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Characterization of the heterotrimeric G-protein family and its transmembrane regulator from capsicum (*Capsicum annuum* L.)☆

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ABSTRACT

Throughout evolution, organisms have created numerous mechanisms to sense and respond to their environment. One such highly conserved mechanism involves regulation by heterotrimeric G-protein complex comprised of alpha (Gα), beta (Gβ) and gamma (Gγ) subunits. In plants, these proteins play important roles in signal transduction pathways related to growth and development including response to biotic and abiotic stresses and consequently affect yield. In this work, we have identified and characterized the complete heterotrimeric G-protein repertoire in the *Capsicum annuum* (Capsicum) genome which consists of one Gα, one Gβ and three Gγ genes. We have also identified one RGS gene in the Capsicum genome that acts as a regulator of the G-protein signaling. Biochemical activities of the proteins were confirmed by assessing the GTP-binding and GTPase activity of the recombinant Gα protein and its regulation by the GTPase acceleration activity of the RGS protein. Interaction between different subunits was established using yeast- and plant-based analyses. Gene and protein expression profiles of specific G-protein components revealed interesting spatial and temporal regulation patterns, especially during root development and during fruit development and maturation. This research thus details the characterization of the first heterotrimeric G-protein family from a domesticated, commercially important vegetable crop.

1. Introduction

The Solanaceae family consists of more than 3000 species and is the third most economically important plant taxon [1]. The family exhibits extreme diversity with respect to its geographical distribution and ecological niches as well as its agricultural utility. Many of the common vegetables such as potato, tomato, peppers and eggplant are the members of Solanaceae family. The genus Capsicum of this family consists of at least 25 species, five of which are domesticated [2] including *Capsicum annuum* (pepper) which is economically most important [2,3]. *C. annuum* is a self-pollinating diploid ($2n = 24$), and is highly related to other vegetable crops in its genome sequence and organization [4]. However, in contrast to some members of the Solanaceae family that are highly favored models for specific research questions, *e.g.* tobacco for defense response [5–9], petunia for anthocyanin pigmentation [10–12] and tomato for fruit ripening and defense response [13–15]; research on Capsicum species remains relatively limited. Multinational efforts are underway to generate critical genomic and functional information on economically important members of this family to help improve their environmental adaptation and yield.

Signal transduction processes mediated by heterotrimeric guanine-nucleotide-binding proteins (G-protein, hereafter) constitute one of the important regulatory mechanisms for plants' adaptation to their environment [16,17]. The G-protein complex consists of alpha (Gα), beta (Gβ) and gamma (Gγ) subunits and operates *via* a switch-like mechanism to transduce the signal [18]. The Gα subunit, in its GDP-bound conformation, remains associated with the Gβγ subunits and represents the inactive signaling

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; AGB, Arabidopsis G-protein β subunit; AGG, Arabidopsis G-protein γ subunit; BLAST, Basic Local Alignment Search Tool; BODIPY-GTP-FL, boron-dipyrromethene-guanosine 5′-O-(3-thiotriphosphate)-fluorescence; GAP, GTPase-activity accelerating protein; GDP, guanosine diphosphate; GPA, Arabidopsis G-protein α subunit; GTP, guanosine triphosphate; RGS, regulator of G-protein signaling.

☆ The GenBank accession numbers of capsicum G-protein genes are: CaGα: KJ914669; CaGβ: KJ921707; CaGγ1: KJ921708; CaGγ2: KJ921709; CaGγ3: KJ921710 and CaRGS: KJ921711.

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state. Signal-dependent GDP to GTP exchange on the Gα subunit leads to the dissociation of the complex into GTP-Gα and Gβγ. Both these entities can interact with downstream effectors to transduce the signal. This represents the active state of the G-protein signaling. The Gα subunit, due to its inherent GTPase activity, hydrolyzes the bound GTP, regenerating the GDP-bound Gα, which re-associates with the Gβγ dimer to return to its inactive conformation [18,19]. The GTP-hydrolyzing (GTPase) activity of Gα proteins is accelerated by the regulator of G-protein signaling (RGS) proteins [20,21]. This signaling mechanism is conserved in all eukaryotes from yeast and humans to green algae and higher plants, representing its evolutionary breadth [22–26]. In contrast to metazoans, where each of the G-protein subunits is represented by multiple genes (*e.g.* 23 Gα, 5 Gβ, 12 Gγ and 37 RGS domain containing proteins in human), most plants contain simpler repertoire of G-protein genes. In both Arabidopsis and rice, single Gα and Gβ together with few Gγ proteins represent the G-protein networks [27–30]. Plants such as soybean that have undergone recent whole genome duplication events seem to retain most copies of the duplicated G-protein genes as 4 Gα, 4 Gβ, 12 Gγ and 2 RGS proteins represent G-protein network in the soybean genome [31–33].

In higher plants, G-proteins have been extensively characterized from Arabidopsis, rice and soybean, and to some degree from maize, pea and tobacco, where they have been shown to regulate signaling pathways involved during important growth and development processes [28–30,32,34–40]. The roles of G-proteins have been identified in integrating a multitude of signals such as light, nutrient ions, hormones, drought stress, pathogen attack and protein ligands to diverse intracellular changes in processes such as ion flux, enzymatic activity, protein proximities and trafficking [38,39,41–43]. At the whole plant level, these changes result in control of growth and cell proliferation including meristem development, lateral root formation, hypocotyl elongation, apical hook opening, leaf expansion, nodule organogenesis, stomatal movements, sugar sensing, hormonal responses and response to bacterial and fungal pathogens [28–30,32–36,39–61].

Interestingly, mutations in the specific G-protein subunits in different plant species do not always result in similar phenotypes. For example, rice plants expressing reduced levels of Gα or Gβ proteins exhibit dwarfism and changes in internodal distance [38–40]; phenotypes not shared by Arabidopsis mutants devoid of Gα or Gβ genes. Similarly, tobacco plants expressing reduced levels of Gβ proteins exhibit aberrant pollen development, germination and inflorescence architecture [57]. These phenotypes are not exhibited by either Arabidopsis or rice Gβ mutants. Moreover, altered expression of a Gγ homolog (DEP1) leads to changes in panicle branching in rice, a phenotype not observed in the Arabidopsis mutants in homologous genes [28–30,52]. In addition to these developmental phenotypes, the Gα mutants of Arabidopsis and rice (*gpa1* and *d1*, respectively) also show profound differences among them during response to several pathogens. While the rice *d1* mutants are highly susceptible to a fungal pathogen, *Magnaporthe grisea*, the Arabidopsis *gpa1* mutants have lower susceptibility to several pathogens [5,24,62–65]. Observations such as these suggest that despite the conservation of the core G-protein components, their signaling mechanisms might have evolved to play distinct roles related to the environmental adaptation of specific plant species. Therefore, characterization of G-protein complexes from additional non-model plant species is required to understand their importance in controlling species-specific adaptive responses.

Toward this, we have identified the complete repertoire of G-protein genes from the Capsicum genome, encoding for one Gα, one Gβ and three Gγ proteins. We have also identified a RGS protein that regulates the Gα protein activity. The Capsicum G proteins exhibit the signature characteristics, *i.e.* specific GTP-binding and -hydrolysis by Gα protein, the ability of Gα protein to functionally complement yeast mutants lacking Gpa1, interaction of Gα protein with Gβ protein and with RGS protein, and interaction of Gβ proteins with each of the three Gγ proteins. Expression analysis of specific G-protein genes and proteins suggest their developmental and temporal regulation and their involvement during Capsicum fruit maturation. These results constitute the necessary foundation for studying the role of G-proteins during critical developmental processes in an important vegetable crop.

2. Materials and methods

2.1. Plant material and growth conditions

Capsicum plants (cv California wonder) were grown in a greenhouse (26/23 °C, day/night temperature). Tissue samples from different parts of Capsicum plants or fruits of different developmental stages were collected, immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Identification and cloning of Capsicum G-protein genes

Capsicum G-protein genes were identified by BLAST analysis of the latest Capsicum genome assembly (http://Capsicumgenome.snu.ac.kr) using Arabidopsis *(Arabidopsis thaliana*) G-protein sequences as queries. Full length G-protein genes were amplified from seven days old Capsicum seedlings cDNA, using gene specific primers (Table S1). cDNA sequences corresponding to all sixth G-protein components (1Gα, 1 Gβ, 3 Gγ and 1 RGS) were cloned into pCR8/GW/TOPO vector (Life Technologies) and confirmed by sequencing.

2.3. Phylogenetic analysis

Capsicum G-protein component genes were aligned using Clustal W with corresponding sequences from other plant species. MEGA 6 was used to evaluate the evolutionary relationship analysis using the Neighbor-Joining method [66]. Evolutionary distances were computed using the p-distance method. All positions containing gaps and missing data were eliminated.

2.4. RNA isolation and quantitative transcript level analysis

Total RNA was isolated from different Capsicum tissues using Trizol reagent (Invitrogen). Genomic DNA was removed from the RNA using Turbo DNA-free (Ambion). Four micrograms of RNA was used for cDNA synthesis using Superscript III cDNA synthesis kit (Invitrogen). The cDNA samples were diluted to 1:50 with sterile water. Quantitative real-time PCR (qRT-PCR) was performed with transcript-specific primers (Table S1) as previously described [32]. Capsicum *α-tubulin* gene (EF495257.1) was used as a normalization control. The qRT-PCR was repeated three times and data were averaged. Melting curve analysis of the amplicons was performed to confirm their specificity.

2.5. Protein extraction and western blotting

Total protein was extracted from Capsicum flowers and different stages of developing and mature fruits. Frozen tissue was ground extensively in liquid N_2 in a mortar and pestle and resuspendend in extraction buffer [50 mM Tris, pH 7.5, 10 mM EDTA, 10% glycerol, 1 mM DTT (dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1× plant protease inhibitor cocktail; Sigma]. Samples were spun at 12,000 × *g* for 30 min at 4 °C. The supernatant was further spun at 49,000 × *g* for 1 h at 4 °C in an ultracentrifuge. The supernatant was discarded and the pellet (total microsomal

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