose membrane on a micro-filtration apparatus, and incubated with [^{35}S]GTP-γ-S to test the GTP-binding activity of these proteins. As shown in Fig. 5, the recombinant protein encoded by *arf-001* showed an appreciable binding activity toward GTP-γ-S, however, the activity was markedly reduced when the protein was pre-incubated with non-labeled GTP. In contrast, the binding activity was, essentially, not inhibited upon the pre-treatment of the protein with ATP, GMP or UTP. Similar results were also obtained in the assay for *arf-002* protein.

3.4. Expression of arf-001 and arf-002 genes

Tissue specific expression and the possible change in the transcriptional levels upon the treatment with various stimuli of these *arf* genes were examined by RT-PCR analysis (Fig. 6). The appreciable expression of *arf-001* was detected in leaf and stem tissues while *arf-002* appeared to be mainly expressed in roots of carrot seedlings. It seemed that the expression of *arf-001* was transiently enhanced by the exposure of carrot to ethylene, and the intensity of the band of the amplified cDNA fragment was significantly increased after 3–6 h of the treatment. However, treatment of carrot seedlings with oligogalacturonides, the specific inducer for plant defense responses, appeared not to affect the transcriptional level of *arf-001* (Fig. 6). Chill (4 °C), high temperature (42 °C) and salt stress (400 mM NaCl) also did not show the notable effects on the expression activity of *arf-001* in carrot seedlings. Unlike in

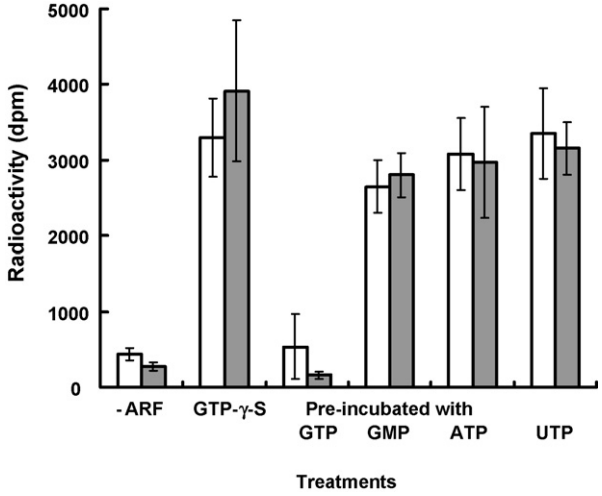

Fig. 5. GTP-binding activity of recombinant ARF proteins. Recombinant proteins for *arf-001* (white columns) and *arf-002* (gray columns) were incubated with [^{35}S]GTP-γ-S, and, after several washings, radioactivities associated with the proteins were determined. In some experiments, the protein samples were pre-incubated with non-labeled GTP, GMP, ATP or UTP, respectively. The results were presented as the means and standard deviations obtained from five replicate experiments.

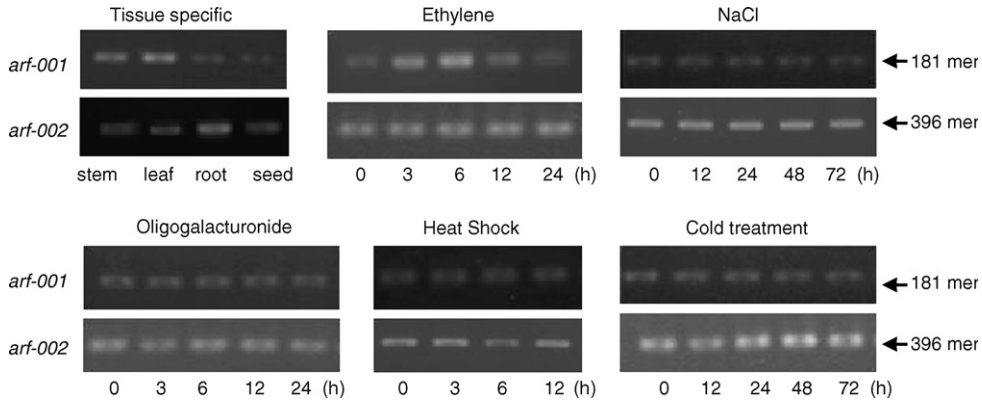

Fig. 6. RT-PCR analyses of the expression of *arf-001* and *arf-002* genes. RNA samples were prepared from various tissues of carrot seedlings and the expression levels of *arf-001* and *002* were tested by RT-PCR using appropriate primer pairs. The possible changes in the transcriptional activities of these two *arf* genes were also examined after the treatment with various stimuli, temperature and salt stresses. At regular intervals, RNAs were prepared from the treated seedlings, and, after RT reaction, the transcriptional levels of these two genes were determined by PCR amplification.

arf-001, *arf-002* appeared to be a house-keeping gene, and its expression levels were maintained at almost constant levels as far as tested. The set of the experiments was repeated at least three times to confirm the reproducibility, and the similar results were obtained.

4. Discussion

Recently, many low molecular weight GTP-binding proteins have been isolated from higher plant sources [22], however, at present, only very limited information is available about the properties and functions of these monomeric G-proteins in plant cells. Among these small GTP-binding proteins, ARFs have been considered to be involved in the vesicle-mediated intracellular trafficking of biopolymers around Golgi apparatus [23,24] as in microorganisms and animal cells [6,7]. In the present study, we have isolated two cDNA clones, *arf-001* and *arf-002*, presumably encoding ARF proteins from carrot seedlings (Fig. 1). Kiyosue and Shinozaki [18] isolated an ARF gene from cDNA library from embryogenic cells of carrot, and, from genomic Southern analysis, they assumed that at least two more *arf* genes could be present in carrot genome. Therefore, the present study should be consistent with their previous work.

In Southern blot hybridization of genomic DNA, both of *arf-001* and *002* showed one dominant band with several weak signals (Fig. 3), suggesting that *arf-001* and *002* are organized as a small or multi-gene family in carrot genome. Similar results were obtained for several other plants [19–21], and therefore, this class of genes might be present in plant genomes in a same manner.

Although *arf-001* was found to be expressed mainly in stem and leaf tissues, a marked transcriptional activity of *arf-002* was observed in roots of carrot seedlings (Fig. 6). In addition, it appeared that expression level of *arf-001* transiently increased by the exposure of carrot to ethylene, while *arf-002* did not respond to external stimuli or physiological stresses, as far as tested. These observations, together with the result of the molecular phylogenetic tree analysis shown in Fig. 2, suggest that *arf-001* and *002* play the distinct physiological roles in carrot cells. A marked increase in the expression levels of *arf* gene after the treatment with plant hormones was reported in *Avena fatua* [25]. These results also suggest that some of ARF proteins function in cellular events evoked by plant hormones, and the expression levels of the corresponding genes should transiently increase by the treatment with these stimuli. It was reported [26] that the increase in transcriptional activity of *arf* gene of rice was observed at 1 h after the treatment at low temperature, however, either *arf-001* or *002* of carrot did not respond to these environmental changes such as chilling, high temperature and salt stress. Possibly, unknown *arf* gene(s) of carrot would mediate these stress-induced responses of this plant.

To further elucidate the regulation of cellular events by plant small G-proteins, more extensive work on the identification and characterization of small GTP-binding protein belonging to deferent groups is required.

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