

Cloning and Expression of *Dcga* Gene Encoding α Subunit of GTP-Binding Protein in Carrot Seedlings

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A homology-based cloning strategy yielded a cDNA clone designated *Dcga*, presumably encoding α subunit of GTP-binding protein, from carrot (*Daucus carota*) seedlings. Molecular phylogenetic tree analysis of G protein α subunits from various biological sources suggested that, unlike in animal cells, classification of G α proteins into specific subfamilies could not be applicable to the proteins from higher plants. The restriction digests prepared from genomic DNA of carrot showed one or two hybridized signals in Southern blot analyses, and the expression level of *Dcga* was appreciably decreased upon the exposure of carrot to high temperature or the prolonged treatment with salt. These results suggest that *Dcga* occurs as single or double copy genes in carrot genome, and its transcript might play specific roles in heat- and salt-induced responses of the plant.

Key words GTP-binding protein; α subunit; hetero-trimer complex; gene cloning; *Daucus carota*

Transmembrane signaling mediated by heterotrimeric GTP-binding proteins is one of the most conservative mechanisms in eukaryote cells, and these G proteins transmit the numerous external stimuli to the activation of appropriate intracellular functional proteins called effectors.^{1,2)} It has been well known¹⁻³⁾ that G proteins are consisted of three subunits, α , β , γ , and α subunit is dissociated from the heterotrimeric complex upon the activation by G protein-coupled receptor. The dissociation of α subunit results in the activation of several functional proteins called effector, including adenylyl cyclase, guanylyl cyclase, phosphodiesterase and phospholipase, to evoke various cellular responses. Multiple α subunits of GTP-binding proteins have been reported from mammalian cells, and these G α subunits are appropriately classified into several subfamilies such as Gs, Gi, Gq and Go according to their target effector proteins and cell physiological functions.³⁾

It has been demonstrated that GTP-binding proteins of higher plants also play important roles in various cellular processes including blue or red light-mediated responses,⁴⁾ plant hormone signaling,⁵⁾ regulation of ion channels⁶⁾ and pathogen-induced resistance mechanisms.⁷⁾ However, only very limited information is available about the properties of G proteins and their target effector molecules in plant cells. Several cDNAs encoding G α subunit have been isolated from some 'model plants' such as rice,⁸⁾ potato,⁹⁾ *Arabidopsis thaliana*,¹⁰⁾ however, unlike in mammals, the possibility has been suggested that plant G protein α subunits might be encoded by very small numbers of genes.⁸⁻¹⁰⁾

We have previously shown that GTP-binding protein(s) mediates an inducible plant defense response, phytoalexin biosynthesis, in carrot cells triggered by elicitor-active chemicals.¹¹⁾ In order to understand the properties and functions of α subunit of GTP-binding protein in the induced plant defense response, in the present experiments, we attempted to isolate cDNA that encodes α subunit of the mediator protein from carrot. The properties of the isolated G α gene, such as transcriptional regulation and genomic organization, were also examined.

MATERIALS AND METHODS

***Dcga* Genes from Carrot** Carrot seedlings were grown in a green house of Experimental Station for Medicinal Plant Research in University of Toyama, and total RNA was isolated from the plant with RNeasy Plant Mini Kit (Qiagen). The RNA obtained (approximately 1 μ g) was subjected to the rapid amplification of cDNA end (RACE) methods using the GeneRacer Kit (Invitrogen) after the generation of cDNA templates by reverse-transcription (RT) reaction with AMV-RT. The 3'-RACE was performed with GeneRacer Oligo dT as the reverse primer, and 5'-TTY ACN GTN TGG GAY GTN GGN GGN CAR GA-3', 5'-GCC AGG ACA AGA TCC GTC CTT T-3' and 5'-GGG TAA TGA ACT CCA AGT TCC TGA CTG C-3' as the gene-specific forward primers for polymerase chain reaction (PCR) amplification of the DNA fragments. The 5'-RACE was carried out with GeneRacer RNA Oligo as the forward, and 5'-CAT GTC CTG TTT GTT TGC AAA CAC CAA-3', 5'-AGC AGC ATT CAT TGC ATT TGG AAG AT-3' and 5'-G CTC TCT TGT CTC CAT CAT TCT GTT CCG-3' as the reverse primers, respectively. The DNA fragments obtained were subcloned into the pCR2.1-TOPO vector (Invitrogen), and the 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).

Southern Blot Hybridization of *Dcga* Gene Genomic DNA was isolated from carrot seedling using Nucleocon Phytopure (Amersham Biosciences) according to the instruction manuals,¹²⁾ and the restriction digests were prepared using *Eco*RI, *Eco*RV and *Hind*III (Takara). They were electrophoresed on a 0.8% agarose gel, and the separated samples were transferred onto an Immobilon-NY+(Millipore). The DNA fragment containing the translatable regions near stop codon of *Dcga* (455 nucleotides) was amplified by PCR and was directly labeled with AlkPhos Direct Labeling and Detection System (GE Healthcare Bio-Science) to be used as the probe. The membrane was hybridized with the probe for 24 h at 55°C in a solution containing 6 \times SSC, and, after

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appropriate washings, the filters were dried and exposed to an X-ray film for 3 h at room temperature.

Expression of *Dcga* Gene The expression level of *Dcga* was analyzed semi-quantitatively using RT-PCR. Carrot seedlings (3-week-old) were incubated with oligogalacturonides or 2-chloroethylphosphonic acid (Sigma), an ethylene-generating reagent, according to the method described previously.¹²⁾ The seedlings were also incubated at low or high temperature (4°C or 42°C), or in the presence of 400 mM NaCl. At regular intervals, 100 mg of the samples were harvested, and total RNA was isolated according to the method described above. Aliquots of RNA solutions (approximately 0.5 µg RNA equivalent) were added to the RT-PCR mixture prepared from OneStep RT-PCR Kit (Qiagen), and after the RT reaction, PCR was carried out with the primer pair, 5'-GAG AAC CGG AAC AGA ATG ATG GAG AC-3' as the forward and 5'-GTG TTC CCA CCT CAC TGC AGG AAT C-3' as the reverse primer (457 mer as the product), respectively. In the parallel experiments, 5'-GGG AAT GGA GTC TGC TGG AAT CCA TGA -3' and 5'-GGA CCT GAT TCG TCA TAT TCA CCC TTC G-3' were used as the primer pair for the amplification of actin gene fragments of carrot (GenBank accession number X17525, 306 mer as the product) as the controls. In the separate experiments, total RNA was also prepared from the roots, leaves, stems of carrot seedlings and from the seeds to test the tissue specificity of the expression level. Since transcriptional activity of either *Dcga* or actin gene was very low in seeds as compared with other tissues, RT-PCR reactions were performed with various amounts of RNA prepared from carrot seeds. The result was presented in which amounts of actin transcript appeared to be the comparable level with those of leaf, stem and root tissues.

RESULTS AND DISCUSSION

Cloning of *Dcga* from Carrot Seedlings Based on the reported nucleotide sequences of GTP-binding proteins from various biological sources, we isolated a cDNA clone encoding α subunit of G protein complex from carrot seedlings by means of RACE method. The 3'RACE was carried out with GeneRacer Oligo dT and the appropriate gene-specific forward primers, and 5'RACE was performed with GeneRacer RNA Oligo and gene-specific reverse primers, respectively. The cDNA clone thus obtained (GenBank accession number EF095216, 1679 nucleotide) contains an open reading frame of 383 amino acid residues. The primary sequences of the putative amino acids encoded by *Dcga* showed the significant homology with those of $G\alpha$ proteins from various biological sources, and 83.8 and 74.2% amino acids of *Dcga* were identical to the α subunits from *Arabidopsis thaliana*¹⁰⁾ and *Oryza sativa*,⁸⁾ respectively (Fig. 1). The five characteristic motifs unique to GTP-binding protein superfamily involved in GTP-binding and/or hydrolysis¹⁴⁾ are well conserved (Fig. 1), and putative N-terminal myristoylation site (glycine at position 2) of *Dcga* product, an important lipid modification site for binding to $G\beta\gamma$ subunit complex,¹⁴⁾ was also highly conserved as MGXXXS in the listed $G\alpha$ subunits. The *Dcga* product contains three effector-binding domains (EBD 1–3) which are the common structure of $G\alpha$ subunits, and the receptor-binding domain (RBD) is also found to locate near the C-terminus of the proteins.¹⁴⁾ Although arginine residues

at position 190 as a potential site for ADP-ribosylation by cholera toxin are conserved in the α subunits listed,¹⁵⁾ *Dcga* is lacking in the C-terminal cysteine, a target of ADP-ribosylation by pertussis toxin which is known as a unique modification site in several $G\alpha$ proteins, Go, Gi and Gt.^{14,15)}

The deduced amino acid sequence of *Dcga* translate was further compared with other $G\alpha$ proteins from various biological sources by molecular phylogenetic tree analysis (Fig. 2). It is well known^{1–3)} that more than 20 α subunits of GTP-binding proteins have been isolated from human, and they are classified into several subfamilies according to the amino acid sequences, specificity toward effector proteins and the physiological functions. In sharp contrast, however, plant α subunits including *Dcga* appeared to be very close each other, and only very low divergency was observed in the members of this protein group in plants (Fig. 2A). It appeared, however, that differences might be sufficient between α subunits from monocot and dicot plants (Fig. 2B).

Southern Blot Analysis of *Dcga* In order to characterize the genomic organization of isolated *Dcga* gene in carrot, Southern hybridization analysis was carried out employing a probe containing a part of the translatable region of *Dcga* immediately upstream of the stop codon (455 nucleotides). As shown in Fig. 3, the labeled probe for *Dcga* hybridized to the restriction digests of carrot genomic DNA, and two signals were observed in the DNA fragments hydrolyzed with either *EcoRI* or *EcoRV*. In contrast, only one major band was observed in the samples digested by *HindIII* (Fig. 3). From these results, we assumed that α subunit of trimeric G protein of carrot is encoded by single or double copy genes, and therefore, the result obtained in the present study would be analogous to those reported for several other plants.¹⁶⁾

Expression of *Dcga* Gene Tissue specific expression and the possible change in the transcriptional levels of *Dcga* gene upon the treatment with various stimuli were examined by RT-PCR analysis (Fig. 4). The appreciable expression of *Dcga* was detected in leaf, root and stem tissues while the transcription of this gene was significantly low in carrot seeds.

It seemed that the expression of *Dcga* in carrot seedlings was transiently decreased by the treatment with high concentration of NaCl (400 mM), and the intensity of the amplified cDNA band was almost disappeared after the incubation for 72 h. Transcriptional level of *Dcga* was also decreased by the exposure of carrot to high temperature (42°C), and a marked reduction of the amplified signals were observed after 3–6 h of the treatment even if the level of actin controls might drop slightly. In contrast, incubation of carrot seedlings at low temperature (4°C) appeared not to affect the expression level of *Dcga*, and the treatment with ethylene or oligogalacturonide, the specific inducer for plant defense responses,¹³⁾ also did not show the notable effects on the expression activity of the gene in carrot seedlings. These results suggest the possibility that *Dcga* might play some specific role in carrot cells toward salt- and heat-induced stresses. The reduction of the transcriptional level of *Dcga* in carrot seedlings might be, possibly, one of the physiological events in down-regulation of cellular processes against these stresses though no direct evidence is available at present. We reported previously¹¹⁾ that GTP-binding protein functions in the induction of defense-related secondary metabolism in carrot cells, how-

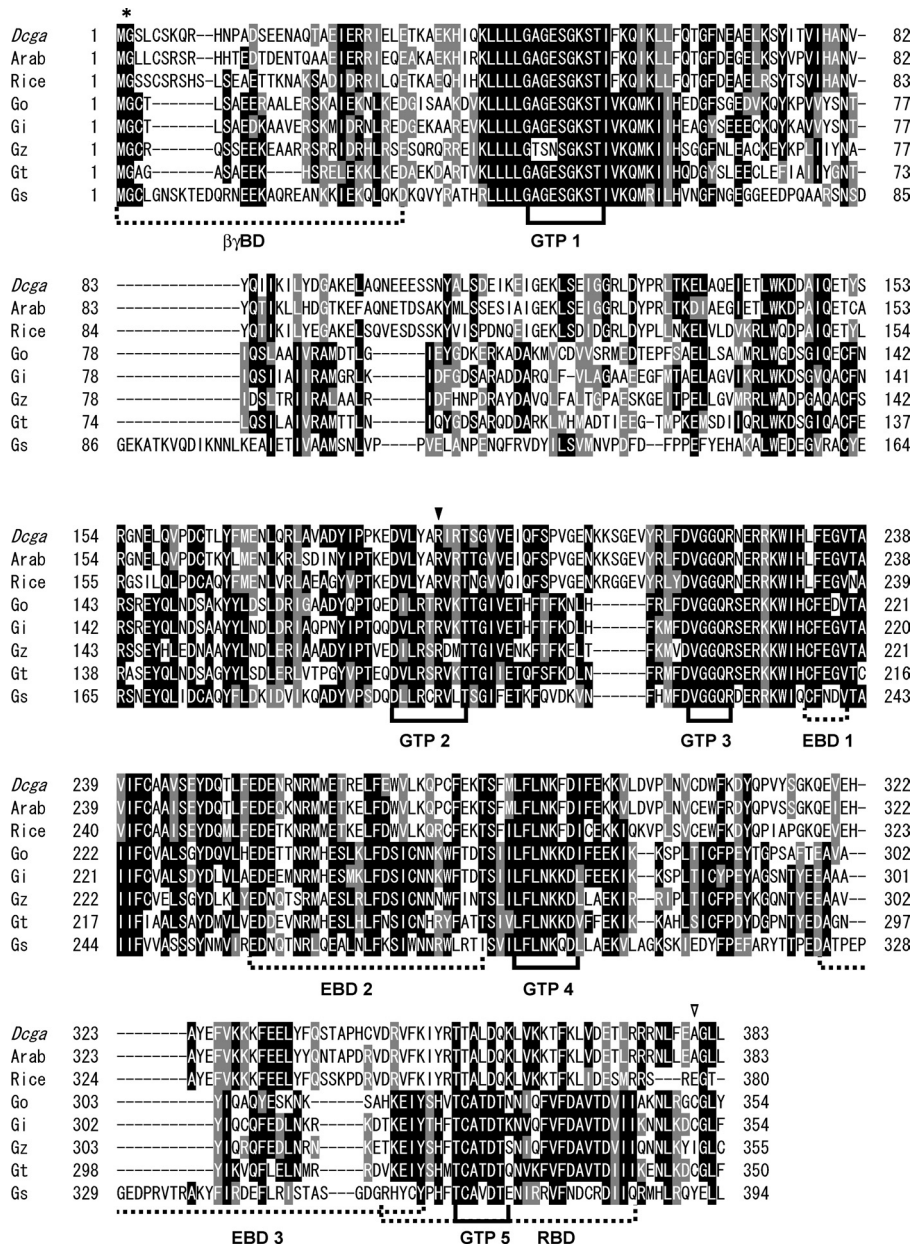


Fig. 1. Alignment of Amino Acids Sequences of α Subunits of GTP-Binding Protein from Various Biological Sources

A cDNA clone encoding α subunit of GTP-binding protein, *Dcga*, was isolated from carrot seedlings, and the deduced amino acid sequences was compared with the proteins from various biological sources (Arab, *Arabidopsis thaliana*; Rice, *Oryza sativa*; Go, Gi, Gz, Gt and Gs are α subunit proteins from human). Their GenBank accession numbers were shown in Fig. 2, and the identical and resemble amino acids were presented as black and gray background, respectively. The specific domains for binding to $G\beta\gamma$ -subunit complex ($\beta\gamma$ BD), GTP (GTP 1–5), effectors (EBD 1–3) and receptor structures (RBD) were highly conserved in the listed $G\alpha$ -subunits of plant and human sources. Putative sites for *N*-myristoylation (*), ADP-ribosylation by cholera toxin (▼) and pertussis toxin (▽) were also presented.

ever, as shown in Fig. 4, expression level of *Dcga* did not respond to the treatment of carrot with oligogalacturonides. These results imply that carrot G-protein would participate in the plant defense responses by a certain mechanism which does not accompany the change in transcriptional level of the gene.

In the present study, it has been demonstrated that i) *Dcga* encoding $G\alpha$ subunit occurs as one or two copy genes in carrot genome; ii) plant $G\alpha$ proteins appeared to show only very low diversity; iii) transcriptional level of *Dcga* is usually maintained at constant level except salt- and heat-stress conditions. In contrast, as described above, it has been widely accepted that numerous α subunit proteins with specific structures and functions occur in animal cells, and they are

the important mediators in signal transduction processes in the cells.^{1–3} These facts strongly suggest that cell physiological functions and mode of action of $G\alpha$ subunits of plant and animal cells should be quite different although structural organization of heterotrimeric complex and mode of the interaction between α , β and γ subunits of plant GTP-binding proteins are considered to be highly conserved.¹⁶ Since carrot gene(s) encoding either $G\beta$ or $G\gamma$ subunit has been isolated not yet, there is no guarantee that *Dcga* product functions in the cells as a component of heterotrimeric complex. However, biochemical responses of carrot toward several reagents, which should perturb the activities of heterotrimeric GTP-binding proteins,¹¹ suggest the possibility that, as in animal cells and several model plants, *Dcga* product

would play physiological roles as a member of heterotrimer G protein complex.

Recently, Ellis and Miles have proposed¹⁷⁾ that external stimuli and their receptor structures are not directly connected to a specific G protein in higher plant cells, and, there-

fore, the results obtained in the present experiments would, at least partly, support their hypothesis that G α subunits are not the key determinants in transmembrane signaling mechanisms in plants. How does GTP-binding protein participate in the signal transduction processes in plant cells? It might be possible that plant G-protein functions as a non-specific transducer or an enhancer of external signals, and a certain functional protein downstream of the signaling cascade, such as small GTP-binding proteins¹⁸⁾ and protein kinases,^{17,19)} should play rather specific roles which triggers appropriate cellular responses in plants instead of trimeric G proteins.

Further studies are in progress in our laboratory to elucidate the physiological significance of GTP-binding proteins and the detail mechanisms of signaling pathway mediated by G protein in higher plant cells.

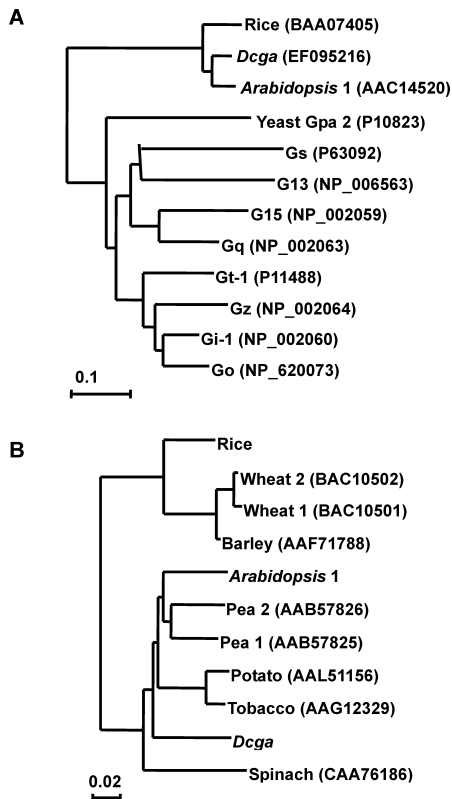


Fig. 2. Phylogenetic Tree Analysis of α Subunits of GTP-Binding Proteins from Various Biological Sources

Phylogenetic tree for the amino acid sequences of G α subunits from various biological sources were constructed by NJplot. GenBank accession numbers of the corresponding genes were shown in the parentheses. The results obtained for plants and human (A), and for monocot and dicot plants (B) were presented.

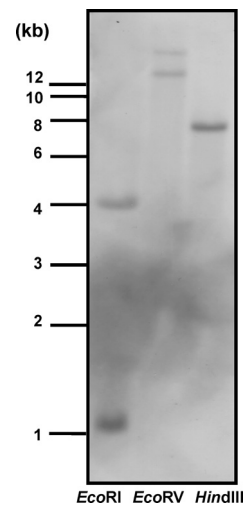


Fig. 3. Genomic Southern Blot Analyses of *Dcga*

Genomic DNA of carrot was digested with *EcoRI*, *EcoRV* or *HindIII*, and, after the separation by electrophoresis, the DNA fragments were probed and visualized.

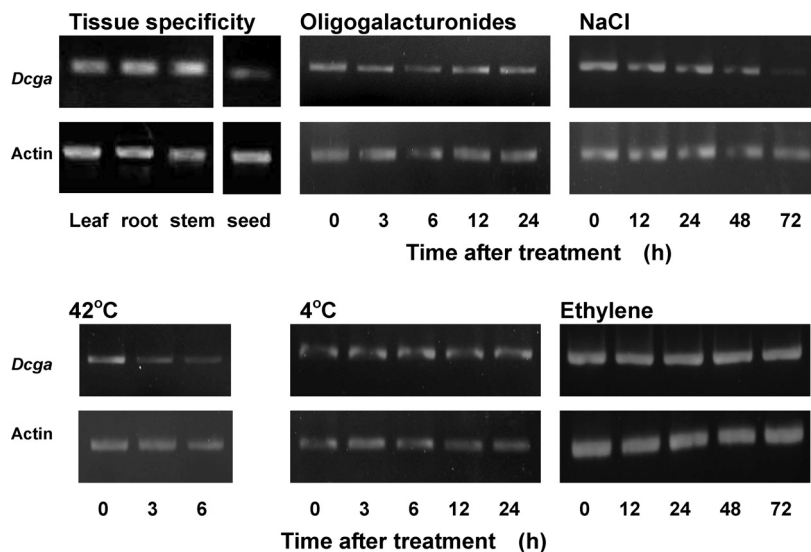


Fig. 4. Semi-quantitative Analyses of the Expression of *Dcga* in Carrot Seedlings Using RT-PCR

For the analyses of tissue-specific expression (A) of *Dcga*, total RNA was prepared from 100 mg of tissues of roots, stems, roots, leaves and seeds, and RT-PCR was performed employing the appropriate primer pair as described in the text. Changes in the expression levels of *Dcga* in carrots were also examined after the incubation with oligogalacturonides, NaCl, ethylene, or at high and low temperature. The DNA fragment of carrot actin gene was also amplified and presented as the control.

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